ISOLATION, PURIFICATION, CHARACTERIZATION AND BIOLOGICAL SPECIFICITY OF SNTI

2.1 INTRODUCTION
From past century it is known that plant materials possess different types of protease inhibitors (Osborne et al., 1917). Physiological function for isolated PIs can be assessed for the proteins only with high purity. Hence, the crude samples which Once inhibitory activity is perceived in a crude sample, it is important to accomplish the purification. However, the presence of iso-inhibitors with different properties have been reported in some tissues (Joubert, 1982; Richardson, 1977; Vogel et al., 1968a). These multiple molecular forms often present difficulties in the preparation of the inhibitors. For comparative biochemical studies of these iso-inhibitors, pure preparations of the inhibitors are essential. Plant pigments also interfere with the purification procedure and complicate the isolation of protease inhibitors. In order to achieve the purified compound, the crude sample is treated with organic solvent or polyethylene glycol, followed by ammonium sulphate saturation and dialysis. Further purification techniques are employed to obtain a homogenous preparation of the inhibitor.

Most of the natural inhibitors of proteases are proteins with molecular size ranging from 3,000-80,000 Da. Kasymova and Yuldashev have developed a method of isolation slightly different from the most adopted method (Kasymova and Yuldashev, 1996). For isolating inhibitors from seeds different chromatography techniques are employed. The most pertinent method of protease inhibitor purification is the use of affinity ligands. In the absence of affinity purification, conventional purification procedures like ion-exchange chromatography, gel filtration chromatography, High Performance Liquid Chromatography (HPLC), Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC), Fast Protein Liquid Chromatography (FPLC),
Isoelectric focusing and preparative gel electrophoresis are used. To establish effective purification, it is important to determine the specific activity of the inhibitor sample in each step of purifications (Hideaki and Guy, 2001). In the last stages of purification Kasymova and Yuldashev have used gel filtration on Acrilex, which permits the elimination of pigments and substances of low molecular weight, while the use of affinity sorbents enabled the inhibitors to be obtained with a high degree of purity (Kasymova and Yuldashev, 1996).

The first purified inhibitor was obtained in 1945 from soybeans by Kunitz (Kunitz, 1945). Since then, several protease inhibitors from plant seeds such as Lima bean (Fraenkel-Conrat et al., 1952; Haynes and Feeney, 1967), soy bean (Frattali and Steiner, 1968; Rackis and Anderson, 1964; Yamamoto and Ikenaka, 1967), finger millet (Manjunath et al., 1983), Kidney bean (Jacob and Pattabiraman, 1986), Faba beans (Asao et al., 1991), gourd seeds (Hamato et al., 1995), ground nut seeds (Arachís hypogaea) (Tur-Sinal et al., 1972), Indian mustard (Brassica juncea), Sunflower seeds (Helíanthus annuus) (Tacon, 1997) and soybean (Dipietro and Liener, 1989) have been isolated in pure form. Trypsin inhibitors (TIs) were extracted and determined spectrophotometrically from seed meals of African bread fruit (Treculia africana), African walnuts (Coula edulis Bail), Calabash (Lagenaria sicerania) and Castor (Ricinus communis) (Uzogara et al., 1987). Isolation and characterization of protease inhibitors from cotton seeds was done by Kasymova et al., (Kasymova and Yuldashev, 1996).

Protease inhibitors isolated from kidney bean, Jack fruit seeds and soap nut seeds exhibited differences in their physico-chemical properties due to source variation (Annapurna et al., 1991; Bhat and Pattabiraman, 1989; Jacob and Pattabiraman, 1986; Kundu and Sinha, 1989; Pusztai, 1968; Rachel et al., 2012). A comprehensive scheme for the isolation of trypsin inhibitors and the agglutinin from soybean seeds was given by Arpad Pusztai, et al., in 1991 (Pusztai et al., 1991). Tania Maria Serigado Antunes gave a detailed account on the isolation and purification of a cystatin from rice (Maria and Antunes, 1998).
2.1.1 Biological Specificity of a protease inhibitor

Protease inhibitors play an important role in the protection of plant tissues from pest and pathogen attack by virtue of an anti-nutritional interaction. Numerous studies have been conducted focusing attention on the anti-nutritional value of protease inhibitors and on the adverse effects of these proteins on insects. A positive correlation existed between the plants with higher levels of trypsin and chymotrypsin inhibitors and their resistance towards insects and pathogens. Pathogens, on the contrary, developed resistance against chemical agents when exposed continuously. Together these agents might provide more protection to plants against invaders. Generation of a protein with protease inhibitory, antimicrobial and lectin activities would be advantageous to the host against pathogen attack. Serpins have been reported to possess antimicrobial (Kim et al., 2009) and anti-insecticidal activities (Macedo et al., 2011; Sudha, 1999).

Trypsin inhibitors from Chinese white cabbage (Brassica chinensis) seeds and Bottle Gourd (Lagenaria siceraria) seeds are reported to possess antibacterial activities (Muni Kumar and Siva Prasad, 2014; Ngai and Ng, 2004; Shee et al., 2009). A trypsin-chymotrypsin inhibitor from Broad Beans showed antifungal activity towards Mycosphaerella arachidicola, Fusarium oxysporum, and Botrytis cinerea (Ye et al., 2001). Protease inhibitor, Mungoin from mung bean (Phaseolus mungo) seeds and Potide G from potato tubers possessed both antifungal and antibacterial activities (Kim et al., 2006; Wang and Ng, 2006).

Soap nuts are reported to possess antimicrobial activity and the pericarp is known to be rich in secondary metabolites such as saponins, isoflavones, tannins, terpenoids etc. These are reported to have antimicrobial activities (Soetan et al., 2006; Vijaya et al., 2013). Soap nuts are used in cosmetics and detergents, among many other products. According to Rachel et al., Soap nut seeds (Sapindus trifoliatus) are rich in trypsin inhibitory activity and also possess antimicrobial activity (Rachel et al., 2012). In-order to gain more insight into the chemical nature, structure, specificity and mode of interaction with trypsin it is thought worthwhile to isolate and purify the trypsin inhibitor from the seeds of soap nuts. The present investigation was carried out to study the biological specificity of SNTI against bacterial, dermatophytes, insect and human gut proteases.
2.2 MATERIALS AND METHODS

2.2.1 Materials

Bovine pancreatic α-chymotrypsin, N-Acetyl-L-tyrosine ethyl ester (ATEE), dimethyl sulfoxide (DMSO), elastase, elastin congo red, pronase, papain, pepsin, thermolysin, α-amylase, protein ladder, α-chymotrypsin and trypsin from human pancreas and casein were procured from Hi-Media chemicals, India. CM- Cellulose was procured from Sigma-Aldrich, USA. Sephadex G-100 and G-150 were obtained from GE Healthcare Life Sciences, United Kingdom. Luria-Bertani broth, Agar, Dextrose, Neopeptone, actidione were procured from Hi-Media. All chemicals that are used for purification and for biological specificity are of analytical grade.

The bacterial strains Bacillus subtilis (MTCC 441), Staphylococcus aureus (MTCC 7443), Escherichia coli (MTCC 727), Klebsiella pneumonia (MTCC 9544), Proteus vulgaris (MTCC 742) and fungal species Malassezia fur fur (MTCC 1374), Trichophyton rubrum (MTCC 8477), Trichophyton mentagrophytes (MTCC 8476) and Microsporum gypseum (MTCC 4476) were procured from MTCC, Chandigarh, India.

Bacteria were sub-cultured in Luria-Bertani media and the fungal species in Sabouraud’s Dextrose media and Emmon’s modification sabouraud’s media.

2.2.2 Methodology

2.2.2.1 Sample Collection

Sapindus trifoliatus trees bearing soap nuts were selected from Annavaram, East Godavari District, Andhra Pradesh, India. Ripened fruits were collected from the selected trees and seeds were removed from the fruits and preserved for extraction of proteins. The endosperm collected after the removal of the hard seed coat was used for the isolation and purification of protease inhibitor.

2.2.1.2 Extraction of SNTI

The protease inhibitor was isolated and purified according to the methodology followed by Rachel et al., (Rachel et al., 2012). The endosperm was collected from the seeds after the removal of the hard coat and 25 g of the endosperm was homogenized with 200ml of 0.1M sodium phosphate buffer, pH 7.6 and then made up to 250ml with the same buffer. The extract was then centrifuged at 2500 rpm for
15min at 4°C and the supernatant was used for further steps. The supernatant obtained was treated with 50% ice cold acetone (1:5 v/v) and the resultant mixture was centrifuged at 2500 rpm for 15min at 4°C. The precipitate was then re-suspended in 0.1M sodium phosphate buffer, pH 7.6.

2.2.1.3 Purification of SNTI

Purification of the inhibitor is carried out by 50% Ammonium Sulfate precipitation and dialyzed against PB (Phosphate Buffer). Further purification is achieved by ion exchange and gel permeation chromatography. Purity is assessed by native PAGE.

2.2.1.3.1 Ammonium Sulfate precipitation

The supernatant obtained after acetone fractionation was subjected to Ammonium Sulfate precipitation. Solid Ammonium Sulfate was added gradually with constant stirring at 4°C to obtain 50% saturation. The mixture was allowed to stand overnight at 4°C.

2.2.1.3.2 Dialysis

The precipitate from 50% Ammonium Sulfate precipitation was collected by centrifugation at 2500rpm for 15min at 4°C, then dissolved in 0.1M sodium phosphate buffer pH 7.6 and dialyzed against the same buffer for 12hrs at 4°C. The dialysate was further purified by column chromatography for separation of the inhibitor protein from the mixture of molecules based on charge and size using CM-cellulose and Sephadex G-100 columns respectively.

2.2.1.3.3 Ion Exchange Chromatography

The dialyzed sample was loaded on a CM-Cellulose column (2x30cm) previously equilibrated with 0.1M sodium phosphate buffer pH 7.6. After washing with the equilibration buffer, stepwise elution was performed with increasing concentrations of 0.1M, 0.2M, 0.3M, 0.4M and 1.0 M NaCl and the respective fractions were collected. These fractions were monitored for protein by measuring their absorbance at 280nm and fractions of each peak are pooled. Each pooled fraction samples were tested for the inhibitory activity against trypsin.
2.2.1.3.4 Gel Filtration Chromatography
The method of Andrews (Andrews, 1964) was used to determine the molecular weight of the inhibitor by molecular sieve chromatography on Sephadex G-100. Sephadex G-100 was swollen in 0.1M Phosphate buffer, pH 7.6 and packed in a column (2×30cm). The pooled fraction exhibiting inhibitory activity was loaded on Sephadex G-100. The column was equilibrated and developed with the same buffer. The fractions were collected and the protein was monitored by measuring the absorbance at 280nm. The fractions from a single peak were pooled, dialyzed against phosphate buffer at 4°C and lyophilized.

2.2.1.3.5 High Performance Liquid Chromatography (HPLC)
The column fraction with SNTI activity was then separated by reverse-phase HPLC, as described by Macedo et al., on a C18 column (Shimadzu) that was previously equilibrated with water and >5% acetonitrile. The SNTI active fraction was finally purified by re-chromatography in a reverse phase HPLC with a flow rate of 1.0ml/min for 35min by isocratic elution. Proteins were monitored for absorbance at 280nm (Macedo et al., 2011).

2.2.1.3.6 Native PAGE
Polyacrylamide gel electrophoresis was carried out as described by Reisfeld et al., (Reisfeld et al., 1962) and followed by Fairbanks (Fairbanks et al., 1971). PAGE was carried out under non-denaturing condition using 12 % slab gels. About 50µg of protein was layered on the gel. After the electrophoretic run, proteins were fixed in 10% TCA. Proteins were visualized using coomassie brilliant blue according to the method of Fairbanks (Fairbanks et al., 1971).

2.2.1.4 Characterization of SNTI
2.2.1.4.1 Determination of Molecular weight
SDS Polyacrylamide gel electrophoresis was carried out using Phosphorylase-b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (22.1 kDa) and lysozyme (14.3 kDa) as standard proteins for calibration. The molecular weight of the inhibitor was determined by constructing a calibration curve.
2.2.1.4.2 Effect of pH on the stability of the inhibitor
Solutions of the inhibitor (1mgml⁻¹) in 10mM buffers of five different pH values (pH 3.0, glycine-HCl; 5.0, sodium citrate; 7.0, sodium phosphate; 9.0, Tris-HCl; 12.0, glycine-NaOH) were kept at 5°C for 24h. Aliquots of the inhibitor were diluted with 0.1M phosphate buffer, pH 7.6 and assayed for trypsin inhibitory activity (TIA).

2.2.1.4.3 Effect of temperature on the stability of the inhibitor
The inhibitor solutions (100µgml⁻¹) were separately incubated in a water bath at various temperatures for 10min and then quickly cooled in ice and appropriate aliquots were assayed for trypsin inhibitory activity (TIA).

2.2.1.4.4 Kinetic measurements
The amidolytic activity of trypsin (50µg) was determined with various concentrations of BAPNA (1.2-5.0µmol) in the absence of the inhibitor. The assays were then repeated in the presence of 5 and 15µg of the inhibitor. The Ki values were calculated from Lineweaver-Burk plots.

2.2.1.4.5 Competition experiments
50µg trypsin was separately incubated with 5, 10, 15 and 20µg of SNTI for 10min at 37°C in 1ml phosphate buffer, pH7.6. Suitable aliquots of all the samples were taken and assayed for residual trypsin activity using BAPNA as a substrate.

2.2.1.4.6 Studies on complex formation
The trypsin-SNTI complex was isolated by gel filtration on Sephadex G-100. To form the complex, 2mg of SNTI was incubated with 5mg of trypsin at 37°C for 15 min. Excess trypsin was used to make sure that all the inhibitor is complexed, such a mixture was applied onto a column of Sephadex G-100 at 5°C previously calibrated with SNTI and trypsin run separately and the absorbance was monitored at 280nm. Trypsin and Trypsin inhibitory activities are monitored in the fractions collected.

2.2.1.4.7 Fourier Transform Infra-Red Spectroscopy (FTIR)
The solid state FTIR spectra are recorded in the middle infrared (4000 cm⁻¹ to 400 cm⁻¹) on Perkin Elmer. The sample for FTIR analysis are prepared by grinding the dry blended powders of trypsin inhibitor with powdered KBr and then compressed to form discs.
2.2.1.5 Specificity of the inhibitor

2.2.1.5.1 Mechanistic group specificity of the inhibitor

The inhibition spectrum of SNTI was established by assaying the protease or esterase activity of the enzyme on an appropriate substrate and then incubating a fixed amount of the enzyme with various amounts of the inhibitor and assaying the residual enzyme activity.

2.2.1.5.1.1 Estimation of chymotrypsin activity

Chymotrypsin activity was estimated using casein and ATEE as the substrates. Chymotrypsin was dissolved in 1mM HCl containing 20mM CaCl₂ to obtain a concentration of 100μg per ml.

2.2.1.5.1.2 Caseinolytic activity

Caseinolytic activity of chymotrypsin was determined by the method of Sumathi and Pattabhiraman (Sumathi and Pattabhiraman, 1977). To 3ml of the chymotrypsin solution (containing up to 40μg of the enzyme) in 100mM sodium phosphate buffer, pH 7.6, 2ml of 1% casein solution in phosphate buffer, pH 7.6 was added. After incubation at 37°C for 10min, the reaction was stopped by addition of 5ml of 5% TCA. The precipitate was filtered after standing for one hour at room temperature and the absorbance of the filtrate was read at 280nm in Hitachi spectrophotometer against a blank prepared by adding casein solution to the incubation mixture after TCA.

2.2.1.5.1.3 Esterolytic activity

Esterolytic activity of chymotrypsin was assayed by the method of Prabhu and Pattabhiraman using ATEE as the substrate (Prabhu and Pattabhiraman, 1977). Chymotrypsin was dissolved in 1mM HCl containing 20mM CaCl₂ to obtain a concentration of 25μg per ml. The substrate solution was prepared by dissolving 45mg of ATEE in 2ml of methanol and the solution was made up to 20ml with 100mM sodium phosphate buffer, pH 7.5.

Aliquots of chymotrypsin solution containing 0.2-2.0μg of the enzyme in 1 ml of 100mM phosphate buffer, pH 7.5 was incubated with 1ml of ATEE solution at 37°C for 10 min and the reaction was stopped by addition of 5ml of ethyl acetate. 0.5ml of the aqueous layer was assayed for N-Acetyl-L-tyrosine by the method of Lowry et al., (Lowry et al., 1951).
2.2.1.5.1.4 Assay of chymotrypsin inhibitory activity

For the assay of chymotrypsin inhibitory activity, 30µg of chymotrypsin was pre-incubated at 37°C for 10min with aliquots of the inhibitor extract and then the residual enzyme activity was determined as described above. The decrease in the proteolytic/esterolytic activity of the enzyme was taken as an index of the inhibitory activity. Suitable controls were included to correct for the presence of endogenous protease activity.

Units: One chymotrypsin unit (CU) is arbitrarily defined as an increase in 0.01 absorbance unit at 280nm per 10ml reaction mixture as in the case of the caseinolytic method, and at 530nm under the assay conditions of the esterolytic method. One chymotrypsin inhibitory unit (CIU) is defined as the number of chymotrypsin units (CU) inhibited under the same assay conditions (Kakade et al., 1969).

2.2.1.5.1.5 Assay of elastase activity

Elastolytic activity of elastase was assayed according to the method of Naughton and Sanger using elastin Congo red as the substrate (Naughton and Sanger, 1961). The method is based on measuring the release of the dye from elastin congo red by elastase at 495nm.

Elastin congo red (2mg) was suspended in 1.8ml of 0.2M Tris-HCl buffer, pH 8.8 was pre-incubated at 37°C for 30 min. Then 0.2ml of the enzyme solution containing 50µg elastase was added and further incubated at 37°C for 30min. The reaction was terminated by adding 2ml of 0.5M phosphate buffer, pH 6.0. The solution was centrifuged and the absorbance of the supernatant was measured at 495nm against a blank prepared by adding enzyme solution to the incubation mixture after the addition of 0.5M phosphate buffer, pH 6.0.

For the assay of inhibitory activity, 25µg of the enzyme was pre-incubated with aliquots of the inhibitor extract and the residual enzyme activity was assayed as described above.

2.2.1.5.1.6 Assay of pronase

Amidolytic activity of pronase was estimated using BAPNA as the substrate (Kakade et al., 1969) by the same method described for the activity of trypsin.
For inhibitory activity the assay mixture contained varying amounts of the inhibitor and suitable aliquots of the enzyme in 2ml of water. After pre-incubation for 10min at 37°C, the residual enzyme activity was measured as described earlier.

2.2.1.5.1.7 Estimation of subtilisin activity
Caseinolytic activity of subtilisin was assayed according to the method of Vaidyanathan and Virupaksha (Vaidyanathan and Virupaksha, 1991). To 2ml of enzyme (10- 50µg) in 100mM phosphate buffer, pH 7.6 containing 0.15M NaCl (PBS), 2ml of 2% casein solution in PBS was added and incubated at 37°C for 10min and the reaction was stopped by addition of 3ml of 5%TCA. The precipitate was filtered after standing for one hour at room temperature and the absorbance of the filtrate was read at 280nm in Hitachi spectrophotometer against a blank prepared by adding casein solution to the incubation mixture after TCA. Inhibitory activity assay was carried out by incubating 50µg of enzyme with aliquots of the inhibitor extract prior to the addition of the substrate.

2.2.1.5.1.8 Estimation of papain activity
The proteolytic activity of papain was assayed using casein as the substrate (Arnon, 1970). The enzyme was activated with 50mM Cysteine and 20mM EDTA. 1ml of papain (50µg) in 100mM Tris-HCl buffer, pH 8.0 containing 20mM EDTA and 50mM Cysteine was added to 1ml of 1% casein solution in Tris-HCl buffer, pH 8.0 and incubated at 37°C for 10min. The reaction was terminated by adding 3ml of 5%TCA. The precipitate was filtered after standing for one hour at room temperature and the absorbance of the filtrate was read at 280nm in Hitachi spectrophotometer against a blank prepared by adding casein solution to the incubation mixture after TCA. Inhibitory activity assay was carried out by incubating 20µg of the enzyme with aliquots of the inhibitor extract prior to the addition of the substrate.

2.2.1.5.1.9 Estimation of pepsin activity
The activity of pepsin was estimated by the method of Anson using denatured hemoglobin as the substrate (Anson, 1938). 1ml of 1mM HCl containing 30µg of pepsin was incubated with 1ml of hemoglobin solution (20mg/ml in 0.06N HCl) at 37°C for 10min and the reaction was arrested by adding 2ml of 10%TCA. The
solution was filtered and the increase in absorbance over the controls at 280nm was taken as an index of proteolysis.

For the inhibitory activity assay, 25µg of pepsin was incubated with various aliquots of the inhibitor extract in 1ml of 1mM HCl for 10min before the addition of hemoglobin solution.

2.2.1.5.1.10 Assay of thermolysin activity
Caseinolytic activity of thermolysin was assayed according to the method of Matsubara (Matsubara, 1970).

Thermolysin solution - Thermolysin crystals (10mg) were suspended in 4ml of 0.01 M Tris-HCl buffer, pH 8.0 containing 0.01 M CaCl₂ in an ice-bath and dissolved in 0.2 N NaOH (1.0 -1.5ml). The pH of the solution was immediately adjusted to 8.0 with 0.2 N acetic acid (1.0 -1.5ml) and the solution was stored at -10°C until use.

Thermolysin solutions, appropriately diluted with 0.01M Tris-HCl buffer, pH 8.0, were incubated at 35°C with 1ml of 2% casein solution for 10min. The reaction was terminated by adding 2.0ml of 5% TCA, the precipitate was filtered after standing for 30min at room temperature and the absorbance of the filtrate was read at 280nm in Hitachi spectrophotometer against a blank. The blank was prepared by mixing the casein solution with TCA prior to the addition of the enzyme solution. For the assay of thermolysin inhibitory activity, 25µg of thermolysin was incubated with aliquots of the inhibitor extract for 10min and the residual thermolysin activity was assayed as described above.

2.2.1.5.1.11 Assay of protease activity
Trypsin-like protease activity was assayed in the seeds using BAPNA as the substrate. 10% seed extract was prepared in 50mM phosphate buffer, pH 7.6 and aliquots of the extract in 2ml of distilled water were incubated with 7ml of BAPNA solution at 37°C for 20min. The reaction was stopped by adding 1ml of 30% acetic acid. The absorbance of the solution was read at 410nm against a blank prepared by incubation of sample containing 2ml of water instead of the seed extract.
2.2.1.5.1.12 Assay of α-amylase activity
Pancreatic α-amylase was assayed by the method of Saunders and Lang (Saunders and Lang, 1973). 2ml buffer (20mM sodium diethyl barbiturate, pH 6.9 containing 50mM NaCl) containing 1-5µg of pancreatic α-amylase was incubated with 1ml of 1% starch solution for 10min at room temperature. Then 2ml of dinitrosalicylate reagent was added and the mixture was heated in a boiling water-bath for 10min. After cooling, the solution was made up to 20ml with water and the absorbance was measured at 540nm against a blank prepared by incubation of sample containing 2ml of water instead of the enzyme.

For the inhibitory activity assay, 0.1ml of the inhibitor extract was added to the buffer solution containing 5µg of pancreatic α-amylase. The mixture was made up to 2ml with buffer and incubated at room temperature for 30min. The residual α-amylase activity was assayed as described above.

Units: Units for elastase, pronase, subtilisin, papain, pepsin, thermolysin and α-amylase

One enzyme unit is defined as an increase in 0.01 absorbance units over the controls under assay conditions. One enzyme inhibitory unit is defined as the number of enzyme units inhibited under the same assay conditions.

2.2.1.5.2 Biological specificity of the inhibitor against dermatophytic microorganisms
2.2.1.5.2.1 Preparation of SNTI
SNTI (1mg) was dissolved in 1ml of 50mM sodium phosphate buffer, pH 7.6 and was used for studies on antimicrobial activity.

2.2.1.5.2.2 Preparation of Luria-Bertani medium
Luria-Bertani Broth (2g) and Agar (2g) are mixed and dissolved by heating in 100ml of distilled water. The pH was adjusted to 7.5 and sterilized by autoclaving at 121°C for 15min and poured into sterile petri plates. After setting, the media is preserved in refrigerator for use.
2.2.1.5.2.3 Sabouraud’s dextrose Agar medium
Dextrose (2g), neo-peptone (1g), agar (2g) are mixed and dissolved by heating in 100ml of distilled water. pH is adjusted to 5.6, sterilized by autoclaving at 121°C for 15mins and then actidione (10µg) was added. 10-20ml of medium was poured into Petri plates under sterile conditions.

2.2.1.5.2.4 Emmon’s Modification Sabouraud’s dextrose Media
Dextrose (4g), neo-peptone (1g), agar (2g) and butter (0.2g) or few drops of corn oil were mixed and dissolved by heating in 100ml of distilled water. pH is adjusted to 5.5, sterilized by autoclaving at 121°C for 15mins and then actidione (10µg) was added. Then 10 – 20ml of medium was poured into Petri plates under sterile conditions.

2.2.1.5.2.5 Antimicrobial activity by well diffusion method
The bacteria are grown in Luria agar at 37°C for overnight and fungi T. Mentagrophytes, T. rubrum and M. gypseum are cultured in Sabouraud’s Dextrose Agar at 25°C for 7days. And M. fur fur was cultured in Emmon’s modified Sabouraud’s Dextrose Agar at 30°C for 7days. The grown cultures were sub cultured in fresh Luria broth, Sabouraud’s Dextrose and Emmon’s modified Sabouraud’s Dextrose broth and grown to obtain log phase culture. The agar plates are prepared by pour plate method (Vardar-Ünlü et al., 2003).

The sterile medium was cooled at 45°C and 1ml of grown cultures are inoculated in their respective medium. The test organisms consist of 1x10⁶ cells/ml. Then, the culture was poured into sterile petri dishes and allowed to solidify. 3-6mm size wells are made with sterile borer and purified SNTI was added at different concentrations 5, 25 and 50µl respectively. The agar plates were incubated according to their respective temperatures and incubation time. Antibiotics Ampicillin for bacterial species and Flucanazole for fungal species served as standards. The diameters of zones of inhibition were measured in mm. And the results presented are the average values of triplicates.
2.2.1.5.3 Inhibition against commercially available insect larval gut and human gut proteases

The enzyme assay methods were suitably modified to study inhibitory activity of the protease inhibitor. The commercially available insect larval gut and human gut proteases are used to study SNTI specificity against these enzymes. The enzyme assay methodologies are same as mechanistic group specificity methodology. The inhibition spectrum of SNTI was established by assaying the protease or esterase activity of the enzyme on an appropriate substrate and then incubating a fixed amount of the enzyme with various amounts of the inhibitor and assaying the residual enzyme activity.

The activities of trypsin and pronase or their inhibition were assayed by the method of Kakade et al., using either BAPNA or casein as the substrate (Kakade et al., 1969). The inhibitory activity towards chymotrypsin was determined using casein or ATEE as the substrate. The proteolytic activity of papain was assayed using casein as substrate by the method of Arnon (Arnon, 1970). The Esterolytic activity of subtilisin was assayed using ATEE as the substrate. Thermolysin was assayed according to the method of Matsubara (Matsubara, 1970). The method of Saunders and Lang was employed for assaying pancreatic α –amylase (Saunders and Lang, 1973).
2.3 RESULTS

2.3.1 Extraction and Purification of SNTI

The Soap Nut Trypsin Inhibitor was isolated and purified from soap nut seeds (*Sapindus trifoliatus* L.) according to the procedure adopted by Rachel *et al.*, (Rachel *et al.*, 2012) and the results are shown in the Table 2.1.

Table 2.1: Summary of purification of soap nut seed protease inhibitor

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total Trypsin inhibitory activity (Units) TIU$\times10^3$</th>
<th>Specific activity TIA$\times10^2$ (Units/mg) protein</th>
<th>Yield %</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>250</td>
<td>4030</td>
<td>63.10</td>
<td>0.156</td>
<td>100.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Acetone fractionated extract</td>
<td>225</td>
<td>2644</td>
<td>54.81</td>
<td>0.207</td>
<td>86.60</td>
<td>1.33</td>
</tr>
<tr>
<td>Heat treated extract</td>
<td>200</td>
<td>2334</td>
<td>53.10</td>
<td>0.227</td>
<td>84.15</td>
<td>1.44</td>
</tr>
<tr>
<td>50% Ammonium sulphate</td>
<td>30</td>
<td>172.5</td>
<td>23.78</td>
<td>1.370</td>
<td>37.69</td>
<td>8.78</td>
</tr>
<tr>
<td>CM – Cellulose</td>
<td>27</td>
<td>112</td>
<td>14.74</td>
<td>1.440</td>
<td>23.36</td>
<td>9.23</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>12</td>
<td>52</td>
<td>13.20</td>
<td>2.540</td>
<td>20.92</td>
<td>16.28</td>
</tr>
</tbody>
</table>

TIU – Trypsin Inhibitory Units TIA - Trypsin Inhibitory Activity

Legend: Yield and fold purification were calculated on the basis of TIU and TIA respectively.

The extraction procedure was carried out maintaining physiological conditions and ice cold acetone was used to remove lipids. The endosperm was collected from the seeds after the removal of the hard seed coat and 25g of the endosperm was homogenized with 200ml of 0.1M sodium phosphate buffer, pH 7.6 and then made up to 250ml with the same buffer. 230ml of supernatant was obtained on centrifugation of the extract at 2500rpm for 15 min at 4 ºC. The supernatant (230ml) was treated with 50% ice cold acetone (1:5V) and the resultant mixture was centrifuged at 2500rpm for 15min at 4°C to remove lipids. The resultant defatted solution was subjected to ammonium sulphate precipitation.

To the supernatant (200ml) from acetone fractionation, solid ammonium sulphate (62.6g) was added gradually with constant stirring at 4°C to obtain 50% saturation. The mixture was allowed to stand overnight at 4°C. The precipitate was collected by
centrifugation at 2500rpm for 15min at 4°C, then dissolved in 30ml of 0.1M sodium phosphate buffer pH 7.6 and dialyzed against the same buffer. The dialysate was found to contain 172mg of protein.

Proteins have numerous functional groups that can have both positive and negative charges. Ion exchange chromatography separates proteins with regards to their net charge. If a protein has a net positive charge at pH 7, then it will bind to a column of negatively charged beads, whereas a negatively charged protein would not. By changing the pH so that the net charge on the protein is negative, it too will be eluted. The dialyzed sample (172mg) was loaded on a CM-Cellulose column (2×80cm) previously equilibrated with 0.1M sodium phosphate buffer pH 7.6.

After washing with 250ml of the equilibration buffer, stepwise elution was performed with 200ml each of 0.1, 0.2, 0.3, 0.4 and 1.0 M NaCl in 0.1 M phosphate buffer pH 7.6. Fractions of 5ml were collected at a flow rate of 60 ml per hour. These fractions were assayed for protein by measuring their absorbance at 280nm as well as the inhibitory activity against trypsin using BAPNA as the substrate. The elution profile of CM-Cellulose chromatography for the inhibitor is shown in figure - 2.1. The fractions 42-48 found to contain trypsin inhibitory activity. Fractions 42-48 were pooled and dialyzed against distilled water at 4°C and lyophilized. The protein yield from ion exchange chromatography was 112 mg.
Figure 2.1 shows the following
- 172 mg of the ammonium sulphate fractionated sample (0-50%) was applied on to the column (2x80cm) in 0.1 M sodium phosphate buffer (pH 5.8) and the adsorbed proteins were eluted with stepwise gradient in the buffer.
- Fractions of 5 ml were collected at a flow rate of 60 ml per hour. The protein was monitored by absorbance at 280 nm.
- When the elution was done with a stepwise gradient of 0.1 to 1.0M NaCl.

The sample from ion exchange chromatography (110mg) was dissolved in 0.1 M phosphate buffer pH 7.6 and was loaded on Sephadex G-100 column (1.8×30cm) which was previously equilibrated with 0.1M phosphate buffer, pH 7.6. The inhibitor was eluted with the same buffer. 2 ml fractions were collected at a flow rate of 12ml per hour and the protein was monitored by measuring the absorbance at 280nm. The trypsin inhibitory activity of the fractions was assayed using BAPNA as the substrate. The elution profile of the gel permeation chromatography is shown in figure - 2.2. A single protein peak with corresponding trypsin inhibitory activity was observed.
Fractions 8-12 containing the trypsin inhibitory activity were pooled, dialyzed against distilled water at 4°C and lyophilized. The yield of protein after gel permeation chromatography was 52mg. This preparation was stored at 0°C. The preparation thus stored, showed full activity even after three months. By this procedure about 52mg of the inhibitor was obtained and the final yield was about 20.9%.

Figure 2.2 shows the following
- 110mg of lyophilized preparation was applied to the Sephadex G-100 column 2x80cm in 0.1M phosphate buffer pH 7.6 and eluted with the same buffer.
- 2ml fractions were collected at a flow rate of 12ml per hour.
- The protein was monitored at 280nm. Protease inhibitory activity was determined using BAPNA as the substrate.
SNTI was analyzed using reverse phase HPLC to confirm its purity. HPLC analysis of SNTI revealed a major portion of peak with 2.141/45796 mAU with shoulder 2.232/46390 mAU at high intensity of 17.459 mAU/mV (Figure – 2.3).

**Figure 2.3: HPLC Chromatogram of SNTI**

Figure 2.3 shows the following

- HPLC elution profile of SNTI monitored at 290nm.
- A major peak with 2.141/45796 mAU with shoulder 2.232/46390 mAU.

The methodology followed in the purification procedure resulted in high purification with a 20.92 % yield. A sharp band was obtained on 12 % slab gel at pH 8.3 signifying the homogeneity of the purified SNTI (Figure 2.4). SNTI did not respond to PAS (Periodic Acid Schiff’s) stain suggesting it to be a non-glycoprotein.
Figure 2.4: Polyacrylamide Gel Electrophoresis of SNTI

Figure 2.4 shows the following

- 1 – Crude Extract;
- 2 – Acetone Fractionated
- 3 – Dialysate form Ammonium Sulphate precipitation
- 4 – Sephadex Purified Sample

2.3.2 Characterization of SNTI

Figure 2.5a shows the protein band pattern of the inhibitor on 12% SDS slab gels when stained with coomassie brilliant blue. Silver staining of SNTI showed a sharper band on SDS-PAGE. From the plot of distance migrated in cm versus log molecular weight for standard proteins (Figure 2.5b), the inhibitor showed a molecular weight of 29kDa.
Figure 2.5: (A) SDS – PAGE

Figure 2.5 (A) shows the following

- Direction of migration is from top (cathode) to bottom (anode)
- 1. Molecular weight markers: Phosphorylase b (97.4 kDa), Bovine serum albumin (66kDa), Ovalbumin (43kDa), Carbonic anhydrase (29kDa), Lysozyme (14.3kDa)
- 2. Purified SNTI (coomassie brilliant blue stained)
- 3. Purified SNTI (silver stained)
*1 to 3 were kept at 100°C for 3min with SDS and β-mercaptoethanol

Figure 2.5: (B) Molecular weight determination of SNTI by SDS PAGE on 12% slab gel

Figure 2.5 (B) shows the following

- Plot of distance migrated against log molecular weight of standard proteins BSA-66KDa; Ovalbumin - 43KDa; Carbonic anhydrase - 29KDa; Lysozyme - 14.3KDa
When subjected to Gel filtration on Sephadex G-150, SNTI eluted out as a single protein with a corresponding activity peak (Figure 2.6a). The plot of elution volume versus log molecular weight of the calibrating proteins is shown in Figure 2.6b. The molecular weight of SNTI calculated from the plot was 28.5 kDa.

![Absorbance at 280 nm vs. Elution volume (ml)](image)

**Figure 2.6: (A) Gel filtration of SNTI on Sephadex G-150**

Figure 2.6 (A) shows the following
- Elution profile of SNTI on a calibrated column of Sephadex G-150.
- 10 mg of purified SNTI was applied to the column in phosphate buffer pH 7.6 containing 20mM NaCl and eluted with same buffer.
- Fractions of 4 ml each were collected at flow rate of 12 ml per hour. Protein was monitored at 280 nm.
Figure 2.6 (B) Molecular weight determination of SNTI by gel filtration on Sephadex G-150

Enzyme inhibition studies were carried out to identify the specificity of the inhibitor towards the mechanistic classes of proteases. SNTI was tested for its inhibitory capacity against bovine trypsin using both BAPNA and casein as the substrates. The inhibition patterns of the amidolytic activity of bovine trypsin by SNTI were linear up to 80% inhibition (Figure 2.7). On extrapolation, it was found that 12µg of the inhibitor can totally inhibit amidase activity of 30µg of trypsin. The activity of the SNTI against chymotrypsin, elastase and pronase (Streptomyces griseus protease) subtilisin, papain, pepsin, thermolysin and α-amylase was tested. Except pronase, the rest of the enzymes were not affected by SNTI. The serine proteases trypsin and pronase were inhibited by SNTI. Majority of plant protease inhibitors isolated so far have been found to be specific for serine proteases and there are some reports of these inhibitors inhibiting other classes of proteases. SNTI specifically inhibited serine proteases trypsin and pronase and it has no effect on thiol, acidic, metalloproteases and α-amylase.
Figure 2.7 shows the following:

- 30µg of trypsin was incubated with varying amounts of SNTI for 10min at 37°C.
- The percentage residual enzyme activity was assayed using BAPNA as the substrate.
- The concentration of the inhibitor required to cause 50% inhibition of the enzyme activity was determined from the graph.

### 2.3.3 Reverse zymography

Substrate containing SDS-PAGE enables visualization of trypsin inhibitor. The inhibitory activity produced by SNTI detected using trypsin and gelatin substrate in the gel is shown in Figure 2.8. SNTI showed a single inhibitory band specific to trypsin when subjected to electrophoresis.
Figure 2.8: Detection of protease inhibitor using substrate-containing SDS PAGE

Figure 2.8 shows the following
- Lane 1 - Crude fraction
- Lane 2 - Ammonium Sulphate dialysate
- Lane 3 - CM-Cellulose sample
- Lane 4 - Sephadex purified sample

2.3.4 Mode of inhibition of Trypsin

Trypsin activity in the presence and absence of SNTI was measured at different substrate concentrations. The double reciprocal plot of the kinetic data is shown in Figure 2.9. In the presence of inhibitor, there was a decrease in the $V_{\text{max}}$ and the curves met on the X-axis at a point equivalent to $-1/ \text{km}$. The mode of inhibition of trypsin by SNTI was non-competitive. The $K_i$ value of trypsin for SNTI calculated from Dixon plot was $0.75 + 0.05 \times 10^{-10}$ M.
Figure 2.9: Mode of inhibition of trypsin by SNTI Line weaver – Burk plot

Figure 2.9 shows the following

- Inhibition of amidolytic activity of trypsin by SNTI was done by incubating 30µg of trypsin and BAPNA solution (0.8 to 5.0µ mole) with reaction system containing 2.5 to 7.5 µg of SNTI.

2.3.5 Complex Studies

SNTI was treated with excess trypsin and the mixture was pre-incubated at 37°C for 15min. This mixture when applied onto a column of Sephadex G-150 at 5°C gave rise to two distinct peaks at 280nm (Figure 2.10). Peak-I had an elution volume of 20ml and is higher for free SNTI i.e. 35ml. The binary complex of trypsin - SNTI did not show any trypsin activity or trypsin inhibitory activity. The molecular weight calculated for trypsin - SNTI complex on Peak-I based on the calibration curve for standard proteins (Figure 4b) gave a value of 68.9 kDa. This would mean a mole/mole interaction of SNTI with trypsin. Peak-II was small and represented uncomplexed SNTI with corresponding trypsin inhibitory activity. The trypsin left over after the enzyme inhibitor complex formation, was eluted out as peak-III with a corresponding elution volume of 51 ml.
Figure 2.10: Elution patterns of SNTI, Trypsin and Trypsin-SNTI complex on Sephadex G-100 column

Figure 2.10 shows the following

- (---) Absorbance, (o--o) Protease inhibitory activity, (x--x) Absence of activity
- Elution patterns of free trypsin
- Elution patterns of trypsin - SNTI complex
- Elution patterns of free trypsin, free SNTI & trypsin - SNTI complex on Sephadex G – 100.
- Protein was monitored at 280 nm.

2.3.6 Fourier Transform Infra-Red Spectroscopy (FTIR)

IR spectroscopic studies elucidate functional groups in a molecule. The IR peak at 3399 (broad) and 2939 cm\(^{-1}\) can be assigned to OH of carboxylic group and asymmetric CH\(_3\) stretching. The over ton peak is observed at 2074 cm\(^{-1}\). The peaks at 1642 and 1423 cm\(^{-1}\) could be due to amide C=O (CONH\(_2\)) and CH\(_3\) bending vibrations. The peaks observed at 995 and 925 could be attributed to OH bending vibrations. The presence of amide and carboxylic groups are confirmed from the above peaks (Figure 2.11).
Figure 2.11: Fourier Transform Infra-Red Spectroscopy

Figure 2.11 shows the following

- FTIR peaks representing presence of amide and carboxylic groups
2.3.7 Inhibition against Bacteria

The purified SNTI inhibited the growth of various bacteria and fungi. SNTI showed inhibition against *Escherichia coli*, *Bacillus subtilis*, *Klebsiella pneumonia*, *Proteus vulgaris* and *Staphylococcus aureus*. SNTI exhibited a maximum zone of inhibition of 21mm against *S. aureus* followed by *B. subtilis* (20mm), *P. vulgaris* (17mm), *E. coli* (16mm) and *K. pneumonia* (15mm).

**Table 2.2: Inhibitory activity of SNTI against bacteria**

<table>
<thead>
<tr>
<th>Concentration of SNTI</th>
<th>Zone of inhibition in mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli</td>
</tr>
<tr>
<td>5µg</td>
<td>10mm</td>
</tr>
<tr>
<td>25µg</td>
<td>14mm</td>
</tr>
<tr>
<td>50µg</td>
<td>16mm</td>
</tr>
</tbody>
</table>

Figure 2.12 shows the following

- With increase in concentration of SNTI size of zone of inhibition were increased.
- SNTI has shown highest inhibition against *S. aureus*
2.3.8 Antifungal activity

SNTI showed antifungal activity against the dermatophytic fungi like *Malassezia furfur*, *Trichophyton rubrum* *T. Mentagrophytes* and *M. gypseum*. SNTI showed inhibition against both the fungi and exhibited a zone of inhibition of 16mm against *M. furfur* and 14mm for *T. rubrum*. The study revealed that purified SNTI was effective against fungi implicated in causing dandruff.

Table 2.3 Inhibitory activity of SNTI against dermatophytic fungi

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Concentrations of SNTI</th>
<th>Concentrations of Standard synthetic antifungal drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 µg</td>
<td>25µg</td>
</tr>
<tr>
<td><em>M. furfur</em></td>
<td>11mm</td>
<td>19mm</td>
</tr>
<tr>
<td><em>T. rubrum</em></td>
<td>9mm</td>
<td>15mm</td>
</tr>
<tr>
<td><em>T. Mentagrophytes</em></td>
<td>6mm</td>
<td>10mm</td>
</tr>
<tr>
<td><em>M. gypseum</em></td>
<td>4mm</td>
<td>7mm</td>
</tr>
</tbody>
</table>

Figure 2.13 Inhibitory activity of SNTI against dermatophytic fungi

Figure 2.12 shows the following

- With increase in concentration of SNTI size of zone of inhibition were increased.
- SNTI has shown highest inhibition against *M. furfur*
SNTI exhibited antimicrobial activity against various bacterial strains and dermatophytic fungi. The study revealed that SNTI was a better antifungal agent compared to standard synthetic antifungal drugs. Further changes in hyphal morphology are to be checked by electron microscopic studies.

2.3.9 Inhibition of SNTI against commercially available insect larval gut and human gut proteases

The inhibitory activity of PMSF and SNTI at varying concentrations was carried out against commercially available larval gut and human gut proteases at 40°C for 10min, casein served as substrate. Decrease in enzymatic activity was considered as index of inhibitory activity (Kakade et al., 1969). The inhibitory activity of SNTI against insect larval proteases was comparatively higher than that of standard PMSF. SNTI at 100µg concentration has shown 83% inhibition against *H. armigera* and 75.65% against *S. frugiperada*. The inhibitory activity of SNTI against human gut protease was comparatively low compared to PMSF. At 60µg of SNTI the maximum inhibition was observed, whereas for PMSF the maximum inhibition was at 40µg. 100% of inhibition was not achieved with both SNTI and PMSF. Further, interaction studies and the inhibitory potential against different biological protease sources are carried out using *in silico and in vitro* approaches.
2.4 DISCUSSION

The observation that the trypsin inhibitory activity in the crude extracts of the seeds is stable at 70°C for 10min has led to the use of this treatment as the first step in the purification of the inhibitor. This step also helps in the removal of the endogenous proteolytic activity present in the seed extracts (Kanamori et al., 1974). About 50% of the proteins present in the crude extract were removed by this step. When ammonium sulphate fraction was subjected to CM-cellulose column chromatography, protease inhibitory activity was found to be associated with the protein eluted from the column by 0.3M NaCl. SNTI has been found to be homogeneous by the criteria of native PAGE and gel filtration. Most serine proteinase inhibitors from seeds have been isolated and characterized from the families Leguminosae, Cucurbitaceae, Solanaceae and Graminae. However, there are few reports on the purification and characterization of these inhibitors from other plant families, such as Rutaceae and Euphorbiaceae (Macedo et al., 2011). No protein has previously been characterized from Sapindus trifoliatus.

HPLC analysis revealed major portion of peak 2.141/45796 mAU with shoulder 2.232/46390 mAU represents SNTI. Protease inhibitors have been separated by several gel electrophoretic methods. Anionic inhibitors have been examined by the Davis method (Davis, 1964), cationic inhibitors by the Reisfeld method (Reisfeld et al., 1962) and neutral inhibitors by Weber and Osborn (Weber and Osborn, 1969) and Laemmli (Laemmli, 1970) methods. While these methods have proved useful for establishing the purity and determining the molecular weight of proteins, they cannot be used to distinguish iso-inhibitors or compare the activity of the particular inhibitor against different proteolytic enzymes. SNTI in all the fractions during its purification showed a single inhibitory band specific to trypsin on gelatin PAGE. A single inhibitory band also signifies the absence of isoforms which are common in many sources (Richardson, 1977; Robert and Wong, 1986; Vogel et al., 1968a). The molecular weight of SNTI as determined by SDS-PAGE was 29kDa. This is close to the value 28.5kDa obtained for the inhibitor by gel filtration on Sephadex G-100.

Some protease inhibitors have exhibited anomalous behavior on Sephadex gel columns due to the existence of oligomeric forms of inhibitors arising from monomer– dimer – trimer equilibrium (Gatehouse et al., 1980; Gennis and Cantor,
The subunit nature of SNTI has been analyzed by the SDS – PAGE technique. The inhibitor showed a single sharp band on SDS – PAGE when stained with silver supporting the monomeric nature of the protein. Further SNTI did not give positive result with PAS stain suggesting it free from carbohydrate moieties. Most of the trypsin inhibitors are non-glycoproteins. Papain inhibitor from potato tubers is a glycoprotein with a molecular weight of 80 kDa (Rodis and Hoff, 1984). Shakuntala first identified a glycoprotein trypsin inhibitor from Jack fruit seeds and to possess lectin activity (Shakuntala, 1996). The unusual stability of protease inhibitors, in general, is their most remarkable property. SNTI showed similarities to other protease inhibitors from soy bean (Edelhoch and Steiner, 1963) in their stability. The low Cysteine content (Rachel et al., 2012) in these inhibitors negates the possibility of the stability of the inhibitors rendered due to extensive intra-peptide cross-linking. However, the unusual stability of the inhibitor may be due to strong hydrophobic interactions forming an inner core in the protein.

The result of the investigation of the inhibitory specificity of SNTI has shown it to be a serpin and is strongly active against bovine trypsin and porcine elastase. SNTI was ineffective against other proteases such as papain (thiol), pepsin (carboxyl) and thermolysin (metallo). Majority of plant protease inhibitors isolated so far have been found to be specific for serine proteases (Chiche et al., 2004). However, there are reports of plant protease inhibitors inhibiting other classes of proteases. The trypsin/chymotrypsin inhibitor from broad beans inhibits the sulphhydrlyl enzyme papain (Warsy et al., 1974). Serine protease inhibitors such as barley subtilisin inhibitor (Mundy et al., 1983) and wheat germ protease K inhibitor (Zemke et al., 1991) are found to be active against α–amylases. The human LEKTI has 15 domains and inhibits plasmin, trypsin, elastase, subtilisin A and cathepsin G (Mitsudo et al., 2003).

As regards the mechanism of action, SNTI has shown a non-competitive type of inhibition. Although a few like soybean trypsin inhibitor has shown the competitive type of inhibition, the majority of the inhibitors follows non-competitive inhibition kinetics (Vogel et al., 1968a). Jack fruit seed protease inhibitor isolated by Annapurna et al., (Annapurna and Prasad, 1991) also showed non-competitive enzyme inhibition but the one isolated by Bhat exhibited uncompetitive inhibition (Bhat and
Pattabiraman, 1989). The Ki value of SNTI was found to be $0.75 \pm 0.05 \times 10^{-10}$ M. The low Ki value indicates high affinity of SNTI towards trypsin. The formation of stable trypsin inhibitor complex has been demonstrated by Sephadex G-100 gel filtration studies. The results obtained suggest that the inhibitor binds to trypsin in a 1:1 molar ratio. SNTI is a monoheaded inhibitor with a site for trypsin. Double-headed inhibitors with overlapping or non-overlapping binding sites are reported from plant sources (Annapurna and Prasad, 1991; Shakuntala, 1996).

The fruits of *Sapindus trifoliatus* are found to exhibit remarkable medicinal properties. Seed extract was found to be effective in inhibiting standard strains of bacteria and dermatophytic fungi. Plant protease inhibitors are known to play an important role in the innate host defense mechanisms in living organisms. They are also known to possess effective antibiotic activity against bacteria, fungi, and even certain viruses.