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ANTIMICROBIAL ACTIVITY OF PRICKLY CHAFF (*ACHYRANTHES ASPERA*) SEED TRYPSIN INHIBITOR

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ABBREVIATIONS:
AATI: Achyranthes aspera trypsin inhibitor**, MIC:** Minimum inhibitory concentration**, DEAE:** diethylaminoethyl**, BSA:** bovine serum albumin, **TIU:** trypsin inhibitory units, **BAPNA:** α-N-benzoyl-DL-arginine-p-nitoanilide HCl, **SDS:** sodium dodecyl sulphate, **ATEE:** N-acetyl-L-tyrosine ethyl ester, **JSTI:** Jack fruit seed trypsin inhibitor, **PBS:** Phosphate-buffered saline, **PAGE:** Polyacrylamide gel electrophoresis

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ABSTRACT

Trypsin inhibitor (AATI) was purified from the seeds of *Achyranthes aspera* to homogeneity by conventional methods and its antimicrobial activity was tested on selected pathogenic microbes. The inhibitor significantly affected the growth of *Proteus vulgaris* followed by *Bacillus subtilis, Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumonia* with zones of inhibition recorded as 28mm, 26mm, 25mm, 20mm and 14mm respectively. No inhibition, however, was observed with *Staphylococcus pneumonia* strain. Minimum inhibitory concentration (MIC) values of AATI supported that *Proteus vulgaris and Bacillus subtilis* were the most sensitive strains against the inhibitor. AATI did not differentiate gram positive and gram negative bacteria in its antibacterial activity. The trypsin inhibitor was ineffective against fungal strains *Asperigillus niger, Fusarium oxysporum, Alternaria alternate* and *Candida albicans* tested.

INTRODUCTION: Proteinase inhibitors (PIs) comprise one of the most abundant classes of proteins in plants accounting 1-10% of total proteins in storage organs such as seeds and tubers [1, 2, 3]. These inhibitors are specific for each of the four mechanistic classes (Serine, Cysteine, Aspartyl and Metallo) of proteolytic enzymes [4, 5, 6].

Functionally, these proteins are concerned with the regulation of endogenous protease activities and supplying amino acids as storage proteins apart from protecting plant tissues from pest and pathogen attack. A positive correlation existed between plants with higher levels of trypsin and chymotrypsin inhibitors and their resistance towards pathogens. Hilder *et al* [7] reported that transgenic plants with double headed inhibitors provided more resistance against pathogens compared to those with mono headed inhibitors. Protease inhibitors exhibiting lectin activity from the seeds of northern beans, jack fruit, taub, cowpea have been reported [8, 9, 10].

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Some of the serpins, cystatins, pepstatins and metallo protease inhibitors have been reported to possess antimicrobial activities [11]. A protease inhibitor with antimicrobial and lectin activities would be advantageous to the host against a pathogen attack.

Even though AATI is found to be very active against trypsin, its influence on the growth of bacteria and fungi is not yet examined. This paper, therefore, deals with the effect of AATI on the growth of selected microbes.

Achryanthes aspera Linn.*,* known as Uttarane in Telugu, belongs to family Amaranthaceae. It is a weedy perennial herb growing to the height of 1-3 feet found usually in moist or shaded places. The flowering and fruiting season of the plant is from September to December. Seeds are rich in protein and are used in culinary preparations. Seed extracts are reported to have medicinal properties in reducing appetite, treatment of bleeding piles, brain diseases. The seeds are employed as an emetic, purgative, and cathartic, in gonorrhea, for insect bite and in hydrophobia, cough including whooping cough, as an anti-asthmatic.

MATERIALS AND METHODS: Seeds of *Achyranthes aspera* were removed from the ripened spikes and were used for the isolation and purification of trypsin inhibitor.

Purification of Achyranthes aspera Trypsin Inhibitor (AATI): This was followed by the method of Annapurna *et al.,* [12]. Dehusked seeds (20g) of *Achyranthes aspera* were homogenized with 200 ml of 0.1M sodium phosphate buffer, pH 7.6 and the extract was then centrifuged at 10,000 *g* for 15 min at 5°C. The supernatant was dialyzed against the buffer for 24h in the cold, and rapidly heated to 60°C and maintained at this temperature for 10 min. The extract was quickly cooled in ice and then centrifuged at 10,000 *g* for 15 min at 4°C. To the supernatant, solid ammonium sulfate was added to 60% saturation with constant stirring at 4°C. The suspension was centrifuged at 10,000 *g* for 15 min and the precipitate was dissolved in 0.1 M sodium phosphate buffer, pH 7.6 and dialyzed against the same buffer for 48h. The dialyzed sample was loaded on a DEAE-cellulose column (2.2 x 34 cm) and the elution was performed with 0.1- 0.3M NaCl in the buffer.

Fractions of 8 ml were collected at a flow rate of 60 ml/h. Fractions containing trypsin inhibitory activities were pooled, concentrated, dialyzed against distilled water for 24h and lyophilized.

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, dialysed against distilled water at 4-6°C and then lyophilized. The purified inhibitor was stored as a dry powder at 0-4°C.

Protein Estimation: Protein was estimated by the method of Lowry *et al*., [13] using BSA as the standard.

Determination of Molecular weight: Molecular weight of the inhibitor was determined by SDS-PAGE using the method of Laemmli [14] and also by gel filtration on Sephadex G-200 column.

Measurement of Trypsin and Trypsin Inhibitory Activity: The inhibition of AATI 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.,* [15] 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.

Hemagglutination Assays: Sheep blood was obtained from the slaughter house. Human blood belonging to different groups was collected from healthy volunteers. JSTI was a gift from Dr. Shakuntala.

Erythrocyte agglutinating activity of AATI was determined according to the method of Paulova *et al*., [16]. Both normal and trypsin treated erythrocytes from sheep and human subjects were used in these assays.

The hemagglutination assay was carried out in a plexi-plate with a concave wells using the two fold serial dilution technique. 0.2 ml of saline was added to each well followed by 0.2ml of AATI solution (2mg/ml PBS) to the first well. The sample was mixed and was serially diluted each time transferring 0.2ml to each well. Finally 0.2 ml of erythrocyte suspension (4% (v/v)) was added to each of the wells, gently mixed and incubated for 90 min at 37^{o}C and the agglutination of the cells was examined visually. The control had no AATI in the system. JSTI was present in the positive control.

The highest dilution which showed positive hem-agglutination was taken as the titre. The amount of protein present at this dilution represents the minimum quantity of the lectin necessary for agglutination under the experimental conditions and is defined as one hemagglutinating unit.

Determination of Antimicrobial Activity: The microbial strains, *Bacillus subtilis* (MTCC 121), *Escherichia coli* (MTCC 118), *Proteus vulgaris* (MTCC 426), *Staphylococcus aureus* (MTCC 96), *Klebsiella pneumonia* (MTCC 2405), *Streptococcus pneumoniae* (MTCC 2672), *Asperigellus niger* (MTCC 2723)*, Fusarium oxysporum* (MTCC 1755), *Alternaria alternate* **(**MTCC 1362) and *Candida albicans* (MTCC 227) were procured from Microbial Type Culture Collection (MTCC), Chandigarh.

Active cultures were generated by inoculating a loopful of culture in separate 100ml nutrient/potato dextrose broths and incubating on a shaker at 37^{o}C overnight. The cells were harvested by centrifuging at 10,000*g* for 5 min, washed with normal saline, spun at 10,000*g* for 5 min again and diluted in normal saline to obtain 5 x 10^{5}colony formation units/ml.

Antibacterial Activity: AATI was subjected to antibacterial assay using the agar well diffusion method of Murray *et al*., [17] as modified by Olurinola [18]. 20ml of nutrient agar was dispensed into sterile universal bottles 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 three uniform wells in each petri dish. The wells were filled with buffer containing, varying concentrations of AATI and allowed for diffusion of the inhibitor for 45 min. The plates were incubated at 37°C for 24 h for bacteria. Streptomycin was included in the positive control. The inhibition zones were measured with antibiotic zone scale in mm and experiment carried out in triplicate.

Antifungal Activity: AATI was subjected to antifungal assay using the agar well diffusion method of Perez *et al.*, [19]. The cultures of 48h old grown on potato dextrose agar (PDA) were used for inoculation of fungal strains on PDA plates. An aliquot (0.2ml) 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 filled with the buffer containing various concentrations of AATI and allowed for diffusion of the inhibitor for 45 min. The plates were incubated at 25°C for 48 h. The fungicide, Flucanozole, was used in the positive control. The zones of inhibition were measured.

Minimum Inhibitory Concentration (MIC) Assays: Minimum Inhibitory Concentrations (MIC) of the trypsin inhibitor was determined according to the method of Elizabeth [20]. Different concentrations of AATI ranging from 25-1000µg/ml were added to the wells of each petri dish. Inhibition of the growth of the organism in the plates containing AATI was judged by comparison with the growth in the control plates. The MICs were determined as the lowest concentration of AATI inhibiting visible growth of each organism on the agar plate.

RESULTS:

Isolation, Purification and Properties: AATI was purified by ammonium sulphate fractionation and chromatography on DEAE-cellulose and Sephadex G-100. Protein eluted with 0.1 M NaCl on DEAE-cellulose

column showed antitryptic activity. When the lyophilised active fractions are subjected to Sephadex G-100 column chromatography, the inhibitor eluted out as a single peak with corresponding trypsin inhibitory activity (**Fig. 1**). AATI is 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**.**

Recoveries and relative purification at each step for a typical purification from 20 g seeds are shown in **Table 1**. By this procedure, about 32 mg of the inhibitor is obtained. The final yield of the inhibitor was about 15%. The inhibitor gave a single sharp band on SDS-PAGE even in the presence of 2-mercaptoethanol supporting the monomeric nature of the protein. Molecular weight of the inhibitor was determined to be 20 kDa on SDS-PAGE (**Fig 2**). The results obtained indicate the absence of multiple forms of protease inhibitors in *Achyranthes aspera seeds*. The inhibitor was found to be glycoprotein with a neutral sugar content of 2.5%. It was devoid of amino sugars and free sulfhydryl groups. The absence of thiol groups indicates that cysteines in protease inhibitor form disulphide bonds. AATI was found to be ineffective against α-chymotrypsin when tested with both casein and ATEE as substrates of the enzyme.

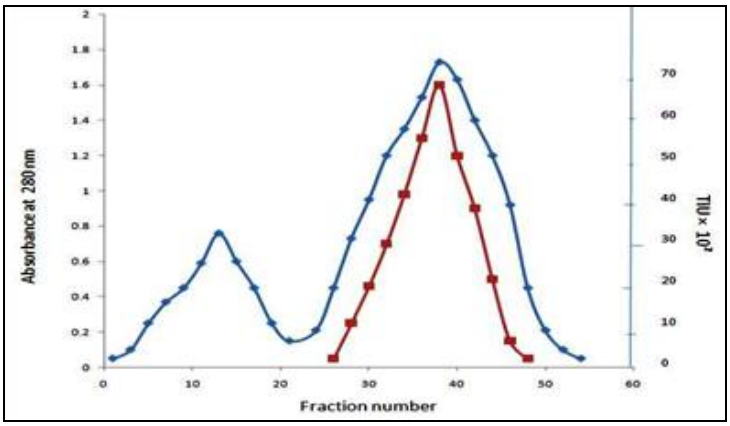
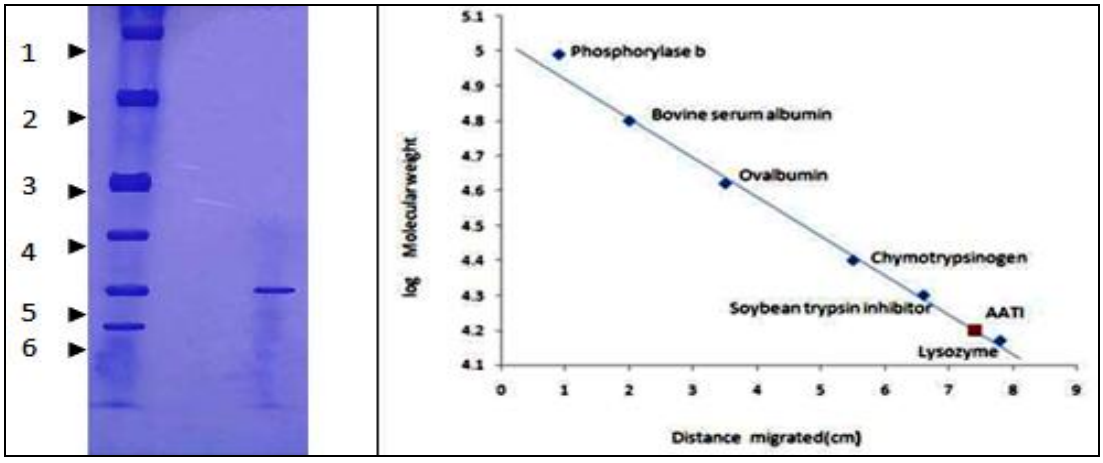


FIG. 1: GEL FILTRATION ON SEPHADEX G-100 OF THE DEAE-CELLULOSE PREPARATION. 62mg of the lyophilized preparation was applied to the column (1.9 x 63) in 0.1 M phosphate buffer, pH 7.6 and eluted with the same buffer. Fractions, each 2 ml, were collected at a flow rate of 12 ml/h. Protein was monitored by absorbance at 280 nm (♦---♦). TIA (■---■)



(a) (b)

FIG 2. (A) SDE-PAGE ON A 5 - 20% GRADIENT SLAB GEL. Lanes: (a) Standard proteins; 1) Phosphorylase b, 97kDa; 2) BSA, 67kDa; 3) Ovalbumin, 44kDa; 4) Chymotrypsinogen A, 25kDa; 5) Soybean trypsin inhibitor, 20.1 kDa ;6) Lysozyme, 14kDa. (b) AATI kept at 100°C for 2 min with SDS and 2-mercaptoethanol. **(B) MOLECULAR WEIGHT DETERMINATION OF AATI BY SDS-PAGE.** Plot of distance migrated against log molecular weight of standard proteins (♦) and AATI (■).

TABLE 1: SUMMARY OF PURIFICATION OF PROTEINASE INHIBITOR FROM 20g SEEDS OF *ACHYRANTHES ASPERA*

Preparation	Volume (ml)	Total Protein (mg)	Total activity (units) TIUx10^3	Specific activity(units/mg of protein) TIA x10^2	Yield %	Fold* Purification
Crude extract	200	1040. 0	300.55	2.89	100.0	1.00
Heat treated extract	160	456.5	271.40	5.94	90.30	2.05
60% Ammonium sulfate	50	135.6	192.11	14.16	63.91	4.898
DEAE – cellulose	100	62.0	154.31	24.88	51.33	8.592
Sephadex G-100	60	32.5	140.31	43.17	46.67	14.90

*Yield and fold purification were calculated on the basis of TTU and TTA respectively; TIU - Trypsin inhibitory units, TIA - Trypsin inhibitory activity

Hemagglutinating Activity: AATI did not cause any hemagglutination with the normal and trypsin treated erythrocytes of sheep even when the amount of the inhibitor was raised up to 200 µg. The inhibitor also did not show any agglutination with any of the four human blood groups (**Table 2).** JSTI, on the other hand, agglutinated both normal and trypsin treated erythrocytes of rabbit, rat, human and sheep but with different efficiencies. As low as 6.25 µg of the inhibitor was sufficient to cause visible agglutination reaction with trypsin treated rabbit erythrocytes. The titre values obtained for JSTI are in agreement with those reported earlier by Shakuntala, 1996. The seed extract of *Achyranthes aspera* also did not cause any hemagglutination with normal and trypsin treated erythrocytes indicating the absence of lectins in the seed. It was observed that AATI did not cause any hemolysis of erythrocytes during the experimental period.

TABLE 2: AGGLUTINATION OF HUMAN AND ANIMAL ERYTHROCYTES BY AATI AND JSTI

Origin of erythrocytes		Minimal hemagglutinating dose			
		AATI (µg)		JSTI (µg)	
		Normal	Trypsin - treated	Normal	Trypsin - treated
Rabbit		00	00	50	6.25
Rat		00	00	100	6.25
Sheep		00	00	200	100
Human	A	00	00	100	50.00
	B	00	00	100	50.00
	AB	00	00	100	50.00
	O	00	00	100	50.00

0.2ml of AATI/ JSTI (2mg/ml) was serially diluted with 0.9%NaCl and 0.2ml of untreated or trypsin – treated erythrocytes suspension (4%) was added and hemagglutination assays were carried.

Antimicrobial Activity: Table 3 shows the effect of AATI on the growth of gram positive and gram negative bacteria. The inhibitor significantly affected the growth of *Proteus vulgaris* followed by *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli* with zones of inhibition recorded as 28mm, 26mm, 25mm and 20mm respectively. The growth of *Klebsiella pneumoniae* was weakly affected by the inhibitor with a zone of inhibition 14mm. AATI had no effect on *Streptococcus pneumoniae* at the levels tested. Streptomycin (10µg), on the other hand, produced an inhibition zone of 30-34 mm in the control. **Fig. 3** shows the zones of inhibition of AATI for the above bacterial strains. Minimum inhibitory concentrations of AATI for the antibacterial activities are presented in **Table 4**. For *Proteus vulgaris*, the MIC was found to be 5µg/ml. In the case of *Bacillus subtilis* and *Staphylococcus aureus*, the MIC was determined to be 7.5 µg /ml for both strains. With *Escherichia coli*, the MIC was observed to be 8µg/ml and for *Klebsiella pneumoniae* the MIC was found to be 35µg/ml. AATI did not discriminate gram positive and gram negative bacteria in its antibacterial activity.

The trypsin inhibitor was also tested for its antifungal activity on *Asperigellus niger, Fusarium oxysporum, Alternaria alternate* and *Candida albicans* in the range 500-2000 µg/ml along with the positive control containing flucanazole. The radial growth of the organisms was unaffected by AATI and in the positive controls; their growth was significantly decreased with inhibition zones measured as 20-22mm (**Table 5**).

TABLE 3: EFFECT OF AATI ON BACTERIAL GROWTH

Name of the bacterial strain	Zone of Inhibition (Diameter in mm)		
	25 µg AATI	50 µg AATI	Control (C), 20µg Streptomycin
Gram positive			
Bacillus subtilis	13	26	32
Staphylococcus aureus	15	25	30
Streptococcus pneumoniae	00.00	00.00	32
Gram negative			
Escherichia coli	14	20	34
Klebsiella pneumoniae	00.00	14	32
Proteus vulgaris	18	28	32

Bacterial strains were spread on agar plates. Different amounts of AATI (25 µg and 50 µg) were placed in the wells. Controls contained Streptomycin (10 µg) in place of AATI. The incubation period was 24 h at 37°C. Zone of inhibition was measured.

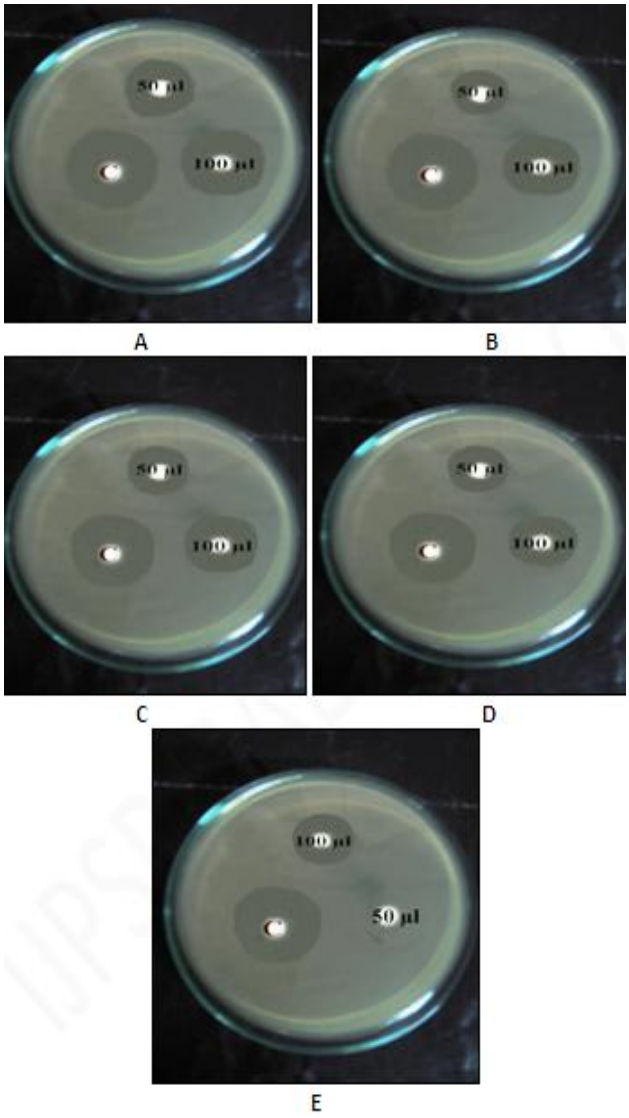


FIG. 3: ANTIBACTERIAL ASSAY OF AATI AGAINST BACTERIAL STRAINS

A - *Proteus vulgaris;* B - *Bacillus subtlis;* C - *Staphylococcus aureus;* D - *Escherichia coli*; E - *Klebsiella pneumonia*

TABLE 4: MINIMUM INHIBITORY CONCENTRATIONS OF AATI

Name of the bacterial strain	Minimum Inhibitory Concentration (µg/ml)
Gram positive	
Bacillus subtilis	7.5
Staphylococcus aureus	7.5
Streptococcus pneumonia	–
Gram negative	
Escherichia coli	8.0
Klebsiella pneumonia	35.0
Proteus vulgaris	5.0

Bacterial strains were spread on agar plates. Different concentrations of AATI (0.025-1 mg/ml) were placed in the wells. Controls contained Streptomycin (10 µg) in place of AATI. The incubation period was 24 h at 37°C. Minimum inhibitory concentrations of AATI were determined.

TABLE 5: EFFECT OF AATI ON FUNGAL GROWTH

Name of the fungal strain	Zone of Inhibition (Diameter in mm) AATI			Control (C)
	25µg	50µg	250 µg	10 µg Fluconazole
Asperigillus niger	-	--	--	22
Fusarium oxysporum	--		--	20
Alternaria alternate	--	--	--	22
Candida albicans	--	--	--	21

Fungal strains were spread on potato dextrose agar plates Different amounts of AATI (25 µg and 50 µg) were placed in the wells and allowed for diffusion. Controls contained Fluconazole (10 µg) in place of AATI. The incubation period was 48 h at 25°C. Zone of inhibition was measured

DISCUSSION: Trypsin inhibitor has been obtained from *Achyranthes aspera* seeds in an apparently pure state. The observation that trypsin inhibitory activity in the crude extracts of the seeds is stable at 60°C for 10 min has led to the use of this treatment as the first step in the purification of the inhibitor. About 55% of proteins present in the crude extract were removed by this step. The inhibitor also eluted out as a single protein with corresponding trypsin inhibitory activity when subjected to ion exchange chromatography and Sephadex G-100 gel filtration. The final yield of the inhibitor was about 15%. Based on its ability to inhibit trypsin, its low half cysteine content and molecular weight of 20 kDa, AATI may be placed in the group of Kunitz type of inhibitors.

It is known that protease inhibitors belonging to Bowman Birk and Kunitz families exhibit antimicrobial activities. AATI has been shown to possess antibacterial activity without differentiating gram positive and gram negative bacteria. The trypsin inhibitor appears to be similar to napin from chinese white cabbage (*Brassica chinensis*) seeds and one from bottle gourd (*Lagenaria siceraria*) seeds in possessing antibacterial activity [21, 22].

In contrast to its effect on bacteria, AATI did not inhibit the growth of fungal strains tested. The protease inhibitors from broad bean, buckwheat and Acacia plumosa seeds showed antifungal activities [23-25]. Potamin -I isolated from the tubers of potato inhibited the growth of fungal and bacterial strains [26]. AATI differs from these inhibitors in having high molecular weight (20 kDa) and carbohydrate moieties. Reports on protease inhibitors exhibiting hem- agglutinating activity are available in the literature [10, 27] and AATI has been examined for similar activity using erythrocytes from different animals and human beings.

Even though AATI contained 2.5% carbohydrate content, it did not exhibit any lectin activity when tested towards normal and trypsinised erythrocytes of four different groups of human beings, sheep, rat and rabbit. This is in contrast to JSTI which was reported to exhibit strong hemagglutinating activity towards erythrocytes from different animals and human beings. Jack fruit seeds are rich in lectins, particularly galactose specific ones and possibility of JSTI possessing lectin activity through gene fusions of protease inhibitor and lectin cannot be ruled out. This double-headed inhibitor with a molecular weight of 28kDa containing 5% carbohydrate gave a titer value corresponding to an amount of 6.25µg.

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. AATI might exert its effect in both ways. Further work on the effect of AATI on a wide range of bacteria and fungi and examining its interaction with intracellular and secretary proteases of microbes will throw more light on understanding its antimicrobial property.

CONCLUSION: The results of the present investigation clearly demonstrate AATI as a potential antibacterial agent. In the medical front, it can be used as a therapeutic agent for skin and wound infections caused by bacteria such as *Staphylococcus aureus* and *Proteus vulgaris.* The inhibitor can also be explored in the agricultural front after carrying out extensive *in vitro* and *in vivo* studies on insect midgut proteases.

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