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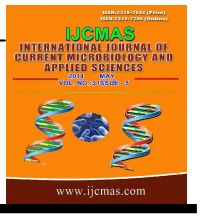

Original Research Article

Antimicrobial activity of a trypsin inhibitor from the seeds of *Abelmoschus moschatus* .L

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Keywords

Trypsin inhibitor, *Abelmoschus moschatus*, antibacterial, antifungal, protein purification.

A B S T R A C T

A novel trypsin inhibitor (AMTI-II), with both antibacterial and antifungal activities, was purified to homogeneity following conventional methods of protein purification from the seeds of *Abelmoschus moschatus*. The trypsin inhibitor has been found to be homogenous by the criteria of native PAGE and gel filtration and its molecular weight was determined to be 21.2 kDa by SDS-PAGE and Sephadex G-200 gel filtration. AMTI-II exerted strong inhibition towards bovine pancreatic trypsin and it showed moderate inhibition towards elastase. The inhibitor was found to be stable under conditions of extreme of pH (3.0 to 12.0), at high temperatures and in the presence of denaturing agents, urea and SDS and it was devoid of free thiol groups. AMTI-II exhibited potent antibacterial activity towards *Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Bacillus subtilis, Streptococcus pneumoniae, Bacillus cereus* and it was moderately active against *Klebsiella pneumoniae, Pseudomonas aeruginosa, Pseudomonas syringae and Streptococcus pyogenes.* The trypsin inhibitor also moderately affected the growth of fungal species, *Candida albicans, Candida tropicalis, Asperigillus flavus, Saccharomyces cerevisiae, Candida glabrata and Asperigellus niger.* Results obtained in the present study suggest that AMTI-II may serve as an antimicrobial agent active against pathogenic microbes.

Introduction

Protease inhibitors, a class of proteins which reversibly, stoichiometrically and competitively inhibit the catalytic activity of proteolytic enzymes, are widespread in plants, animals and microorganisms. They are abundant in the reproductive and storage organs and vegetative tissues of most plant families (Ryan, 1990).

These proteins, apart from regulating proteolytic processes, are also involved in defense mechanisms against insects and other pathogenic microorganisms (Kim et al., 2006). A positive correlation existed between plants with higher levels of trypsin and chymotrypsin inhibitors and their resistance towards pathogens. Several

studies on protease inhibitors were published with the aim of investigating enzyme mechanisms of controlling disease and pathological processes using genes encoding protease inhibitors (Brogden, 2005).

In recent years, appearance of new mutant strains of microorganisms resistant to commonly used antibiotics have stimulated a systematic analysis of natural products for bactericidal and fungicidal properties having therapeutic applications. Recently, protease inhibitors have also been recognized as potential drugs for controlling retroviral infections.

Many phytopathogenic bacteria and fungi are known to produce extracellular proteinases (Kalashnikova et al., 2003) which may play an active role in the development of diseases caused by them (Sara and Heale, 1990). In response to such attack by proteinases, plants synthesize inhibitory polypeptides that can suppress the enzyme activities. This phenomenon was first recorded in tomatoes infected with *Phytophthora infestans* (Woloshuk, 1991). In this case, increased levels of trypsin and chymotrypsin inhibitors correlated with the plant resistance to the pathogen.

Some of the serpins, cystatins, pepstatins and metallo protease inhibitors have been reported to possess antimicrobial activities (Kim et al., 2009). Trypsin inhibitors from the seeds of chinese white cabbage and bottle gourd are reported to possess antibacterial activities (Ngai and Ng, 2004; Shee Chandan et al., 2009). Double-headed inhibitors from broad beans and potato tubers showed antifungal activity (Ye, et al., 2001; Kim et al., 2005). Proteinase inhibitors, Mungoin from mung bean and Potide G from potato tubers, exhibited both antifungal and antibacterial activities (Wang et al., 2006; Kim et al., 2006).

Proteinase inhibitors have also been studied as model systems for elucidating proteinase inhibition mechanisms, as well as protein - protein associations (Oliva and Sampaio, 2009). In pharmacological and medical fields, investigations have been made into the potential of these inhibitors as therapeutic agents in the treatment of wide range of disorders associated with enhanced proteolytic activities like pancreatitis, shock, allergy, inflammation etc. (Richardson, 1977). They also find application in HIV therapy (Martin et al., 1995) and cancer (Kim et al., 2009).

Abelmoschus moschatus (L.) Medic, family *Malvaceae*, is an aromatic and medicinal plant popularly known as Mushkdana / Kasturi bhendi. The seeds are rich in protease inhibitors and they are used to check excessive thirst, cure for stomatitis, dyspepsia, urinary discharge, gonorrhea, leucoderma and itchiness.
Not much work has been done on protease inhibitors from these seeds including their influence on the growth of bacterial and fungal strains. This paper, therefore, deals with the isolation, purification of a trypsin inhibitor from the seeds of *Abelmoschus moschatus* and to examine its effects on the growth of selected bacterial and fungal strains.

Materials and Methods

Source

Abelmoschus moschatus plants bearing pods of uniform size were selected in and around Visakhapatnam district. Pods were collected at the ripening stage and seeds removed from the pods were used for the

isolation and purification of trypsin inhibitor.

Chemicals

Bovine pancreatic trypsin (1 x crystallized, DCC-treated, type XI), bovine serum albumin (BSA), porcine pancreatic elastase type I, chymotrypsinogen A, ovalbumin, lysozyme, catalase, phosphorylase b, soybean trypsin inhibitor (type I-S) were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

N-acetyl-DL-phenylalanyl-β-naphthylester (APNE), α-N-benzoyl-DL-arginine-p-nitroanilide HCl (BAPNA), blue dextran, DEAE-cellulose, N, N-dimethylsulfoxide, N,N'-methylene bis acrylamide, sodium dodecyl sulfate (SDS) were also from Sigma Chemical company, St. Louis, Missouri, U.S.A.

Sephadex G-100 and Sephadex G-200 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. 2-mercaptoethanol was purchased from Fluka, Switzerland.

Guanidine hydrochloride and trichloro acetic acid (TCA) were purchased from Riedel, Germany. Acrylamide was purchased from J.T. Baker Chemical Company, Phillipsburg, N.J., U.S.A. N, N, N', N' – tetramethylene 1,2 diaminoethane (TEMED) was purchased from B.D.H. Chemical Ltd., Poole, England. Casein was purchased from E.Merck, Darmstadt, Germany.

Nutrient agar, Peptone, Beef extract, Agar-agar, Potato dextrose agar (PDA) were purchased from Himedia Pvt Ltd, Mumbai, India. All other chemicals used were of analytical grade.

Purification of *Abelmoschus moschatus* Trypsin Inhibitor (AATI-II)

A procedure has been established for the purification of proteinase inhibitor from the seeds of *Abelmoschus moschatus*. 25 g of the seeds were homogenized with 150 ml of 0.1 M sodium phosphate buffer, pH 7.6 and then made up to 250 ml with the same buffer. The extract was then centrifuged at 5,600 rpm for 15 min at 4°C. The supernatant (250 ml) was dialyzed against the buffer for 24h in the cold and rapidly heated to 70°C and maintained at this temperature for 10 min. The extract was quickly cooled in ice and then centrifuged at 5,600 rpm for 15 min at 4°C. To the supernatant, solid ammonium sulfate was added to 60% saturation with constant stirring at 4°C. The mixture was kept overnight at 4°C. The precipitate was collected by centrifugation at 3,000 rpm for 10 min at 4-6°C, dissolved in 0.1 M sodium phosphate buffer, pH 7.6 and dialyzed against the same buffer. The dialyzed sample was loaded on a DEAE-cellulose column (2.2 x 34 cm) and the elution was performed with 0.1- 1.0 M NaCl in the buffer. Fractions of 8 ml were collected at a flow rate of 60 ml/h and were assayed for protein by measuring their absorbance at 280 nm as well as the inhibitory activity against trypsin using BAPNA as the substrate.

Protein from the previous step was loaded on Sephadex G-100 column (1.9 x 63 cm) and eluted with the same buffer. Fractions (2 ml) were collected at a flow rate of 12 ml/ h and the protein was monitored by measuring the absorbance at 280 nm. The trypsin inhibitory activities of the fractions were assayed using BAPNA as the substrate. Fractions containing the trypsin inhibitory activities were pooled, dialyzed

against distilled water at 4-6°C and then lyophilized.

Protein estimation

Protein was estimated by the method of Lowry et al., (1951) using bovine serum albumin as the standard.

Determination of molecular weight

Molecular weight of the inhibitor was determined by SDS-PAGE using the method of Laemmli (1970) and also by gel filtration on Sephadex G-200 column.

Measurement of Trypsin and Trypsin Inhibitory Activity

The inhibition of trypsin activity by the inhibitor was established by first assaying the proteinase 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. Trypsin activity was assayed by the method of Kakade et al., (1969) using BAPNA as the substrate. Trypsin (30μg) in 2 ml water was incubated with 7 ml of substrate solution at 37°C for 10 min. The reaction was stopped by adding 1 ml of 30%(v/v) acetic acid. The absorbance of the solution was measured at 410 nm against an incubated blank containing 2 ml of water instead of trypsin solution.

To determine the inhibitory activities, suitable aliquots of the inhibitor solutions were included in the assay medium to obtain 30-70% inhibition. One enzyme unit is defined as an increase in 0.01 absorbance unit at 410 nm for trypsin under the assay conditions. One enzyme inhibitory unit is defined as the number of enzyme units inhibited under these conditions.

Assay of other proteases

Elastase was assayed using elastin congo red as the substrate by the method of Naughton and Sanger (1961), *Staphylococcus aureus* protease and *Aspergillus oryzae* protease activities were assayed using casein as the substrate following the methods of Drapeau (1976) and Birk (1976) respectively.

Effect of Protein denaturing conditions/agents

Temperature

Three ml samples of 100 μg/ml solution of AMTI-II in 0.1 M sodium phosphate buffer, pH 7.6, were separately incubated in a water bath at different temperatures for 10 min. After the heat treatment, the solutions were quickly cooled in ice and appropriate aliquots were used for the assay of inhibitory activity against trypsin using BAPNA as the substrate.

pH

In order to determine the pH stability of the inhibitor, 1 mg/ml solution of AMTI-II in an appropriate buffer (10 mM) was kept at 5°C for 24 h. The buffers used were 10 mM glycine-HCl (pH 3.0), sodium citrate (pH 5.0), sodium phosphate (pH 7.0), Tris-HCl (pH 9.0) and glycine-NaOH (pH 12.0). Aliquots of the inhibitor were diluted with phosphate buffer, pH 7.6 and assayed as described above for trypsin inhibitory activities using BAPNA as the substrate.

Urea

To determine the stability of AMTI-II in 8 M urea, 1 μg/ml solution of the inhibitor was prepared in 0.1 M sodium phosphate buffer, pH 7.6 containing 8 M urea, and

was incubated 5°C for 24 h. The solutions were diluted to 1 M in urea with 0.1 M phosphate buffer, pH 7.6 and the appropriate aliquot of this diluted solution was used for the assay of trypsin inhibitory activity. The control assay mixture had the same amount of urea as was present in the diluted inhibitor solution.

Sodium dodecyl sulfate

One mg per ml solution of the inhibitor was prepared in 0.1M sodium phosphate buffer containing 1% SDS and was kept at room temperature for 24 h. The solution was dialyzed extensively against the buffer and aliquots were used for the assay of trypsin inhibitory activity using BAPNA as the substrate.

Guanidine hydrochloride

To determine the stability of the inhibitor in 6M guanidine hydrochloride, 1 mg/ml solution of the inhibitor was prepared in 0.1 M sodium phosphate buffer, pH 7.6 containing 6M guanidine hydrochloride and was incubated at room temperature for 24 h. The solution was diluted to 1 M in guanidine hydrochloride with the phosphate buffer and the appropriate aliquot of this diluted solution was used for the assay of trypsin inhibitory activity. The control assay mixture had the same amount of guanidine hydrochloride as was present in the diluted inhibitor solution.

Test organisms

The microbial strains, *Bacillus subtilis* (MTCC 121), *Bacillus cereus* (MTCC 430), *Escherichia coli* (MTCC 118), *Proteus vulgaris* (MTCC 426), *Staphylococcus aureus* (MTCC 96), *Klebsiella pneumoniae* (MTCC 2405), *Streptococcus pneumoniae* (MTCC 2672), *Streptococcus pyogenes* (MTCC 1923), *Pseudomonas aeruginosa* (MTCC 424), *Pseudomonas syringae* (MTCC 1604), *Asperigellus niger* (MTCC 2723), *Asperigillus flavus* (MTCC 4633), *Fusarium oxysporum* (MTCC 1755), *Alternaria alternata* (MTCC 1362), *Candida albicans* (MTCC 227), *Candida glabrata* (MTCC 3016), *Candida tropicalis* (MTCC 184), *Mucor indicus* (MTCC 6333), *Penicillium chrysogenum* (MTCC 161) and *Saccharomyces cerevisiae* (MTCC 2918) were collected from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh.

Determination of antimicrobial activity

Active cultures were generated by inoculating a loopful of culture in separate 100 ml nutrient/potato dextrose broths and incubating on a shaker at 37^{o}C overnight. The cells were harvested by centrifuging at 4000 rpm for 5 min, washed with normal saline, spun at 4000 rpm for 5 min again and diluted in normal saline to obtain 5 x 10^{5} cfu/ml.

Antibacterial activity

AMTI-II was subjected to antibacterial assay using the agar well diffusion method of Murray et al., (1995) as modified by Olurinola (1996).

Nutrient agar (20 ml) was dispensed into sterile universal bottles, these were then inoculated with 0.2 ml of cultures, mixed gently and poured into sterile petri dishes. After setting, a number 3-cup borer (6mm diameter) was properly sterilized by flaming and used to make four uniform wells in each petri dish. The wells were filled with buffer containing 25 µg - 100

µg of inhibitor and allowed for diffusion of the inhibitors for 45 min. The plates were incubated at 37°C for 24 h for bacteria. Rifampicin, Benzyl Penicillin and Tetracycline were included in the positive control. The inhibition zones were measured with antibiotic zone scale in mm and the experiment was carried out in triplicates.

Antifungal activity

The trypsin inhibitor was also subjected to antifungal assay using the agar well diffusion method of Perez *et al.,* 1990.The cultures of 48 h old grown on potato dextrose agar (PDA) were used for inoculation of fungal strains on PDA plates. An aliquot (0.2 ml) of inoculum was introduced to molten PDA and poured into a petri dish by pour plate technique. After solidification, the appropriate wells were made and they were filled with the buffer containing 50 - 100 µg of inhibitor and allowed for diffusion of the inhibitor for 45 min. The plates were incubated at 25°C for 48 h. The fungicides, Flucanazole and Ketoconazole replaced the inhibitor in the positive control. The zones of inhibition were measured as described earlier.

Minimum inhibitory concentration (MIC) assays

Minimum inhibitory concentrations (MIC) of AMTI-II was determined according to the method of Elizabeth (1999). A series of two fold dilution of each inhibitor, ranging from 50-2000 µg/ml, was prepared. After sterilization, the medium was inoculated with the aliquots of culture containing approximately 5×10^5 CFU/ml of each organism of 24 h slant culture in aseptic condition and transferred into sterile 6 inch diameter petri dishes and allowed to set at room temperature for about 10 min and then kept in a refrigerator for 30 min. After the media was solidified, wells were made and different concentrations of inhibitor ranging from 50-2000 µg/ml were added to the wells of each petri dish. The blank plates were without inhibitors. Inhibition of the growth of the organism in the plates containing inhibitor was judged by comparison with the growth in the control plates. The MICs were determined as the lowest concentration of the AMTI-II inhibiting visible growth of each organism on the agar plate.

MICs for fungal strains were also determined in a similar manner by using spores/cultures and incubating for 48 h.

Results and Discussion

Isolation and purification

The major trypsin inhibitor from *Abelmoschus moschatus* seeds was purified by ammonium sulphate fractionation and chromatography on DEAE-cellulose and Sephadex G-100. By employing DEAE- cellulose chromatography, four protein peaks have been resolved by linear NaCl gradient (01M – 1M NaCl in buffer). Protein eluted with 0.1 M NaCl on DEAE-cellulose column showed higher antitryptic activity and seems to be the major potent trypsin inhibitor obtained when compared to the other protein peaks. The inhibitor was designated as *Abelmoschus moschatus* trypsin inhibitor, AMTI-II, in the order of their elution from DEAE-cellulose column. When the lyophilized active fractions were subjected to Sephadex G-100 column chromatography, the inhibitor eluted out as a single peak with corresponding trypsin inhibitory activity (Fig. 1).

Recoveries and relative purification at each step for a typical purification from 25 g seeds are shown in Table 1. By this procedure, about 52.4 mg of the inhibitor was obtained with a final yield of about 17%.

The molecular weight of AMTI-II as determined by SDS-PAGE (Fig-2) was found to be 21.2 kDa which was close to that obtained with gel filtration on Sephadex G-200 (Fig-3). The inhibitor gave a single sharp band on SDS-PAGE even in the presence of 2-mercaptoethanol supporting the monomeric nature of the protein.

The inhibitory specificity of the inhibitor was tested against various serine proteases of bacterial, fungal and mammalian origin. AMTI-II was strongly active against bovine pancreatic trypsin with an IC 50 value of 7.5 µg. It, however, moderately inhibited porcine elastase with an IC50 value of 20.1 µg. *Staphylocccus aureus* protease and *Aspergillus oryzae* protease were weakly inhibited by AMTI-II.

The purified inhibitor (AMTI-II) was quite stable up to 80°C for 10 min. When the incubation was for 10 min at 90^0C, there was about 25% loss of its trypsin inhibitory activity. Further incubation of the inhibitor for 20 min at 90°C resulted in the loss of 40% trypsin inhibitory activity. When kept in a boiling water bath, there was a 70% loss of its trypsin inhibitory activity in 10 min and 85% loss in 20 min. Incubation of inhibitor for 30 min at boiling temperature caused the total loss of trypsin inhibitory activity (Table-2).
Table -3 shows that trypsin inhibitory activity of AMTI-II was not affected at alkaline as well as acidic conditions under the conditions tested. However, when kept at room temperature with 6 M guanidine hydrochloride for 24 h, AMTI-II had lost 35% of trypsin inhibitory activity.

AMTI-II was devoid of free thiol groups and was found to be stable in the presence of denaturing agents, urea and SDS.

Antimicrobial activity

Antimicrobial assay of the purified trypsin inhibitor was examined against various bacterial and fungal strains by assessing the minimum inhibitory concentrations and the microbicidal effect of AMTI-II was further visualized as inhibition zone by treating the test organisms with inhibitor and then spreading the cells on agar plates. AMTI-II strongly affected the growth of *Staphylococcus aureus* followed by *Escherichia coli, Proteus vulgaris, Bacillus subtilis, Streptococcus pneumoniae, Bacillus cereus* and with zones of inhibition recorded as 28mm, 27mm, 26mm, 25mm, 25mm and 24mm at a concentration of 50 µg of the inhibitor. On the other hand, the growth of *Klebsiella pneumoniae, Pseudomonas aeruginosa, Pseudomonas syringae and Streptococcus pyogenes* was affected moderately by the inhibitor with zones of inhibition recorded as17mm, 16mm, 16mm and 15mm at a concentration of 100 µg AMTI-II respectively (Fig-4).

Minimum inhibitory concentrations of AMTI-II for its antibacterial activity were presented in Table- 4. The minimum inhibitory concentrations (MIC) for *Staphylococcus aureus*, *Escherichia coli, Proteus vulgaris, Bacillus subtilis, Streptococcus pneumoniae* was found to be 62.5 µg/ml, for *Bacillus cereus, Klebsiella pneumonia,* the MIC value was 125 µg/ml and for the last three bacterial strains namely *Pseudomonas aeruginosa, Pseudomonas syringae and Streptococcus*

pyogenes was found to be 250 µg/ml of the inhibitor. From the results obtained, it is clear that AMTI-II exerted a significant inhibitory effect on the growth of selected bacterial strains at a concentration of 50 - 100 µg of the inhibitor.

Antifungal activity

The trypsin inhibitor was also tested for its antifungal activity against *Asperigellus niger, Asperigillus flavus, Fusarium oxysporum, Alternaria alternate, Candida albicans, Candida glabrata, Candida tropicalis, Mucor indicus, Penicillium chrysogenum and Saccharomyces cerevisiae* in the range 500-2000 µg/ml along with the positive control containing the fungicides, Flucanazole and Ketoconazole. AMTI-II moderately affected the growth of *Candida albicans, Candida tropicalis, Asperigillus flavus, Saccharomyces cerevisiae, Candida glabrata and Asperigellus niger* with zones of inhibition recorded as 21 mm, 21 mm, 19 mm, 19 mm, 20 mm and 21 mm at a concentration of 100 µg of the inhibitor respectively (Fig-5). The inhibitor did not show any inhibitory effect on the growth of other fungal strains tested.

Minimum inhibitory concentrations of AMTI-II for antifungal activity was presented in Table- 5. Except for *Saccharomyces cerevisiae*, the MIC of AMTI-II for other fungal strains was found to be 250 µg/ml of the inhibitor.

Protease inhibitors are ubiquitous in plants generally acting as storage proteins and wound-induced defensive agents against herbivores and pathogens (Basir et al., 2000). The role of protease inhibitors in plant protection against insects is studied relatively well. On the other hand, data on the role of protease inhibitors against fungal or bacterial infections are very few and need to be provided more.

In the present study, a novel trypsin inhibitor from *Abelmoschus moschatus* seeds have been isolated and purified to homogeneity following conventional methods of protein purification. The observation that trypsin inhibitory activity in the crude extracts of the seeds is stable at 70^{o}C for 10 min has led to the use of this treatment as the first step in the purification of the inhibitor. About 52% of proteins present in the crude extract were removed by this step. When the ammonium sulphate fraction was subjected to DEAE-cellulose column chromatography, trypsin inhibitory activity was found to be associated with protein present in the void volume and also with the proteins bound to the matrix. A weakly bound protein eluted by 0.1 M NaCl showed strong trypsin inhibitory activity and moderately and firmly bound proteins eluted with 0.25 M NaCl and 0.5 M NaCl exhibited both trypsin and chymotrypsin inhibitory activities. The protein obtained with 0.1 M NaCl also eluted out as a single protein with corresponding trypsin inhibitory activity when subjected to Sephadex G-100 gel filtration.

The final yield of the inhibitor was about 17%. AMTI-II was found to be homogenous by native PAGE and gel filtration on Sephadex G-200 column. The coomassie blue stainable protein band corresponded to the specific staining band for the visualization of the trypsin inhibitory activity**.** The molecular mass of AMTI-II was found to be 21.2 kDa as determined by SDS-PAGE and was close to the mass that obtained with gel filtration of Sephadex G-200 column.

The trypsin inhibitor from the seeds of *Abelmoschus moschatus* was found to be stable under conditions of extreme pH, temperature and in the presence of denaturants, 8 M urea and 1% SDS. The unusual stability of the inhibitor could be due to intramolecular disulphide bridges and strong hydrophobic interactions forming an inner core in the protein. In this respect, it resembles proteinase inhibitors from other plant sources such as soybean (Edelhoch and Steiner, 1963), Italian millet (Udupa and Pattabiraman, 1987), *Archidendron ellipticum* (Bhattacharyya et al., 2006), *Calliandra selloi* (Yoshizaki et al., 2007) and *Inga laurina* (Macedo et al., 2007).

It is well known that some plant proteinase inhibitors possessed *in vitro* antibacterial and antifungal activities. In the present study, AMTI-II exhibited antibacterial and antifungal activities with varying degrees in a dose dependent manner. It did not differentiate Gram positive bacteria from Gram negative bacteria in its antibacterial activity.

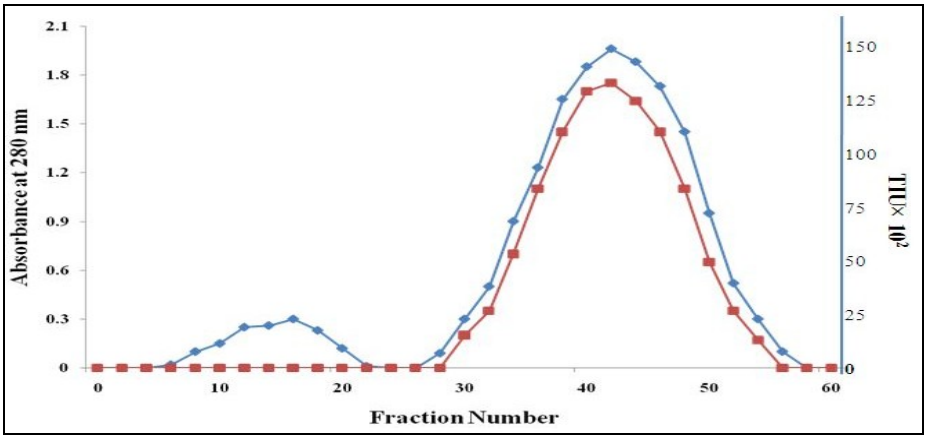
Among all the bacterial strains tested, *Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Bacillus subtilis, Streptococcus pneumoniae, Bacillus cereus* were found to be more sensitive and *Klebsiella pneumoniae, Pseudomonas aeruginosa, Pseudomonas syringae and Streptococcus pyogenes* were found to be less sensitive. However, AMTI-II showed a moderate effect on the growth of fungal species, *Candida albicans, Candida tropicalis, Asperigillus flavus, Saccharomyces cerevisiae, Candida glabrata and Asperigellus niger* and it had no inhibitory effect on the growth of fungi - *Fusarium oxysporum, Alternaria alternata, Mucor indicus and Penicillium chrysogenum* tested.

AMTI-II was similar to napin from chinese white cabbage (*Brassica chinensis*) and trypsin inhibitor from bottle gourd (*Lagenaria siceraria*) in possessing antibacterial activity towards *Pseudomonas aeruginosa, Bacillus subtilis* and *Bacillus cereus* (Ngai and Ng, 2004 and Shee Chandan et al., 2009). It was also close to proteinase inhibitors from broad beans (*Vicia faba*) and buckwheat (*Fagopyrum esculentum*) seeds in its antifungal activity (Ye et al., 2001; Dunaevsky et al., 2001).

Several kunitz proteinase inhibitors have shown potential antimicrobial activity against Gram positive and Gram-negative bacteria and fungi. Inhibitors possessing bactericidal activity include those from the corms of *Xanthosoma blandum,* active against *Staphylocccus aureus, Salmonella typhimurium,* and *Escherichia coli* (Lima et al., 2011) and seeds of *Achyranthes aspera* (AATI) active against *Proteus vulgaris, Bacillus subtilis, Staphylocccus aureus, Escherichia coli* and *Klebsiella pneumonia* (Geetha et al., 2012).

Microbes are known to elaborate proteases into extracellular medium for gaining entry into the host and protease inhibitors by binding to such extracellular proteases could exert antimicrobial effect. Possibility of protease inhibitors entering into microbial cells and interfering with the function of intracellular proteases cannot be ruled out for their antimicrobial activity. Bactericidal proteins are reported to form a channel on cell membrane and cell dies as a result of the out flowing of the cellular contents through a mechanism different from that of antibiotics. Whether protease inhibitors form such a channel is yet to be established. The growth of inhibition of fungi cannot be fully

Fig.1 Gel filtration of AMTI-II on Sephadex G-100 of the DEAE-cellulose preparation

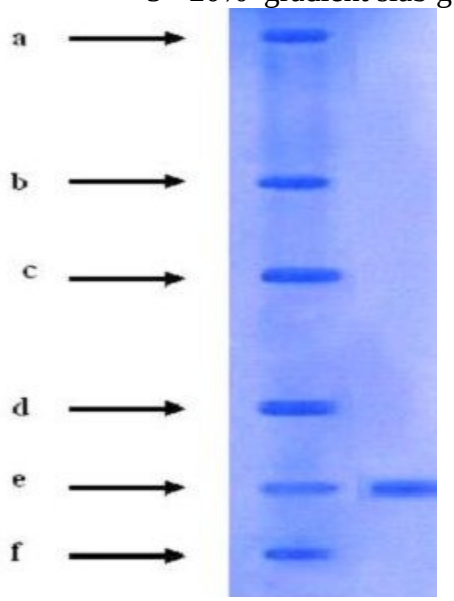


58.8 mg of AMTI-II, was applied on to the column (1.9 x 63) in 0.1 M phosphate buffer, pH 7.6 and eluted with the same buffer. Fractions, each 2 ml, were collected at a flow rate of 12 ml/h.

(♦----------♦) Protein was monitored by absorbance at 280 nm

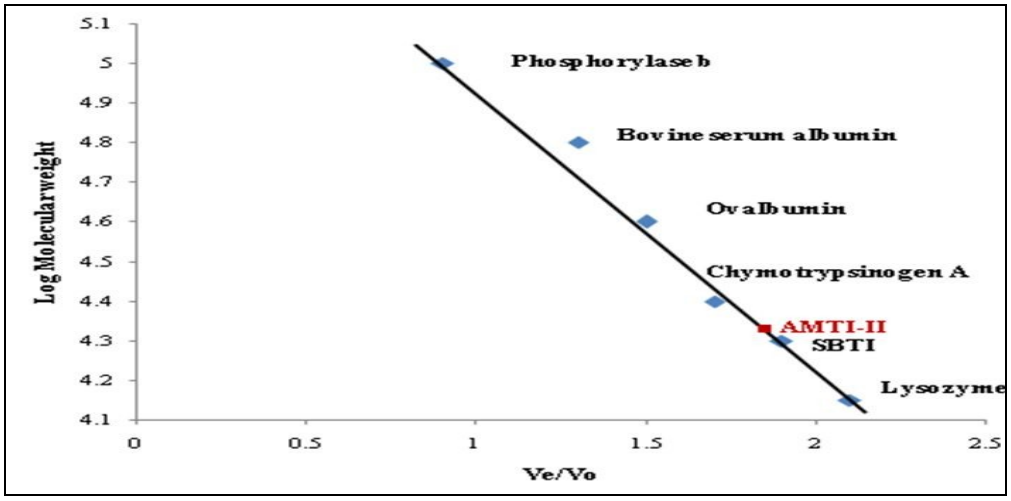
(■---------■) Trypsin inhibitory activity

Fig.2 Molecular weight determination of AMTI-II by SDE-PAGE on 5 - 20% gradient slab gel



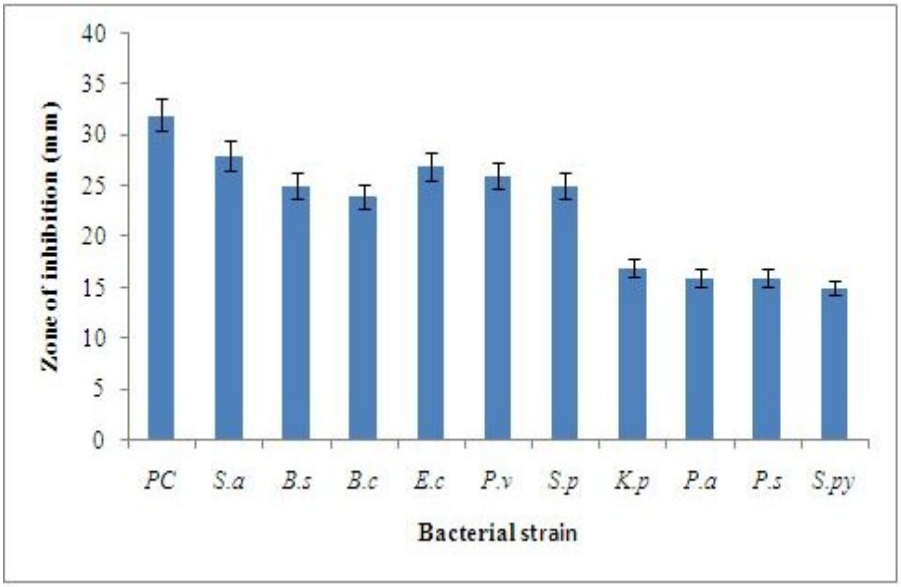
1. Standard proteins
   (a) Phosphorylase b, 97kDa
   (b) Bovine serum albumin, 67kDa
   (c) Ovalbumin, 45kDa
   (d) Chymotrypsinogen A, 25kDa
   (e) Soybean trypsin inhibitor, 20.1 kDa
   (f) Lysozyme, 14kDa
2. Purified AMTI-II

Fig.3 Molecular weight determination of AMTI-II by gel filtration on Sephadex G-200



Plot of elution volume against log molecular weight of standard proteins (♦) and AMTI-II (■).

Fig.4 Antibacterial activity of AMTI-II

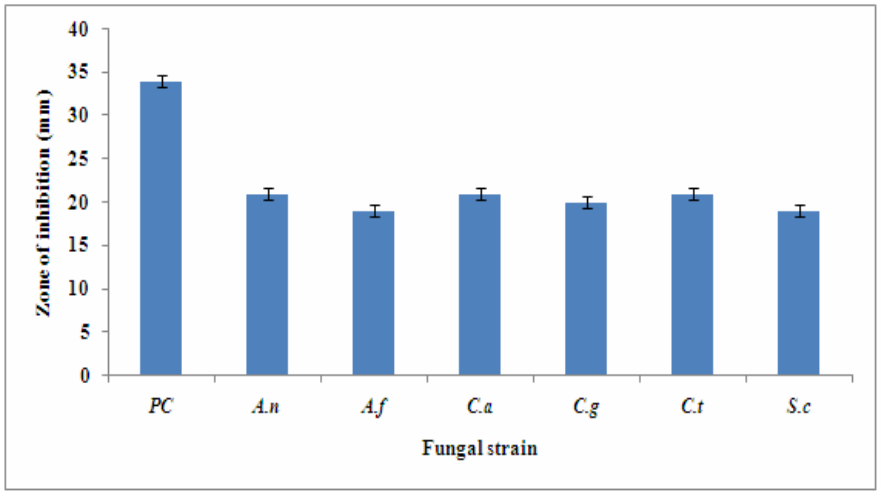


PC- Positive control (Benzyl penicillin)

S.a- Staphylococcus aureus; B.s- Bacillus subtilis; B.c- Bacillus cereus; E.c- Escherichia coli; P.v - Proteus vulgaris; S.p- Streptococcus pneumonia; K.p - Klebsiella pneumonia; P.a - Pseudomonas aeruginosa; P.s - Pseudomonas syringae; S.py - Streptococcus pyogenes

Bacterial strains were spread on agar plates. Different amounts of AMTI-II (50 µg for the first six bacterial strains and 100 µg for the remaining strains) were placed in the wells. Control contained Benzyl Penicillin (20µg) in place of inhibitor. The incubation period was 24 h at37^{0}C. Zone of inhibition was measured.

Fig.5 Antifungal activity of AMTI-II



PC- Positive control (Flucanazole)
A.n - Asperigillus niger; A.f - Asperigillus flavus; C.a - Candida albicans; C.g - Candida glabrata; C.t - Candida tropicalis; S.c - Saccharomyces cerevisiae

Fungal strains were spread on potato dextrose agar plates. AMTI-II (100 µg) was placed in the wells and allowed for diffusion. Control contained Flucanazole (20 µg) in place of inhibitor. The incubation period was 48 h at 25^0C. Zone of inhibition was measured and minimum inhibitory concentration of each inhibitor was determined.

Table-1 Summary of purification of AMTI-II from seeds of *Abelmoschus moschatus*

Preparation	**Volume (ml)**	**Total protein (mg)**	**Total activity units**	**Specific activity Units/mg protein**	**Yield (%)**	**Fold purification**
			TIU×10^3	**TIA×10^2**		
Crude extract	250	2087.5	788.4	3.77	100	1.00
Heat treatment	215	1016.4	626.4	6.16	79.45	1.63
Ammonium sulphate (60%) Fractionation	60	424.8	482.8	11.36	61.24	3.01
DEAE-Cellulose 0.1M NaCl elution	216	58.8	136.8	23.26	17.35	6.17
Sephadex-G-100 fraction	50	52.4	132.6	25.30	16.81	6.71

*Yield and fold purification were calculated on the basis of TIU and TIA respectively.
TIU- Trypsin inhibitory units TIA-Trypsin inhibitory activity

Table-2 Effect of heat on AMTI-II

Temperature °C	Time (min)	AMTI-II TIU/mg of AMTI-II x 10^2
25	10	25.28
37	10	25.34
50	10	25.22
60	10	25.16
70	10	25.18
80	10	25.36
90	10	22.56
90	20	19.84
100	10	7.68
100	20	3.86
100	30	0

* Inhibitory activity at 25°C was taken as 100%
TIU – Trypsin inhibitory units

Table-3 Effect of pH on AMTI-II

pH	Name of the Buffer	AMTI-II TIU/mg of AMTI-II x 10^2
3	Glycine-HCl	25.20
5	Sodium citrate	25.38
7	Sodium Phosphate	25.44
9	Tris-HCl	25.24
12	Glycine-NaOH	25.18

TIU – Trypsin inhibitory units
AMTI-II was incubated for 24 h at 4°C in the respective buffers and assayed for Trypsin inhibitory activity using BAPNA as the substrate.

Table.4 Minimum Inhibitory concentrations (MIC) of AMTI-II on bacterial growth

Name of the bacterial strain	Minimum Inhibitory Concentration (µg/ml) AMTI - II
Staphylococcus aureus	62.5
Bacillus subtilis	62.5
Bacillus cereus	125
Escherichia coli	62.5
Proteus vulgaris	62.5
Streptococcus pneumoniae	62.5
Klebsiella pneumoniae	125
Pseudomonas aeruginosa	250
Pseudomonas syringae	250
Streptococcus pyogenes	250

Bacterial strains were spread on agar plates. Different concentrations of AMTI-II (0.025-2 mg/ml) were placed in the wells. Controls contained Tetracycline, Rifampicin and Benzyl Penicillin (20µg) in place of isoinhibitors. The incubation period was 24 h at 37^0C. Zone of inhibition was measured and minimum inhibitory concentration of inhibitor was determined.

Table.5 Minimum Inhibitory concentrations (MIC) of AMTI-II on fungal growth

Name of the fungal strain	Minimum Inhibitory Concentration (µg/ml)
	AMTI - II
Asperigillus niger	250
Asperigillus flavus	250
Fusarium oxysporum	-
Alternaria alternate	-
Candida albicans	250
Candida glabrata	250
Candida tropicalis	250
Mucor indicus	-
Penicillium chrysogenum	-
Saccharomyces cerevisiae	500

Fungal strains were spread on potato dextrose agar plates Different amounts of the inhibitor (0.05-2mg/ml) were placed in the wells and allowed for diffusion. Controls contained Flucanazole (20 µg) and Ketoconazole (20 µg) in place of inhibitor. The incubation period was 48 h at 25^0C. Zone of inhibition was measured and minimum inhibitory concentration of inhibitor was determined.

explained by trypsin inhibition alone. The antifungal role of trypsin inhibitors has also been attributed to their ability to interfere with chitin biosynthetic process during fungal cell wall development by inhibiting the proteolytic activation of chitin synthase zymogen (Adams et al., 1993).

Some proteinase inhibitors have shown both antibacterial and antifungal activities. Kim et al., (2006) demonstrated that inhibitors from potato tubers strongly inhibited the growth of a wide variety of bacteria, including *Staphylococcus aureus, Listeria monocytogenes, Clavibacter michiganense*, and *Escherichia coli*, and fungi such as *Candida albicans* and *Rhizoctonia solani.*

In conclusion, the purified trypsin inhibitor from the seeds of *Abelmoschus moschatus* is found to be active against selected bacterial and fungal strains with varying efficiencies. AMTI-II can find application in the medical front as therapeutic agent for infections caused by specific bacterial and fungal strains and it can be explored in the agricultural front for developing transgenics after carrying out extensive *in vitro* studies against midgut proteases of insect pests.

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