*International Journal of Pharmaceutical Science Invention*
*ISSN (Online): 2319 – 6718, ISSN (Print): 2319 – 670X*
*www.ijpsi.org Volume 2 Issue 5 ‖ May 2013 ‖ PP.55-65*

# Trypsin inhibitors from *Lavatera cashmeriana* Camb. seeds: isolation, characterization and *in-vitro* cytoxicity activity

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**ABSTRACT**: *Protease inhibitors (PIs) are small proteins that are quite common in nature. PIs are present in multiple forms in numerous tissues of animals and plants as well as in microorganisms. PIs are emerging with promising therapeutic uses in the treatment of diseases like cancers. Working under the same trend we isolated and characterized protease inhibitors from Lavatera cashmeriana Camb. to check their in-vitro cytoxicity activity.*

*Four serine protease inhibitors named LC-pi I, LC-piII, LC-pi III and LC-pi IV were purified to electrophoretic homogeneity from seeds of Lavatera cashmeriana Camb. by procedure that entailed ammonium sulphate precipitation, anion exchange chromatography and gel filtration on Sephadex G-100. The crude protein resolved into four major peaks on ion exchange column. The isolated proteins exerted an inhibitory action against the activity of trypsin, chymotrypsin and elastase. The activity of trypsin and chymotrypsin was inhibited almost completely in a molar ratio of 1:1 whereas elastase was inhibited only in a molar ratio of 0.2:1. The inhibitors appeared as 20.9, 14.1, 16.8 and 7.9 kDa peaks in gel filtration and 10, 14, 16 and 7 kDa bands by SDS-PAGE establishing that LC-pi I is constituted of two similar subunits of 10 kDa. The inhibitors were found to be glycoproteins. The inhibition of trypsin was maximum at pH 7, 8, 7.6 and 8.5 for LC-pi I, LC-pi II, LC-pi III andLC-pi IV respectively. The optimum temperature was found to be 30˚C for all the four inhibitors. We also examined the suppressive activity of LC-pi I against Prostate (PC-3) and Breast (MCF-7), Colon (HCT-15) and Neuroblastoma (IMR-32). The inhibitor was found to suppress in-vitro cell growth of Prostate (PC-3) and Breast (MCF-7) cell lines, but could not inhibit the growth of Colon (HCT-15) and Neuroblastoma (IMR-32) cell lines.*

**Keywords-** *Chromatography, chymotrypsin, cytoxicity, elastase, Lavatera cashmeriana Camb., protease inhibitors, trypsin*

## I. INTRODUCTION

Plants produce a variety of proteins that serve in the defence against pathogens and invading organisms, including ribosome inactivating proteins [1], lectins [2], protease inhibitors [3] and antifungal proteins [4-6]. Among these protease inhibitors are believed to play an important role in the defense against attack of both phytophagous insects and microorganisms [7, 8]. Protease inhibitors are of, very common occurrence and have been isolated from a large number of organisms incuding plants, animals and microorganisms

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[9-19]. Naturally occurring protease inhibitors are essential for regulating the activity of their corresponding proteases and play key regulatory roles in many biological processes e.g. blood clotting, apoptosis, signalling receptor interactions in animals [20]. Recent studies involving in vitro and in vivo model systems and human trials have evidenced that these inhibitors have anti–carcinogenic properties [21-25]. This is supported by the observation that populations consuming relatively high amounts of legume seeds show relatively low incidences of prostate, colon, breast and skin cancers [26].

*Lavatera cashmeriana* Camb. (Malvaceae), which is endemic to Kashmir valley, has great medicinal importance. Its parts are being used to treat sore throatand common cold. In this communication we have reported the isolation and characterization of protease inhibitors from the seeds of this plant which are capable of inhibiting trypsin, chymotrypsin and elastase.

## II. MATERIALS AND METHODS

**a. Seed Collection**

Mature seeds of *Lavatera cashmeriana* Camb. were procured from Department of Botany, University of Kashmir**.** Trypsin, chymotrypsin**,** elastase, N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA), acetyl-L- tyrosine ethyl ester (ATEE), N-succinyl-L-$(ala)_3$–p-nitroanalide, DEAE- cellulose and Sephadex G-100 were purchased from (Sigma Aldrich Company, USA).

**b. Isolation and purification of protease inhibitors from *Lavatera cashmeriana* Camb. seeds**

Twenty five gram dried seeds of *Lavestera cashmeriana* Camb. were soaked in 0.15N NaCl for 16 hours. The swollen seeds were homogenized with 300ml of ice cold saline Tris buffer (20mM, pH 8.0) containing 1mM sodium metabisulfite in a Remi auto mix blender at 4°C for 10 min. The homogenate was filtered through 4 layers of cheese cloth. The filtrate was centrifuged at 12,000g for 20 min, the pellet was discarded and the supernatant was retained and stored at 4°C. From the supernatant, the proteins were precipitated by ammonium sulphate at 90% saturation.Crude extract was further purified by ion exchange chromatography on DEAE-cellulose column using 20mM Tris buffer, pH 8. The protein was eluted using linear sodium chloride gradient from 0.05 to 0.6M. The fractions eluted from ion-exchange column were chromatographed separately on Sephadex G-100 column in 0.1M Tris buffer, pH 8 and 0.4M NaCl. The charge homogeneity of the fractions eluted from gel filtration column was checked by polyacrylamide gel electrophoresis.

**c. Protein estimation**

Protein concentration was determined by the method of Lowry et al. [27] using BSA as the standard protein.

**d. Polyacrylamide gel electrophoresis**

Polyacrylamide gel electrophoresis in presence and absence of SDS was performed at pH 8.3 on 10% gel with a discontinuous buffer system. The gels were stained with Coomassie Brilliant Blue R-250.

**e. Molecular weight determination**

The subunit molecular mass of the protease inhibitors was estimated by discontinuous SDS-PAGE according to method of Lammeli [28]. Native molecular mass was determined by gel filtration on Sephadex G-100 column using the following protein markers: BSA (66kDa), Ovalbumin (45kDa), Pepsin (34.7kDA) and cytochrome c (12.4kDa). The gel filtration data was treated according to the relation given by Andrew [29].

**f. Measurement of antitryptic activity**

The antitryptic activity of inhibitors was estimated by measuring the % inhibition of trypsin, using synthetic substrate benzoyl-DL-arginine-p-nitroanilide [30]. Variable volumes of purified inhibitors were incubated with 10mg of trypsin (diluted in 1mM HCl) and 0.1M Tris-Hcl buffer; pH 8.0, containing 0.01M $CaCl_2$, (final volume 500 µl) for 10 min at $37^0$C. Then 500 µl of BAPNA (1mM in DMSO) was added, incubated for 40 min at $37^0$C and reaction was stopped by the addition of 500µl of acetic acid (30% v/v). The control, which did not contain inhibitor, was prepared under strictly identical conditions. The blank for each volume of the inhibitor tested was obtained by the addition of acetic

acid just before the incubation with the substrate. The intensity of the yellow colour produced was measured by recording the absorbance at 405 nm.

**g. Measurement of antichymotryptic activity**

Different volumes of each inhibitor was pre-incubated with 10µg of enzyme solution for 5min at room temperature in 1 ml of 46mM-Tris, 0.5mM $CaCl_2$ buffer, pH 8.1. The reaction was started by the addition of 3ml of substrate (acetyl-L- tyrosine ethyl ester) solution to pre-incubated enzyme plus inhibitor solution. Blank was prepared for each volume of extract tested by omitting the enzyme from the reaction mixture. The intensity of the yellow color produced was measured by recording the absorbance at 410 nm against an appropriate blank [31]. A control, where chymotrypsin was not pre-incubated with inhibitor was also prepared.

**h. Measurement of antielastase activity**

Different volumes of the purified inhibitors were incubated with the reaction mixture (650µl) containing 100mM Tris-HCl, 10mM $CaCl_2$, pH 7.5, 0.135mM substrate (N-succinyl-L-$(ala)_3$-p-nitroanalide) and elastase (1.13U/ml). The control, which did not contain inhibitor, was prepared under strictly identical conditions [32]. Blank was prepared for each volume of extract tested by omitting the enzyme from the reaction mixture. The reaction was carried out at $36^0$C, and the absorbance was measured at 410nm.

**i. Effect of pH on the stability of the Inhibitor**

Different volumes of the purified inhibitors were incubated with the reaction mixture (650µl) containing 100mM Tris-HCl, 10mM $CaCl_2$, pH 7.5, 0.135mM substrate and elastase (1.13U/ml). The control, which did not contain inhibitor, was prepared under strictly identical conditions. Blanks were prepared for each volume of extract tested by omitting the enzyme from the reaction mixture. The reaction was carried out at $36^0$C, and the absorbance was measured at 410nm.

**j. Effect of temperature on the activity of Inhibitors**

20 µl of the purified samples were incubated at various temperatures from 10 to $70^o$ C, for 10 minutes and activity was determined.

**k. Carbohydrate Analysis**

The purified inhibitors were analysed for carbohydrate content by phenol-sulphuric acid reaction [33] using D-galactose as standard.

**l. Cytotoxicity Assay (SRB assay)**

The cytotoxicity of LC-pi I was determined by SRB assay [34] using Colon (HCT-15), Prostate (PC-3), Breast (MCF-7) and Neuroblastoma (IMR-32). Cells ($3\times10^3$/cells) were plated in 100µl of medium/well in 96-well plates. After incubation overnight, LC-pi I was added in various concentrations (500, 300, 100, 50µg/ml); triplicate wells were included for each concentration. The plates were incubated for 48hrs at $37^0$C and in an atmosphere of 5%n$CO_2$. The cells were then fixed by adding 50µl of ice-cold 50% TCA to each well for 60min in case of adherent cells The plates were washed five times in running tap water and stained with 100ml per well SRB reagent (0.4% w/v SRB in 1% acetic acid for 30min. The plates were washed five times in 1% acetic acid to remove unbound SRB and allowed to dry overnight. SRB was solubilised with 100µl per well 96 well plate 10mM Tris-base, shaken for 5min and the OD was measured at 570nm with reference wavelength of 620nm.

## III. RESULTS

**a. Isolation and Purification**

The crude extract was found to have 85% antitryptic activity, 80% antichymotryptic activity and 94.6 % antielastase activity. On DEAE cellulose major portion of the protein in crude extract passed unbound through the column. The fraction did not show any protease inhibitor activity. The bound protein was eluted using a linear sodium chloride gradient from 0.05 to 0.6 M of 20mM Tris buffer, pH 8 in 5ml fractions at a flow rate of 30ml/hr. At this pH, binding of inhibitors to the ion-exchanger was almost complete. The bound protein eluted into many small minor peaks and four major peaks from the column as shown in Fig. 1. The fractions under minor peaks didn’t show any antiprotease activity whereas fractions under peak I, II, III and IV showed antiprotease activities against trypsin, chymotrypsin and elastase. The fractions under each peak were pooled and

concentrated. The individual peaks were labeled as LC-pi I, LC-pi II, LC-pi III & LC- pi IV and further chromatographed onto Sephadex G-100 column. All the four peaks resolved into single protein peak showing that the preparations were homogenous with respect to size. The results are given in Fig. 2. The fractions under each peak were found to have antitryptic, antichymotryptic and antielastase activity. At each purification steps, the specific activity of inhibitors increased over the homogenate (Table 1).

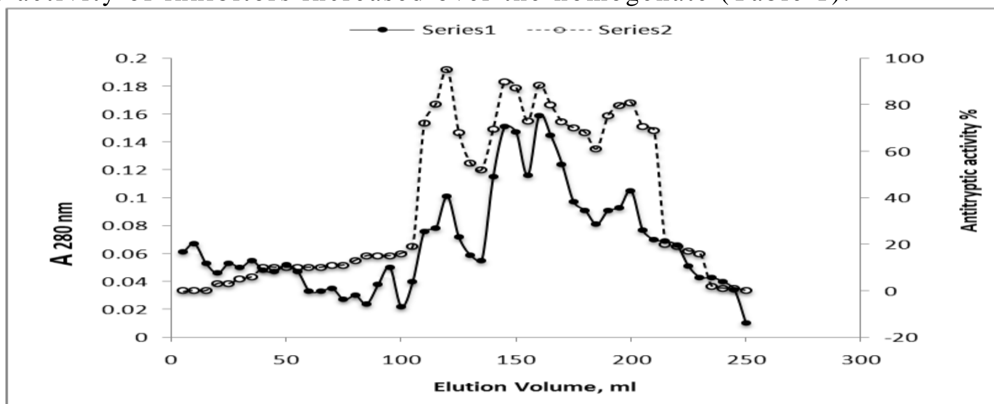



**Fig.1 Elution profile of crude extract on DEAE cellulose column. 300 mg of protein was applied on the column (35×2cm) equilibrated with 20mM Tris buffer, pH8. The protein was eluted using a linear sodium chloride gradient from 0.05 to 0.6M in 5ml fractions at flow rate of 30ml/hr.**

**Series 1 represent absorbance at 280nm and series 2 represent % antitryptic activity.**

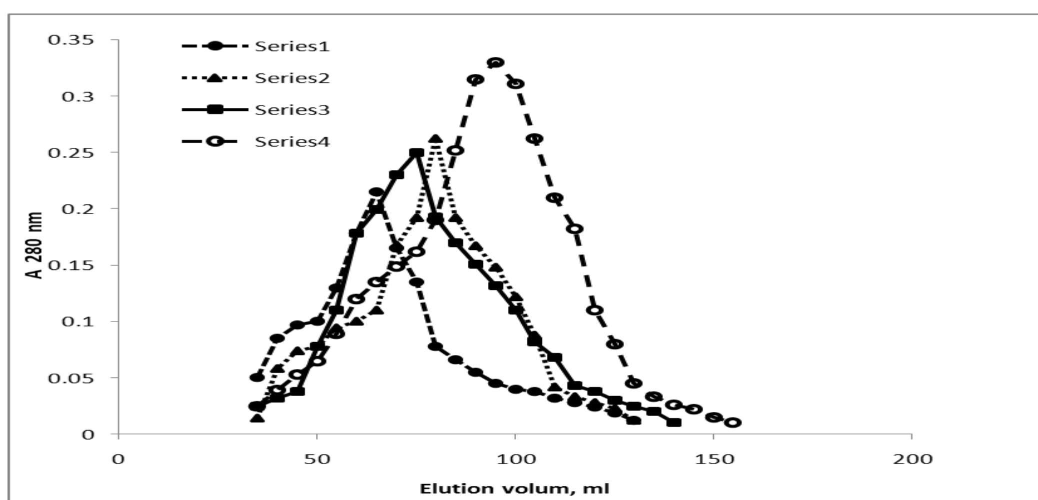



**Fig. 2 Elution profile of DEAE-cellulose fractions on Sephadex G-100 column. Twenty milligram of each protein was applied on the Saphedex G-100 column (40×2cm) equilibrated with 0.1M Tris buffer pH 8, 0.4M NaCl. The protein was eluted at a rate of 30ml/hr in 5ml fractions. The void volume of column was 30ml**

**Series 1 represent absorbance at 280nm of LC-pi I, series 2 of LC-pi II, series 3 of LC-pi III and series 4 of LC-pi IV.**

**Table 1: Purification of protease inhibitors from *Lavatera cashmeriana* Camb. seeds**

| Steps | Total protein (mg) | Antitryptic activity | | Antichymotryptic activity | | Antielastase activity | | Per cent Recovery | Purification factor |
|---|---|---|---|---|---|---|---|---|---|
| | | Inhibitor Units* | Specific activity | Inhibitor Units* | Specific activity | Inhibitor units* | Specific activity | | |
| Crude extract | 160000 | 8000 | 0.05 | 7500 | 0.04 | 8800 | 0.055 | 100 | 1 |
| Ammonium sulphate fraction | 24100 | 5000 | 0.20 | 4000 | 0.16 | 6800 | 0.28 | 75 | 5.09 |
| DEAE-cellulose fraction | | | | | | | | | |
| LC-pi I | 450 | 1000 | 2.2 | 900 | 2 | 2600 | 5.7 | 29.5 | 103.6 |
| LC-pi II | 500 | 950 | 1.9 | 900 | 1.8 | 2400 | 4.8 | 27.2 | 87.2 |
| LC-pi III | 600 | 1000 | 1.6 | 945 | 1.57 | 2500 | 4.1 | 28.4 | 74.5 |
| LC-pi IV | 500 | 900 | 1.8 | 800 | 1.6 | 2250 | 4.5 | 25.6 | 81.8 |
| Gel filteration | | | | | | | | | |
| LC-pi I | 17.5 | 1000 | 57.14 | 950 | 54.28 | 1540 | 88 | 17.5 | 1600 |
| LC-pi II | 26 | 950 | 36.58 | 900 | 34.61 | 1500 | 57.69 | 17 | 1048 |
| LC-pi III | 47 | 980 | 20.85 | 1000 | 21.27 | 1580 | 33.61 | 17.9 | 611 |
| LC-pi IV | 36 | 800 | 22.22 | 800 | 22.2 | 1480 | 41.11 | 16.8 | 747 |

***One inhibitor unit (1 IU) is defined as the amount of inhibitor which suppressed libration of one µmol substrate per min by active enzyme.**

**b. Electrophoretic analysis**

The purified LC-pi preparations were homogeneous with respect to charge as revealed by the single band when subjected to polyacrylamide gel electrophoresis (Fig. 3).

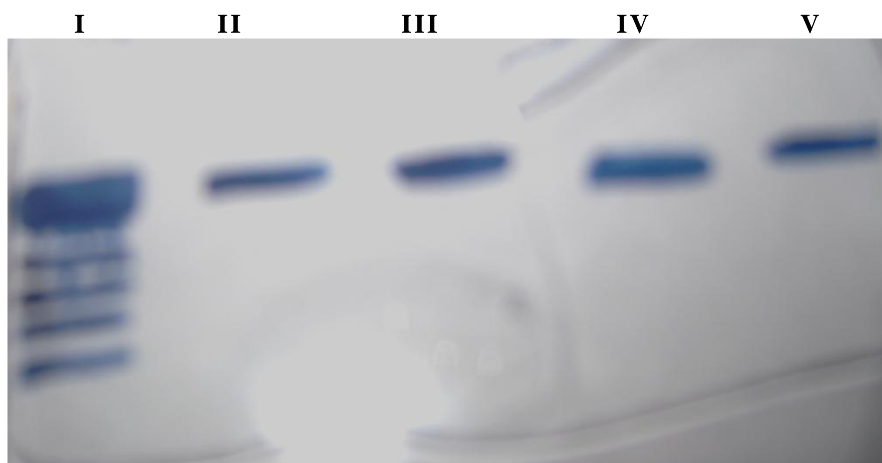



**Fig. 3 Polyacrylamide gel electrophoretic pattern of protease inhibitors from *Lavatera cashmeriana* Camb.**

About 50µg of each protein was applied on polyacrylamide gel at pH 8.3, using 10% separating gel and 4% stacking gel. Current of 8mA per well was applied. The staining solution used was Coomassie brilliant blue R-250. Lane I crude extract; lane II, LC- pi I; lane III, LC- pi,II; lane IV, LC- pi III and lane V, LC- pi IV.

**c. Characterization**

i. **Molecular weight determination**

The molecular weights of *Lavatera cashmeriana* Camb. protease inhibitors as determined by gel filtration chromatography was 20.9, 14.1, 16.8 and 7.9 kDa for LC pi I,

LC pi II, Lc pi III and LC pi IV respectively. Based on SDS polyacrylamide gel electrophoresis data the molecular weight was found to be 10, 14, 16 and 7 kDa for LC-pi I, II, III and IV respectively (Fig 4). The results of SDS-PAGE together with gel filtration data revealed that LC-pi I exists as dimer of two identical subunits of 10 kDa.

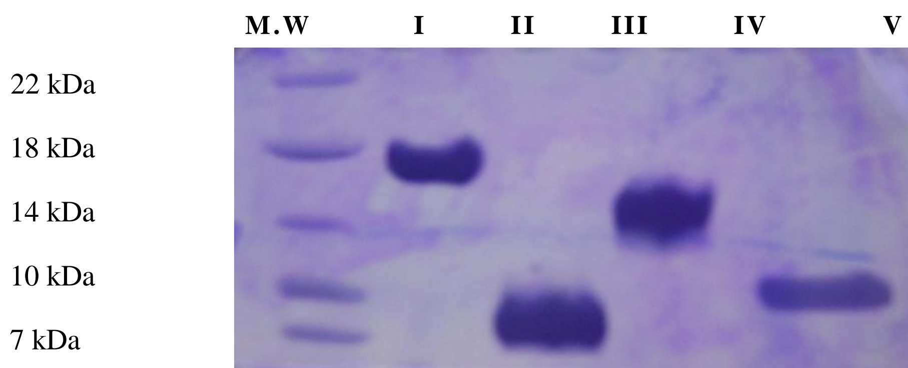
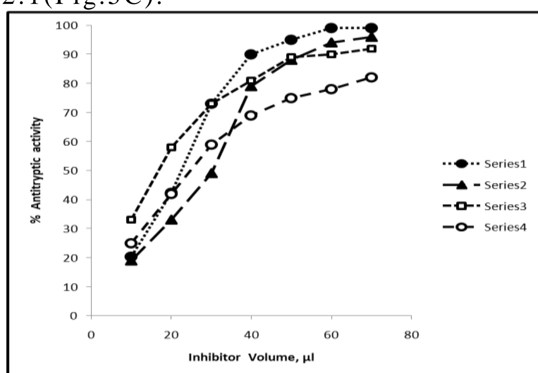



**Fig. 4 SDS-polyacrylamide gel electrophoretic pattern of protease inhibitors.**

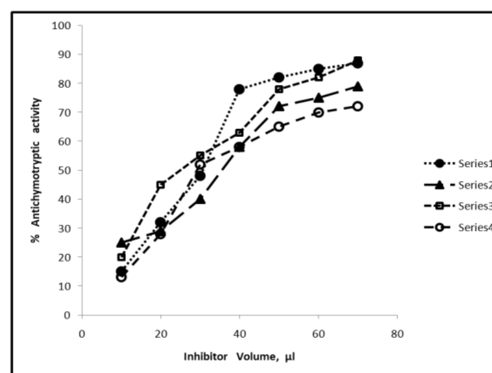
Electrophoresis was carried out in Tris-glycine buffer pH 8.3 at a current of 8mA per well using 10% separating gel and 4% stacking gel. About 80µg of each protein was applied in each wels. Lane I, molecular weight standards; lane II, LC- pi III; lane III, LC-pi IV; lane IV, LC- pi II and lane V, LC- pi I. The gels were stained with Coomassie brilliant blue R-250.

**ii. Specificity**

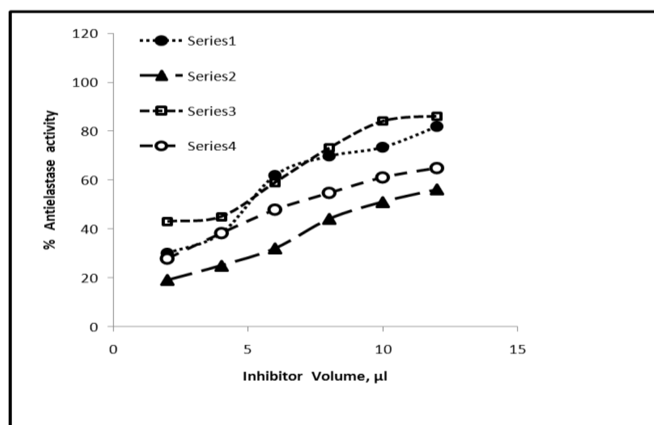
The specificity of purified protease inhibitors was determined against trypsin, chymotrypsin and elastase. LC-pi I, II, III and IV inhibited the activity of trypsin by 99%, 96%, 92% and 82% and of chymotrypsin by 87%, 88%, 79% and 72% respectively at the molar ratio of 1:1 (Fig. 5A and 5B). The activity of elastase was inhibited by 99%, 77%, 91% and 79% respectively by LC-pi I, II, III and IV only at the molar ratio of 0.2:1(Fig.5C).




**(A)**




**(B)**




**(C)**

**Fig. 5 Inhibition of the trypsin (A), chymotrypsin (B) and elastase (C) by *Lavatera cashmeriana* Camb. protease inhibitors. Biological activity of inhibitors was measured by the degree of % inhibition of enzymatic activity, using BAPNA, ATEE and N-succinyl-L-$(ala)_3$-p-nitroanalide as substrate for trypsin, chymotrypsin and elastase respectively.**
**Series 1 represent LC-pi I, series 2 LC-pi II, series 3 LC-pi III and series 4 LC-pi IV.**

### iii. pH optimum

The optimum pH for the maximum antitryptic activity of purified protease inhibitors was 7 for LC-pi I, 8 for LC-pi II, 7.6 for LC-pi III and 8.5 for LC-pi IV (Fig.6).

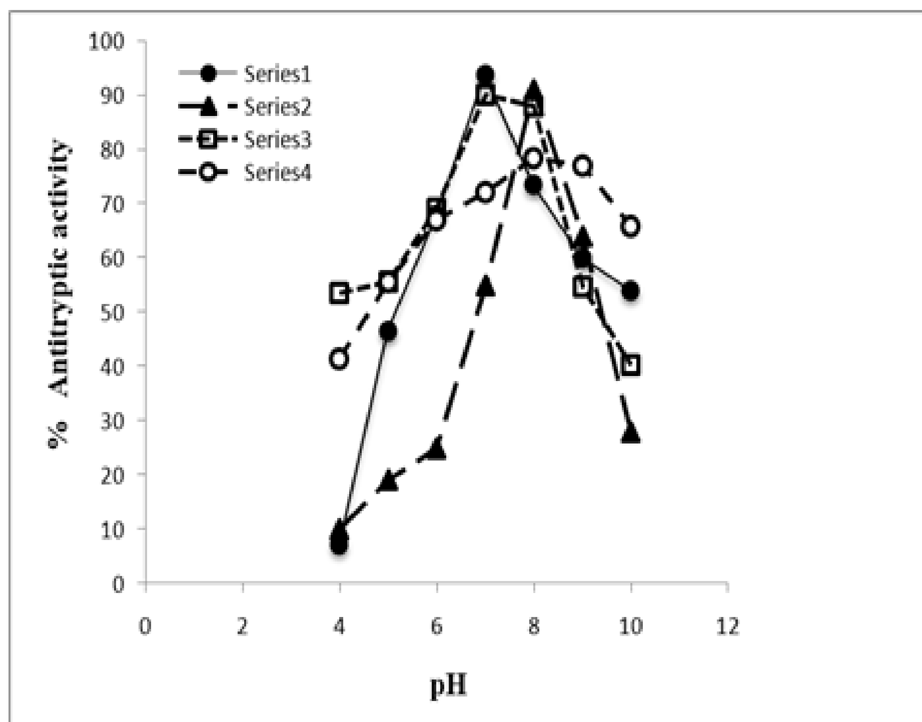



**Fig. 6 Effect of pH on activity of *Lavatera cashmerian* Camb. protease inhibitors.**
**Series 1 represent LC-pi I, series 2 LC-pi II, series 3 LC-pi III and series 4 LC-pi IV**

### iv. Effect of temperature on activity of protease inhibitors

The maximum antitryptic activity of the inhibitors was obtained at 30°C. The inhibitors showed a sharp decrease in activity afterward and were completely inactivated at 70°C (Fig.7).

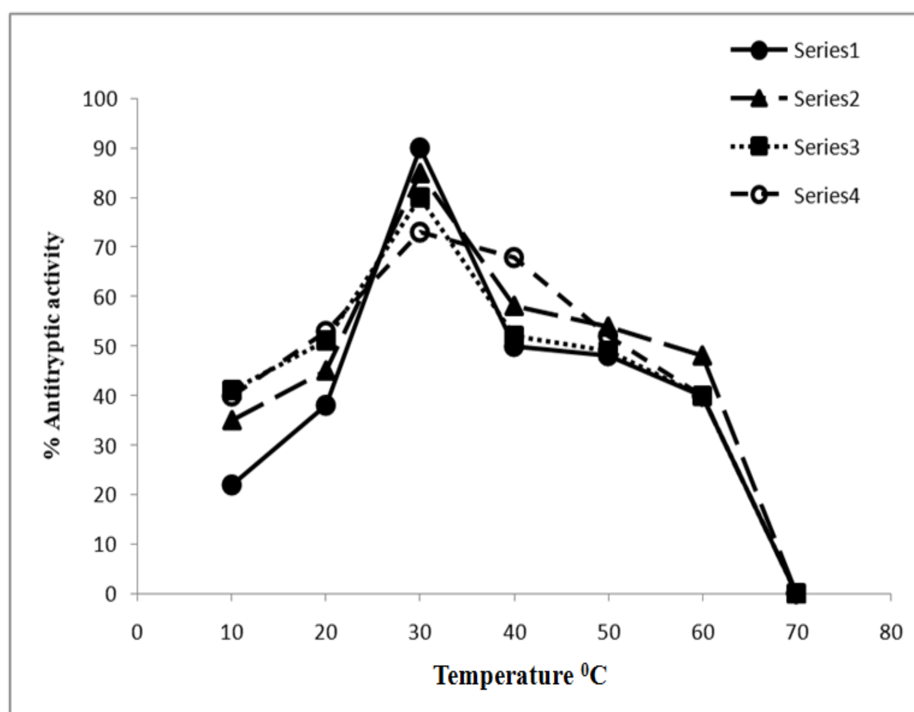



**Fig.7 Effect of temperature on the activity of protease inhibitors**
**Series 1, 2, 3, 4 represent LC-pi I, LC-pi II, LC-pi III and LC-pi IV respectively.**

### v. Carbohydrate Content

The inhibitors were found to be glycoproteins with LC-pi I containing 9.1µg sugar, LC-pi II 7.0µg sugar, LC-pi III 3.8µg and LC-pi IV 20µg of sugar per mg of protein (Table 2).

**Table 2: Carbohydrate content of protease inhibitors**

| Inhibitors | Carbohydrate content (µg/mg of protein) |
|---|---|
| **LC-pi I** | **9.1** |
| **LC-pi II** | **7.0** |
| **LC-pi III** | **3.8** |
| **LC-pi IV** | **20** |

**Analysis of carbohydrate was done by Dubois *et al.*, 1956.**

### vi. Growth inhibition by LC-pi I in human cancer cells

Growth inhibition of Prostate (PC-3) and Breast (MCF-7) cell line by LC-pi I at various concentrations *in-vitro* was determined. The inhibitor showed a dose-dependent inhibitory effect on cell growth. Negligible effect was seen on growth of colon (HCT-15) and Neuroblastoma (IMR-32) cells. The results are given in Table 3.

**Table 3: Cytotoxicity of purified LC-pi I**

| Tissue Type | | | Colon | Prostate | Breast | Neuroblastoma |
|---|---|---|---|---|---|---|
| **Cell line Type** | | | **HCT-15** | **PC-3** | **MCF-7** | **IMR-32** |
| **S. No** | **Code** | **Conc. (µg/ml)** | **% GROWTH INHIBITION** | | | |
| **1.** | **LC-pi I** | **500** | 38 | **89** | **72** | **52** |
| **2.** | **LC-pi I** | **300** | 34 | 32 | **54** | 47 |
| **3.** | **LC-pi I** | **100** | 26 | 11 | 41 | 30 |
| **4.** | **LC-pi I** | **50** | 5 | 5 | 36 | 23 |
| **5.** | **Paclitaxel** | **1µM** | 12 | 34 | 22 | 31 |
| **6.** | **Mitomycin C** | **1µM** | 16 | **76** | 26 | 37 |

**The bold values are shown for those compounds which have proved to be active and those in normal font represent least significant.**

# IV. DISCUSSION

Protease inhibitors were isolated from seeds of *Lavatera cashmeriana* Camb. which is endemic to Kashmir valley of India. It was found to be used by many Kashmiri people for some medicinal purposes. The crude protein separated into four major peaks on DEAE - cellulose column suggesting that four inhibitors exist in *Lavatera cashmeriana Camb.* seeds (LC-pi I, LC-pi II, LC-pi III and LC-pi IV). Similar results were reported by Ogata et al. [35] for okra (*Abelmoschus esculentus* L.) protease inhibitors. The purified inhibitors were homogenous with respect to size and charge as was evident from their gel filtration behavior and polyacrylamide gel electrophoresis in presence and absence of SDS. The molecular weight of the inhibitors determined by SDS-PAGE was about 6% lesser than molecular weights of inhibitors determined by gel filtration data. This is understandable because inhibitors being glycoproteins show anomalous behavior on SDS-PAGE gel [36]. SDS-PAGE also revealed that LC-pi I is composed of two subunits of 10 kDa each. The two-chain structure has been reported previously for 20 kDa Kunitz proteinase inhibitors found in seeds of *Adenanthera pavonina* [37], *Archidendron ellipticum* [38] and potato tubers [39]. Four proteinase inhibitors have also been purified and characterized from *Archidendron ellipticumone* with molecular weight of 20 kDa [38] and *Phaseolus vulgaris L.* [40]. The isolated protease inhibitors were strong inhibitors of elastase than trypsin and chymotrypsin. This is comparable with inhibitors isolated from jackfruit seeds were also fraction II and III inhibited elastase strongly than trypsin and chymotrypsin [41]. The optimum pH of four inhibitors LC-pi I, II, III and IV was found between 7, 8, 7.6 and 8.5 respectively, which is consistent with earlier reports [38, 42]. Singhal [43] reported that the trypsin inhibitor from mungbean seeds was active between pH 4.0 and 10.0. Annapurna [44] reported that the trypsin inhibitor from jack fruit seeds was stable over still a wider range of pH (3.0 to 12.0). In contrast, the trypsin inhibitor from pigeon pea seeds retained its full activity between pH 7.0 and 10.0, but when exposed to acidic pH from 3.0 to 5.0, 20% of the activity was lost [45]. Johnston et al. [46] observed that the midgut of lepidopteran larvae is highly alkaline and the digestive proteases have optimal activity between pH 10.0 and 11.0, while the larvae of phytophagous coleopteran have acidic conditions in their midgut region, with pH optima for digestive enzymes typically in the range 4.0-5.0. The optimum temperature was found to be 30˚C for all inhibitors. However the inhibitors were active up to 60˚C but were completely inactivated at 70˚C. Trypsin inhibitors from okra seeds were also reported to be stable up to 60˚C and rapidly inactivated at 80˚C.

The protease inhibitors isolated from seeds of *Lavatera cashmeriana* Camb. Which inhibited trypsin, chymotrypsin and elastase can be classified as a Kunitz type of inhibitor based on their molecular size. Kunitz-type protease inhibitors having different molecular masses have also been reported in soybean (19 kDa), mustard seeds (20 kDa) and *Cajanus cajan* (14 kDa) [47, 48]. The inhibitors were found to be glycoproteins. The presence of carbohydrates in plant protease inhibitor of other families has been reported earlier also [35, 40 and 41].

In summary, the potential anticancer activity of LC-pi I against these human cancer cell lines was investigated. LC-pi I exhibited a strong inhibitory effect on the proliferation of Prostate (PC-3) and Breast (MCF-7) cancer cell lines in *in vitro*. The anticancer activity of LC-pi I could be attributed in part to its inhibition of proliferation due to its protease inhibitor activity as LC-pi I is strong inhibitor of trypsin, chymotrypsin and elastase.


# ACKNOWLEDGEMENT

The author expresses gratitude to University of Kashmir, Srinagar for providing financial assistance and to the department of Biochemistry for the infrastructure.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