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A PROTEASE INHIBITOR FROM *MOMORDICA CHARANTIA* DIFFERENTIALLY INHIBITS TRYPSIN, ELASTASE AND CATHEPSIN G (SERINE PROTEASES) HAVING REGULATORY ROLE IN INFLAMMATION

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ABSTRACT

Serine proteases are known to have significant role in inflammation. Their dysregulation may result in various inflammatory diseases. They are released from activated leukocytes and mast cells. Effects of serine proteases are mediated by Protease-activated receptors (PARs). Studies have been carried out on serine proteases as therapeutic target for regulation of inflammation by inhibiting serine proteases. Prolonged usage of synthetic drugs shows various side effects. Thus, plants or natural compounds are being explored as an alternative with minimum side effects. The present study focuses on a serine protease inhibitor isolated from the fruit of *Momordica charantia* by ammonium sulphate precipitation method and further purified using gel filtration chromatography on sephacryl S-100. A 3.07 fold purification of serine protease inhibitor with a yield recovery of 12.9% was achieved. The molecular mass of the purified serine protease inhibitor was approximately 28kDa as determined by 12.5% SDS-PAGE. The purified serine protease inhibitor showed 100% trypsin, 91% cathepsin G and 55% elastase inhibition activity. The isolated inhibitor exhibits maximum inhibition activity at 25℃. Thus isolated inhibitor may have potential to be developed as therapeutic against inflammation.

Key words: Inflammation, Serine proteases, Serine protease inhibitor, *Momordica charantia*.

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INTRODUCTION

Inflammation is a non-specific response of the body's immune system towards harmful stimuli such as pathogens, damaged cells, etc. It becomes a natural defense mechanism of the body to fight against foreign particles, however, long-term inflammation can result in various diseases like rheumatoid arthritis, cardiovascular diseases, etc. (Ashley *et al*., 2012). Hence, it is important to control inflammation.

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A majority of immune cells like B-cells, T-cells, neutrophils and mast cells are involved in the process of inflammation (Mizutani *et al*., 1991). Investigations have shown that proteases are an important element of the inflammatory response by regulating the level of production of pro-inflammatory molecules like TNF-α, IL-6, etc. Thus, their main role is to activate cytokines and chemokines through different receptors in order to have a pro-inflammatory effect on the body (Ghayur *et al*., 1997).

Serine proteases are involved at different stages of inflammatory responses and have been shown to have significant role in maintaining a balance between pro-inflammatory and anti-inflammatory state (Sharony *et al*., 2010). One such example is of emphysema, in which deficiency of 1-protease inhibitor, a main inhibitor of

elastase enzyme (serine family) results in increased risk of early onset of the disease (Sharony *et al*., 2010).

Therefore, serine proteases have been studied as a therapeutic target in many inflammatory diseases (Granger *et al*., 2004). The long term usage of anti-inflammatory drugs results in adverse side effects which can further damage the human biological system. So there becomes a need for natural, safe, potent and less toxic anti-inflammatory drug (Verma, 2016).

The regulation of inflammation by plant extracts are a key focus now-a-days and few studies from past have shown the interest in this direction. Various phytochemical compounds have been studied and reported with their role as anti-inflammatory agents (AnilKumar, 2010). Plants with therapeutic properties have started to gain lot of attention as they are less toxic and multi-targeted activities can be possible (Kwatra *et al*., 2016).

Momordica charantia or bitter melon is one of such natural products which has been explored for its medicinal/therapeutic properties due to the presence of bioactive compounds (Grover *et al*., 2004). It has been used in treatment for various diseases including inflammatory diseases (Kwatra *et al*., 2016).

In the present study a serine protease inhibitor has been isolated from the fruit of *Momordica charantia* showing different levels of inhibitory activity towards trypsin, elastase and cathepsin G, the main proteases involved in inflammation (Sharony *et al*., 2010).

MATERIALS AND METHODS

Plant Material

Fresh fruit pulp without the seeds of *Momordica charantia* (Bitter melon) was collected and thoroughly washed with distilled water 2-3 times and used for extraction and purification of protease inhibitor.

Isolation and Purification of Protease Inhibitor

Preparation of Crude Extract

100g of fruit pulp of *Momordica charantia* was taken and washed twice with distilled water. It was then homogenized thoroughly in the presence of liquid nitrogen using 6X volumes (600ml) of extraction buffer (1mM Triton-X 100, 8mM beta-mercaptoethanol, 5mM sodium metabisulphite in 100mM Tris Cl pH 7.6).The homogenate was then centrifuged at 9000rpm for 10 minutes at 4°C. Clear supernatant was collected. Supernatant volume was measured and labeled as crude extract.

Ammonium Sulphate Precipitation

The crude supernatant was subjected to ammonium sulphate precipitation up to 0-30% and 30-65% saturation respectively. For 0-30% ammonium sulphate precipitation, calculated amount of ammonium sulphate salt was added to the crude supernatant in a pre-chilled beaker and mixed thoroughly on a magnetic stirrer at 4°C till the complete solubilization of the salt. Mixture was incubated at 4°C for 30 minutes and then centrifuged at 9000rpm, 4°C for 10 minutes. Pellet obtained was collected and resuspended in minimum volume of buffer (10mM Tris Cl, pH7.6).

The supernatant obtained was further subjected to 30-65% salt precipitation in the same way as mentioned above. Both 0-30% and 30-65% salt precipitated fractions were dialyzed overnight using dialysis buffer (10mM Tris Cl, pH 7.6) at 4°C. After dialysis samples were centrifuged at 9000rpm, 4°C for 10 minutes. Supernatants were collected and protein concentration was estimated following Bradford assay (Bradford, 1976) using BSA as standard.

Trypsin Inhibition Assay

Trypsin inhibition assay was performed by incubating 10µl (10µg) of trypsin with 10µg protein each of crude, 0-30% fraction and 30-65% fraction at 25°C for 15 minutes. These mixtures were then mixed with 3mL of substrate TAME (1mM) solution separately in quartz cuvette and mixed properly. Change in absorbance was measured at 247nm after every 60 seconds for 10 minutes. Decrease in trypsin activity was used to calculate the % trypsin inhibition shown by the samples. Trypsin activity without sample was used as control.

Purification of Trypsin Inhibitor

Ammonium sulphate fraction (0-30%) showing the maximum inhibitory activity was subjected to ultrafiltration using Amicon® ultra centrifugal filters 30 KDa (Merck) to fractionate proteins into less than and more than 30 kDa.

Gel Filtration Chromatography using Sephacryl S-100

Fraction containing less than 30kDa proteins showing the better inhibition was subjected to further fractionation on sephacryl S-100 gel permeation chromatography. After void volume, 2ml fractions were collected. Protein was estimated in collected fractions and fractions showing the presence of proteins were assayed for trypsin inhibition. Fractions showing trypsin inhibition activity were pooled as sephacryl fraction.

Purity of Protease Inhibitor

Native PAGE

The purity of sephacryl S-100 fraction showing trypsin inhibition activity was confirmed on 10% native PAGE. The gel was stained with coomassie brilliant blue (CBB) R-250 and then destained to visualize the protein bands.

SDS-PAGE

Same fraction was subjected to 12.5% SDS-PAGE using protein marker from BIO-RAD to determine the molecular weight and subunit structure of the isolated

trypsin inhibitor. Gel was stained with silver stain for visualization of protein bands.

Inhibition of Elastase and Cathepsin G by Purified Trypsin Inhibitor

Inhibition of Elastase

Isolated trypsin inhibitor was assessed for its potential to inhibit elastase by incubating 0.02 units of elastase (1mg/ml) with isolated protease inhibitor at 25°C for 15 minutes. This mixture was then mixed with 1mL of substrate N-succinyl-ala-ala-ala-p-nitroanilide solution (0.29mM) in quartz cuvette and mixed properly. Change in absorbance was measured at 410nm after every 60 seconds for 10 minutes (Bieth *et al*., 1974). Decrease in elastase activity was used to calculate % elastase inhibition.

Elastase activity without inhibitor served as control. Elastase activity was assessed by incubating 2µl (2µg) of enzyme elastase (1mg/ml) with 1ml of substrate solution in quartz cuvettes and mixed properly. Change in absorbance was measured at 410nm for every 60 seconds for 10 minutes. This was used as the control for elastase assay.

Inhibition of Cathepsin G

Isolated trypsin inhibitor was also checked for its ability to inhibit cathepsin G. 0.03 units of cathepsin G were incubated with isolated protease inhibitor at 25°C for 15 minutes. This mixture was then mixed with 1mL of substrate N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide solution (2.2mM) in quartz cuvette and mixed properly. Change in absorbance was measured at 410nm after every 60 seconds for 10 minutes (Bieth *et al*., 1974). Decrease in cathepsin G activity was used to calculate % cathepsin G inhibition. Cathepsin G activity without inhibitor was used as control.

Optimum Temperature of Inhibition of Various Serine Proteases

Inhibition of Trypsin

Purified protease inhibitor (10µg) was incubated with 10µl of trypsin (10µg) at different temperatures ranging from 4°C to 65°C for 15 minutes. This mixture was then mixed with TAME for inhibition assay. Change in absorbance was measured at 247nm after every 60 seconds for 10 minutes.

Inhibition of Elastase

Purified protease inhibitor (10µg) was incubated with elastase (0.02 units) at different temperatures ranging from 4°C to 65°C for 15 minutes. . This mixture was then mixed with substrate (N-succinyl-ala-ala-ala-p-nitroanilide) for inhibition assay. Change in absorbance was measured at 410nm after every 60 seconds for 10 minutes.

Characterization of Protease Inhibitor at Different pH

With Trypsin Enzyme

Purified protease inhibitor (10µg) was incubated with 10µl of 1mg/mL trypsin enzyme at different pH ranging from pH 2.3 to pH 10.6 for 15 minutes. This mixture was then subjected to normal trypsin inhibition assay as mentioned previously. Change in absorbance was measured at 247nm after every 60 seconds for 10 minutes.

With Elastase Enzyme

Purified protease inhibitor (10µg) was incubated with 10µl (0.02 units) of elastase enzyme at different pH ranging from pH 2.3 to pH 10.6 for 15 minutes. This mixture was then mixed with substrate (N-succinyl-ala-ala-ala-p-nitroanilide) for inhibition assay. Change in absorbance was measured at 410nm after every 60 seconds for 10 minutes.

RESULTS

Purification of Serine Protease Inhibitor from *Momordica charantia*

In order to isolate a plant based inhibitor against proteases involved in inflammation, eight plants were screened for

Fig.1. Trypsin inhibition activity shown by selected plants

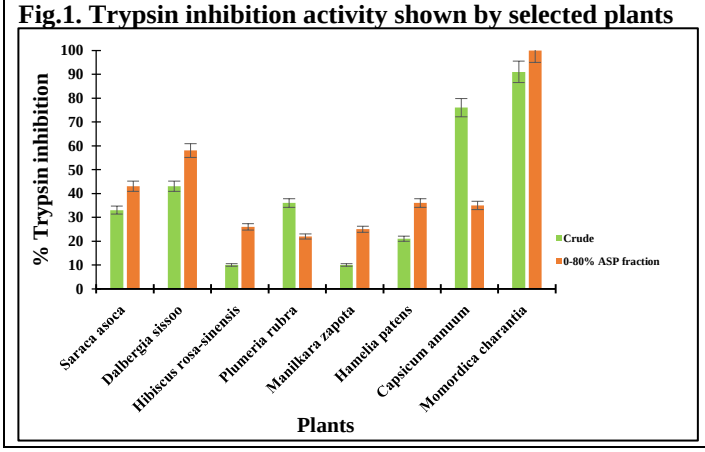


Fig.2. Trypsin inhibition activity shown by different fractions of *Momordica charantia* at 5µg of protein concentration

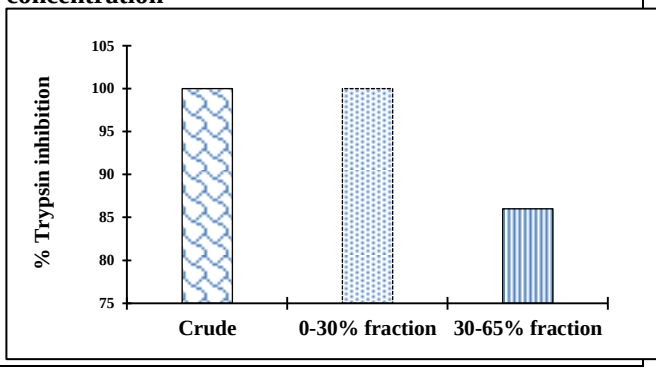


Fig.3. Fractionation of amicon ultra fraction consisting of proteins less than 30kDa on sephacryl S-100 gel permeation chromatography

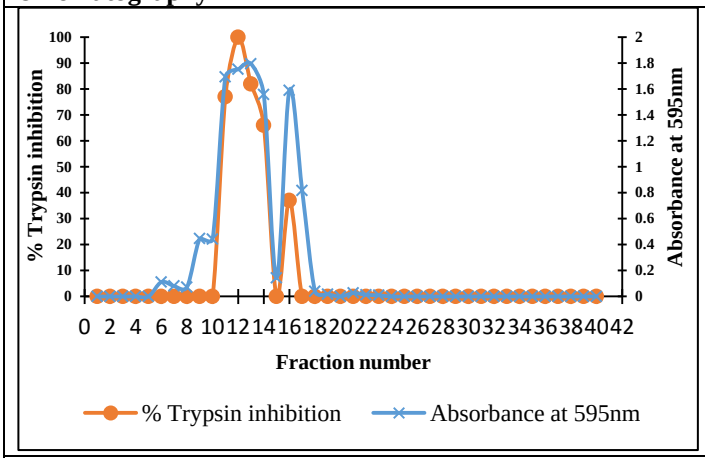


Fig.4. Native-PAGE (10%) of sephacryl S-100 fraction

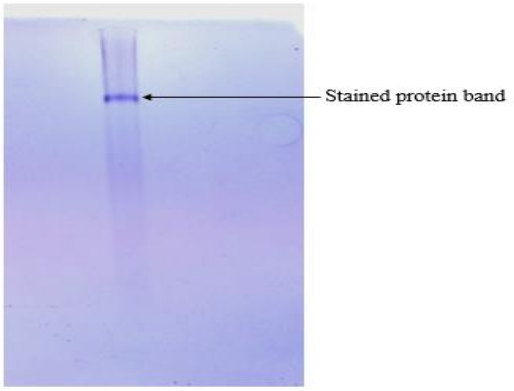


Fig.5. SDS-PAGE (12.5%) of purified protease inhibitor. Lane 1, protein marker and lane 2, purified protease inhibitor

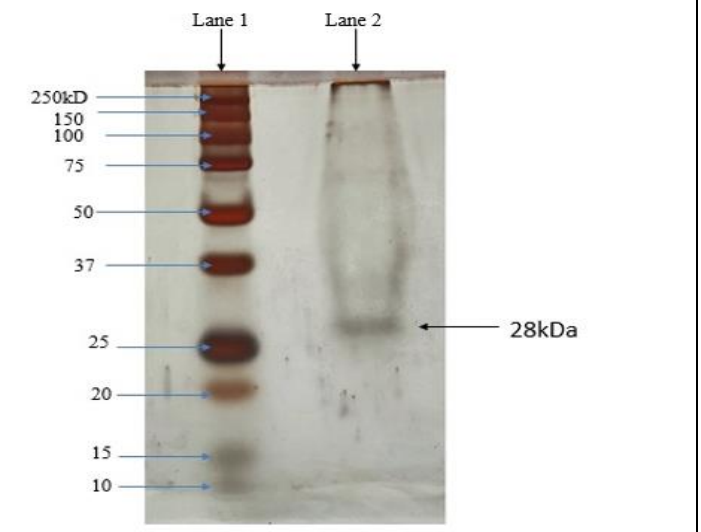


Fig.6 (a) Effect of temperature on trypsin inhibition activity of purified protease inhibitor

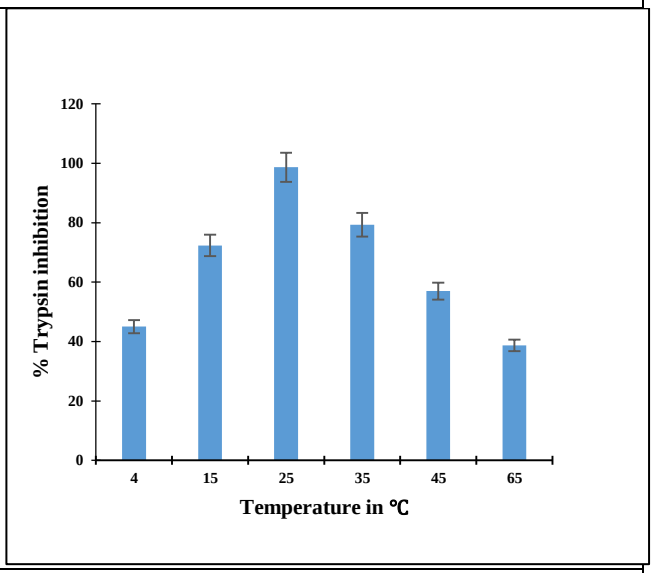


Fig.6 (b) Effect of temperature on elastase inhibition activity of purified protease inhibitor

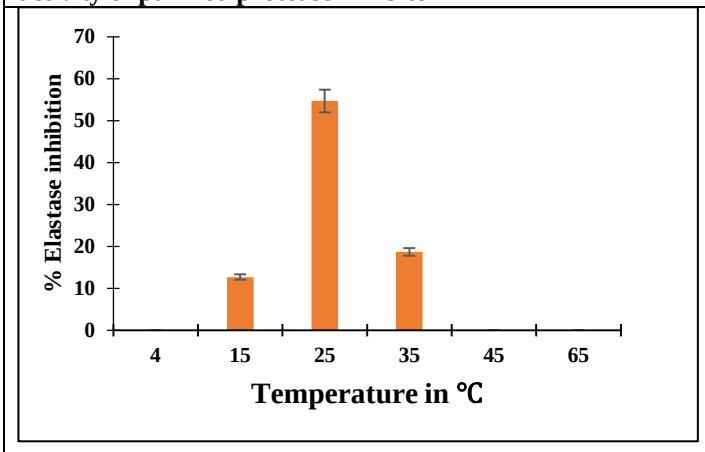


Fig.7 (a) Effect of pH on trypsin inhibition activity of purified protease inhibitor

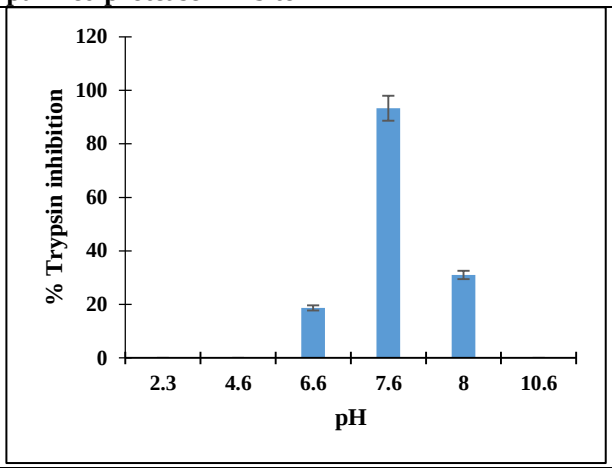


Fig.7 (b) Effect of pH on elastase inhibition activity of purified protease inhibitor

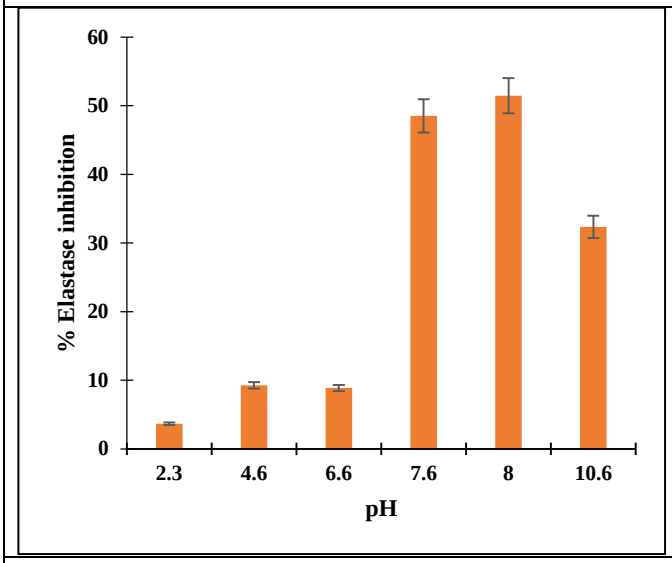


Fig.8. Inhibition of cathepsin G by the purified protease inhibitor. Line 1, cathepsin G activity and line 2, inhibition of cathepsin G activity

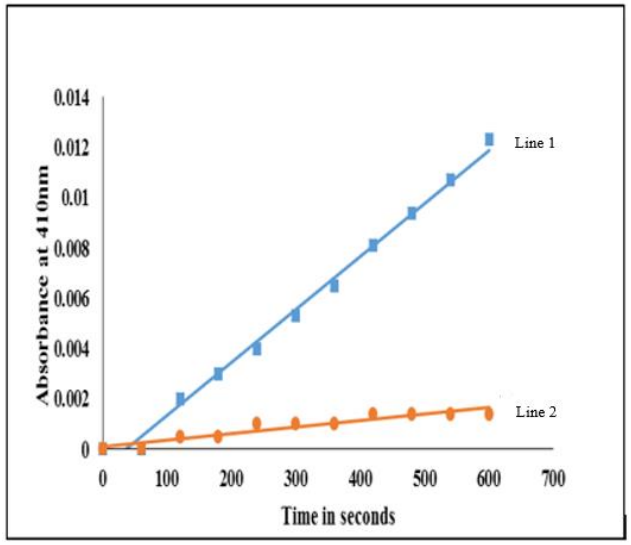
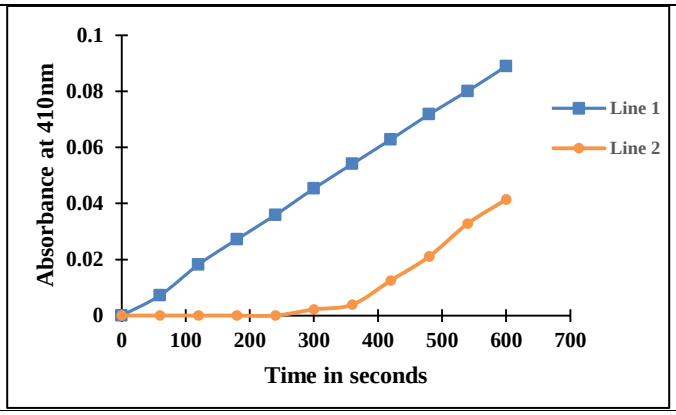


Fig. 9. Inhibition of elastase by the purified protease inhibitor. Line 1, elastase activity and line 2, inhibition of elastase activity



the presence of inhibitor using trypsin as representative serine protease. Different plants showed different levels of trypsin inhibition activity. Out of the screened plants, *Momordica charantia* showed maximum trypsin inhibition activity at crude and ammonium sulphate fraction level (Fig.1).

Hence, it was selected for purification of serine inhibitor. 100g of fruit pulp of *Momordica charantia* was homogenized to extract protease inhibitor. The total protein extracted was found to be 226.2 mg. The crude extract obtained was then subjected to ammonium sulphate precipitation fractionation to achieve 0-30% and 30-65% saturation. The 0-30 % salt precipitated fraction showed better trypsin inhibition activity as compared to 30-65% salt precipitated fraction at 5 µg protein concentration (Fig.2).

Ammonium sulphate fraction (0-30%) showing better trypsin inhibition activity was subjected to ultrafiltration using Amicon® ultra centrifugal filter of 30 KDa to separate proteins into larger than 30KDa and smaller than 30KDa fractions. Trypsin inhibition activity was assessed in both the fractions. Higher trypsin inhibitor activity was found in fraction containing proteins less than 30 KDa. Therefore this fraction was further fractionated on gel permeation chromatography using sephacryl S-100 for the purification of trypsin inhibitor (Fig.3). Fractions showing the maximum trypsin inhibition were pooled and assessed for homogeneity on native PAGE. A single protein stained band was observed thus indicating the homogeneous nature of trypsin inhibitor (Fig.4). It was observed that the GFC purified fraction had protein concentration of 0.95 mg/ml, 17.76 units/mg specific activity with 3.07 fold purification and 100% trypsin Inhibition.

The purified inhibitor was then subjected to 12.5% SDS-PAGE with the protein marker from Bio-Rad. The gel was stained with the silver stain. A single protein stained band was observed corresponding to molecular

weight of 28kDa (Fig.5). The purified serine protease inhibitor was found to be temperature sensitive. Maximum activity was observed at 25℃ which decreases as the temperature increases (Fig.6 (a) and 6(b)). Likewise, it showed maximum activity at pH 7.6 with trypsin and pH 8.0 with elastase (Fig.7 (a) and 7(b)) respectively.

Inhibition of Cathepsin G and Elastase

Inhibitory activity of the purified serine protease inhibitor was assessed for the inhibition of trypsin, elastase and cathepsin G, the proteases that are known to have significant role in inflammation. The purified serine protease inhibitor showed 91% cathepsin G (Fig.8) and 55% elastase inhibition (Fig.9). Thus it was seen that the purified serine protease inhibitor showed maximum inhibition activity for trypsin (100%), followed by cathepsin G (91%) and lastly for elastase (55%).

DISCUSSION

Inflammation is a well-known and important step in order to prevent the microbial invasion or injury to the tissues under different conditions (Ashley *et al*., 2012). It may be caused due to various reasons, including infectious agents, cytotoxic injury, etc. Upon tissue injury, a large number of immune cells with reactive oxygen species and serine proteases lead to the activation of inflammation cascade, as a defensive tool (Soualmia and Amri, 2018). However, chronic inflammation leads to a large number of diseases like rheumatoid arthritis, stroke, autoimmune disorders, etc. Cancer is another major disease condition which is linked to chronic inflammation (Medzhitov, 2010).

Apart from different cells like, cytokines and chemokines, involved in inflammation serine proteases are now being studied as a key element in order to modulate immune response (Soualmia and Amri, 2018). Role of serine proteases in inflammation regulation is being studied. Serine proteases have also been studied as important molecules in apoptosis and tissue remodeling (Sharony *et al*., 2010).

Upon inflammation, the first cells that enter the tissue are the polymorphonuclear cells (PMNs) (Korkmaz *et al*., 2010) and serine proteases like elastase and cathepsin G are present in these cells (Korkmaz *et al*., 2010). They regulate specific enzymes during the course of inflammation and are majorly seen as modulators of the inflammation mechanism as they regulate the production of chemokines and cytokines (Korkmaz *et al*., 2010). During the immune response IL-32, a pro-inflammatory cytokine is produced by NK cells and T-cells and is induced by serine proteases (Novick *et al*., 2006). One of the serine proteases, cathepsin G, triggers the production of IFN-γ and cleaves membrane-bound TNF-α (Mizutani *et al*., 1991) which again act as pro-inflammatory cytokines.

Serine proteases are also known to convert pro-inflammatory interleukins in their active forms (Ghayur *et al*., 1997) which further increases the risk of inflammatory diseases. These proteases mediated mechanisms proceeds via specific protease activated receptors (PARs) (Sharony *et al*., 2010). Hence, regulation of these proteases become important to regulate the inflammatory response.

With increasing side effects associated with the use of current anti-inflammatory agents, need for natural anti-inflammatory agent has been realized. Earlier plants have been shown to be used in the treatment of inflammation related disorders (Okoli *et al*., 2002). These include, *Aloe vera, Saraca asoca, Dalbergia sissoo, Hibiscus rosa-sinensis, Capsicum annuum, Manikara zapota, etc.* The presence of various active compounds and molecules in these plants make them efficient in order to regulate the inflammatory response cascade (Okoli *et al*., 2002). A protease inhibitor isolated from the pulp of *Momordica charantia* showed different levels of inhibition of main proteases involved in inflammation (Pham, 2006).

For thousands of years, bitter melon is being used in Ayurveda also. All the parts of the plant like seed, root, leaves and whole fruit have some or other pharmacological properties (Scartezzini and Speroni, 2000). Whole plant as food is used to treat T2DM (Grover and Yadav, 2004). In Africa, it is shown that bitter melon is used in treatment of worm infections, inflammation, syphilis and skin diseases (Bortolotti *et al*., 2019). The plant is known to have the highest nutritional value among all members of the Cucurbitaceae family. It is a good source of protein, carbohydrates, fiber, vitamins and minerals (Bortolotti *et al*., 2019).

Lifestyle and dietary habits are also responsible for chronic state of low inflammation, which further can alter the immune response of our body. Hence, dietary components play a major role in regulating the inflammation conditions and can also be very helpful when used in therapy (Minihane *et al*., 2015). Therefore dietary plants with potential role in inflammation may be a good agent as therapeutic. Different extracts of bitter melon have been shown to reduce the production of TNF-α. It also leads to decreased expression of different interleukins which are known as pro-inflammatory (Kwatra *et al*., 2016).

CONCLUSION

With the changing lifestyle, herbal medicines are preferred to synthetic drugs as they are natural and less toxic. In the present study, a protease inhibitor isolated from *Momordica charantia* has shown significant inhibition activity towards three major serine proteases i.e. trypsin, elastase and cathepsin G. These serine proteases are known to have pro-inflammatory effect. Hence, the serine protease inhibitor isolated from the fruit pulp of *Momordica charantia* can be a good source of

herbal medicine against inflammation. Further characterization of this serine protease inhibitor may help in designing and development of natural drug against inflammation with minimum or no side effects.

CONFLICTS OF INTEREST
None

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