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A Comparative Study on the Antibacterial Activity of Trypsin Inhibitors from the Seeds of *Abelmoschus Moschatus* L.

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Abstract: ***The aim of the present study is to determine antibacterial activity associated with trypsin inhibitors (AMTI-I, II, III & IV) isolated and purified from the seeds of Abelmoschus moschatus. The inhibitors exerted differential effects on the growth of the bacterial strains tested. AMTI-I and AMTI-II, though moderately active against Klebsiella pneumoniae, Pseudomonas aeruginosa, Pseudomonas syringae and Streptococcus pyogenes, showed a strong effect on the growth of Escherichia coli, Proteus vulgaris, Bacillus subtilis, Streptococcus pneumoniae, Bacillus cereus at a concentration of 50 µg of the inhibitors. AMTI-III and AMTI-IV, on the other hand, exerted a moderate effect in inhibiting the growth of bacterial strains at a concentration of 100 µg of the inhibitors. The inhibitors did not differentiate gram positive bacteria from gram negative bacteria in their antimicrobial activity. Results obtained in the present study suggest that trypsin inhibitors AMTI-I and AMTI-II from the seeds of Abelmoschus moschatus may serve as good candidates for the development of novel antimicrobial agents.***

Keywords: Trypsin inhibitors, antibacterial activity, *Abelmoschus moschatus,* gram positive, gram negative.

1. Introduction

Plants develop a variety of proteins including ribosome-inactivating proteins [1], lectins [2], protease inhibitors [3] and antifungal proteins [4–6] as defensive agents against invading pathogens. Among these, protease inhibitors not only protect plants against attack by both microorganisms and insects, but also serve as storage proteins in addition to regulating endogenous proteases during different stages of development [7].

Many phytopathogenic bacteria are known to produce extracellular proteinases [8] which may play an active role in the development of diseases [9]. In response to such attack by proteinases, plants synthesize inhibitory polypeptides that can suppress the enzyme activities. Some of the serpins, cystatins, pepstatins and metallo protease inhibitors have been reported to possess antimicrobial activities [10]. Trypsin inhibitors from the seeds of chinese white cabbage and bottle gourd are reported to possess antibacterial activities [11]-[12]. Proteinase inhibitors, Mungoin from mung bean and Potide G from potato tubers exhibited both antifungal and antibacterial activities [13]-[14].

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 and serve as cardiac tonic, aphrodisiac, diuretic and antispasmodic agents.

Although *Abelmoschus moschatus* seed proteinase inhibitors were found to be active against trypsin, chymotrypsin and elastase, their differential influence on the growth of bacteria is not yet examined. In our earlier study, we have presented the effect of AMTI-II on the growth of selective bacterial strains [15] and in the present paper, we present the data on their comparative antibacterial effects.

2. Literature survey

The frequency of life-threatening infections caused by pathogenic microorganisms has increased worldwide and is becoming an important cause of morbidity and mortality in developing countries [16]. For a long time, plants have been an important source of natural products for human health. The antimicrobial properties of plants have been investigated by a number of studies worldwide and many of them have been used as therapeutic alternatives because of their antimicrobial properties [17]. Plants have many antimicrobial properties as secondary metabolites such as alkaloids, phenolic compounds, etc. The practice of complementary and alternative medicine is now increasing in developing countries in response to WHO directives culminating in several pre-clinical and clinical studies that have provided the scientific basis for the efficacy of many plants used in folk medicine to treat infections [18]-[19].

Abelmoschus moschatus (L.) Medic, family *Malvaceae,* is an aromatic and medicinal plant popularly known as Mushkdana / Kasturi bhendi. Ambrette is a close relative to Okra and it is cultivated commercially in India (mainly in the Deccan and Carnatic), Java, Madagascar and in parts of Central and South America.

The weedy shrub grows just over a meter in height with soft hairy stems and a long slender tap root. Leaves are alternate, rough, hairy and heart-shaped. Flowers resemble those of the hibiscus and its distinctive seed pods look so similar to okra that the plant is sometimes referred to as “musk okra” or “ornamental okra.” Seeds are contained within hairy

capsules up to 8cm long, which are tough but papery. When mature, the pods split open to reveal kidney-shaped seeds that have a sweet, flowery, heavy fragrance. It is in flower from July to September and the seeds ripen from August to October.

The seeds are added to coffee and unripe pods, leaves and new shoots are eaten as vegetables. Green seed pods are made into pickles and the leaves and shoots are also edible when tender. Seeds contain 11.1% moisture, 31.5% crude fiber; 14.5% lipids, 13.4% starch, 2.3% protein, volatile oil (0.2-0.6%) and ca/ 5% resin.

Seeds of *Abelmoschus moschatus***,** according to Unani system of medicine, check excessive thirst, cure for stomatitis, dyspepsia, urinary discharge, gonorrhea, leucoderma and itchiness. The roots and leaves are administered for gonorrhea. The seeds are also used as stimulant, relaxant and also for casting out the poison of snakes. The seeds serve as cardiac tonic, aphrodisiac, diuretic and antispasmodic agents.

3. Problem definition

Recently, the rapid emergence of microbial pathogens that are resistant to currently available antibiotics has triggered considerable interest in the isolation and investigation of the mode of action of antimicrobial proteins [20]. In recent years, appearance of new mutant strains of bacteria resistant to commonly used antibiotics have stimulated a systematic analysis of natural products for bactericidal properties having therapeutic applications.

Protease inhibitors are ubiquitous in nature and in plants, they are abundant in tubers and seeds [21] and are generally believed to act as storage proteins and as defense mechanism [22]. Protease inhibitors control the action of proteases that are indispensable for the growth and development of the organism. They play an important role in the protection of plant tissues from pest and pathogen attack by virtue of an antinutritional interaction [23].

Generation of a protein with proteinase inhibitory and antimicrobial activities would be advantageous to the host against a pathogen attack. The role of protease inhibitors in protection of plants against insects is studied relatively well. On the other hand, data on the role of protease inhibitors against bacterial infections are very few and need to be provided more.

4. Materials and methods

4.1 Source of trypsin inhibitors

Trypsin inhibitors (AMTI-I, AMTI-II, AMTI-III, AMTI-IV) have been isolated earlier following conventional methods of protein purification – thermal denaturation, ammonium sulphate fractionation, DEAE-cellulose ion exchange chromatography and Sephadex gel filtration. These trypsin inhibitors were used for the antibacterial assays.

4.2 Test pathogens

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) were collected from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh.

4.3 Antibacterial Activity

Trypsin inhibitors (AMTI-I, AMTI-II, AMTI-III and AMTI-IV) were subjected to antibacterial assay using the agar well diffusion method of Murray [24] modified by Olurinola [25].

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 (6 mm) 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 each 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.

4.4 Minimum inhibitory concentration (MIC) assays

Minimum Inhibitory Concentrations (MIC) of four inhibitors were determined according to the method of Elizabeth [26]. A series of two fold dilution of each inhibitor, ranging from 500-2000 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 each inhibitor ranging from 25-2000 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 inhibiting visible growth of each organism on the agar plate.

5. Results and Discussion

In the present study, significant antibacterial activity was exhibited by the four trypsin inhibitors.

Table -1 shows the effect of AMTI-I and AMTI-II on the growth of both Gram positive and Gram negative bacteria. Both the inhibitors strongly affected the growth of *Staphylococcus aureus* followed by *Escherichia coli*,

Proteus vulgaris, Bacillus subtilis, Streptococcus pneumoniae, Bacillus cereus with zones of inhibition recorded as 26mm, 25mm, 25mm, 23mm, 21mm and 20mm for AMTI-I and 28mm, 27mm, 26mm, 25mm, 25mm and 24mm for AMTI-II at a concentration of 50 µg of the inhibitors respectively. The growth of *Klebsiella pneumoniae, Pseudomonas aeruginosa, Pseudomonas syringae and Streptococcus pyogenes* was affected moderately by both the inhibitors with zones of inhibition recorded as 15mm, 15mm, 14mm and 13mm for AMTI-I and 17mm, 16mm, 16mm and 15mm for AMTI-II at a concentration of 100 µg of the inhibitors respectively.

As can be seen from table -2, AMTI-III and AMTI-IV moderately affected the growth of *Staphylococcus aureus* followed by *Escherichia coli, Proteus vulgaris, Bacillus subtilis, Streptococcus pneumoniae, Bacillus cereus* with zones of inhibition recorded as 19mm, 19mm, 18mm, 18mm, 18mm and 15mm for the former and 18mm, 17mm, 17mm, 16mm, 16mm and 14mm for the latter inhibitors at a concentration of 100 µg respectively. The growth of *Klebsiella pneumoniae, Pseudomonas aeruginosa, Pseudomonas syringae and Streptococcus pyogenes* was weakly affected by both the inhibitors with zones of inhibition noted as 9mm, 10mm, 9mm and 10mm for AMTI-III and 10mm, 9mm, 10mm and 11mm for AMTI-IV at a concentration of 100 µg of the inhibitors respectively. Rifampicin (20µg), Tetracycline (20µg), and Benzyl penicillin (20µg), on the other hand, produced an inhibition zone of 30-32 mm as controls (fig.1).

Minimum inhibitory concentrations (MIC) of the four inhibitors for their antibacterial activity were presented in Table- 3. MIC's for *Staphylococcus aureus*, *Escherichia coli, Proteus vulgaris, Bacillus subtilis, Streptococcus pneumoniae, Bacillus cereus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Pseudomonas syringae and Streptococcus pyogenes* were found to be 62.5 µg/ml, 62.5 µg/ml, 62.5 µg/ml, 125 µg/ml, 125 µg/ml, 125 µg/ml, 250 µg/ml, 250 µg/ml, 250 µg/ml and 250 µg/ml for AMTI-I and 62.5 µg/ml, 62.5 µg/ml, 62.5 µg/ml, 62.5 µg/ml, 62.5 µg/ml, 125 µg/ml, 125 µg/ml, 250 µg/ml, 250 µg/ml and 250 µg/ml for AMTI-II respectively.
The MIC's were found to be 125 µg/ml for *Staphylococcus aureus*, *Escherichia coli, Proteus vulgaris*, 250 µg/ml for *Bacillus subtilis, Streptococcus pneumoniae, Bacillus cereus, Klebsiella pneumoniae, Pseudomonas aeruginosa* and 500 µg/ml for *Pseudomonas syringae and Streptococcus pyogenes* for AMTI-III and AMTI-IV.

Table 1: Effect of AMTI-I and AMTI-II on bacterial growth

Name of the bacterial strain	*Zone of Inhibition (Diameter in mm)*						
	AMTI-I		*AMTI – II*		*Positive controls*		
	25 µg	*50 µg*	*25 µg*	*50 µg*	*Tetracycline (20µg)*	*Rifampicin (20µg)*	*Benzyl Penicillin (20µg)*
Staphylococcus aureus	12	26	14	28	32	31	32
Bacillus subtilis	11	23	13	25	31	30	31
Bacillus cereus	10	20	12	24	32	31	32
Escherichia coli	10	25	12	27	31	32	30
Proteus vulgaris	11	25	13	26	29	30	32
Streptococcus pneumoniae	12	21	13	25	29	31	30
50 µg		**100 µg**			**50 µg**	**100 µg**	
Klebsiella pneumoniae	7	15	9	17	31	30	32
Pseudomonas aeruginosa	8	15	8	16	30	31	29
Pseudomonas sringae	8	14	9	16	29	30	31
Streptococcus pyogenes	7	13	8	15	31	30	30

Bacterial strains were spread on agar plates. Different amounts of AMTI- I and AMTI-II (25 µg, 50 µg and 100 µg) were placed in the wells. Controls contained Tetracycline, Rifampcin and Benzyl Penicillin (20µg) in place of inhibitors. The incubation period was 24 h at37^{0}C. Zone of inhibition was measured as described in methods.

Table 2: Effect of AMTI-III and AMTI-IV on bacterial growth

Name of the bacterial strain	Zone of Inhibition (Diameter in mm)						
	AMTI-III		AMTI – IV		Positive controls		
	50 µg	100 µg	50µg	100 µg	Tetracycline 20µg	Rifampicin 20µg	Benzyl Penicillin 20µg
Staphylococcus aureus	10	19	9	18	32	31	32
Bacillus subtilis	9	18	8	16	31	30	31
Bacillus cereus	7	15	7	14	32	31	32
Escherichia coli	11	19	10	17	31	32	30
Proteus vulgaris	10	18	9	17	29	30	32
Streptococcus pneumoniae	11	18	10	16	29	31	30
Klebsiella pneumoniae	5	9	6	10	31	30	32
Pseudomonas aeruginosa	6	10	6	9	30	31	29
Pseudomonas sringae	5	9	5	10	29	30	31
Streptococcus pyogenes	6	10	6	11	31	30	30

Bacterial strains were spread on agar plates. Different amounts of AMTI- III and AMTI-IV (50 µg and 100 µg) were placed in the wells. Controls contained Tetracycline, Rifampcin and Benzyl Penicillin (20µg) in place of AMTI. The incubation period was 24 h at37^{0}C. Zone of inhibition was measured as described in methods.

Table 3: Minimum Inhibitory concentrations (MIC) of AMTI

Name of the bacterial strain	Minimum Inhibitory Concentration (µg/ml)			
	AMTI-I	AMTI – II	AMTI-III	AMTI - IV
Staphylococcus aureus	62.5	62.5	125	125
Bacillus subtilis	125	62.5	250	250
Bacillus cereus	125	125	250	250
Escherichia coli	62.5	62.5	125	125
Proteus vulgaris	62.5	62.5	125	125
Streptococcus pneumoniae	125	62.5	250	250
Klebsiella pneumoniae	250	125	250	250
Pseudomonas aeruginosa	250	250	250	250
Pseudomonas syringae	250	250	500	500
Streptococcus pyogenes	250	250	500	500

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

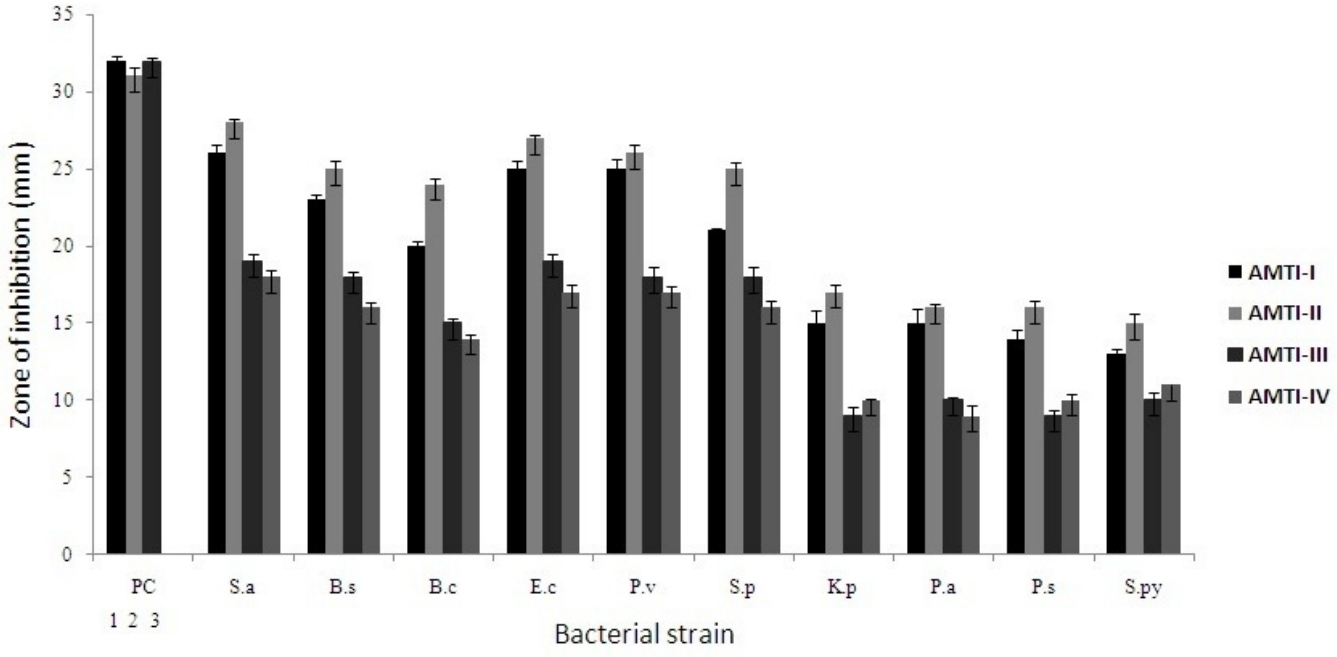

Figure 1: Antibacterial activity of *Abelmoschus moschatus* trypsin inhibitors

PC- Positive controls: 1. Tetracycline. 2. Rifampicin. 3. 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 pneumoniae; K.p - Klebsiella pneumoniae; P.a - Pseudomonas aeruginosa; P.s - Pseudomonas syringae; S.py - Streptococcus pyogenes

Bacterial strains were spread on agar plates. Different amounts of AMTI- I and AMTI-II (25 µg, 50 µg and 100 µg) and AMTI- III and AMTI-IV (50 µg and 100 µg) were placed in the wells. Controls contained Tetracycline, Rifampcin and Benzyl Penicillin (20µg) in place of inhibitors. The incubation period was 24 h at37^{0}C. Zone of inhibition was measured as described in methods.

From the results obtained, it is clear that AMTI-I and AMTI-II exerted a significant inhibitory effect on the growth of selected bacterial strains at a concentration of 50 µg and the other two inhibitors, AMTI-III and AMTI-IV were unable to produce a similar effect even when their concentration was doubled (100 µg). The inhibitors, however, did not discriminate Gram positive and Gram negative bacteria in their antibacterial activity.

It is well known that some plant proteinase inhibitors possessed *in vitro* antibacterial activities. The inhibitors, AMTI-I, AMTI-II, AMTI-III and AMTI-IV have exhibited

antibacterial activities with varying degrees. AMTI-I and AMTI-II significantly inhibited the growth of bacterial strains in a dose dependent manner. The other two inhibitors, AMTI-III and AMTI-IV moderately inhibited the growth of microorganisms tested.

The inhibitors are 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* [12]-[13].

Some proteinase inhibitors have shown both antibacterial and antifungal activities. Kim *et al*. [15] 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.* A protease inhibitor from the leaves of *Coccinia grandis* strongly inhibited the growth of pathogenic microbial strains including *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Escherichia coli*, *Bacillus subtilis* [27]. A trypsin inhibitor from soap nut seeds (SNTI) has been reported to exert potent antibacterial activity against *Staphylococcus aureus, Bacillus subtilis, Proteus vulgaris and Escherichia coli* [28].

Several kunitz proteinase inhibitors have shown potential antimicrobial activity against Gram positive and Gram-negative bacteria. Inhibitors possessing bactericidal activity include those from the corms of *Xanthosoma blandum,* active against *Staphylocccus aureus, Salmonella typhimurium*, and *Escherichia coli* [29] and seeds of *Achyranthes aspera* (AATI) active against *Proteus vulgaris*, *Bacillus subtilis*, *Staphylocccus aureus*, *Escherichia coli* and *Klebsiella pneumonia* [30].

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 forms such channel is yet to be established.

6. Conclusion

In conclusion, the purified trypsin inhibitors from the seeds of *Abelmoschus moschatus* were found to be active against selected bacterial strains with varying efficiencies. These inhibitors did not differentiate gram positive bacteria from gram negative bacteria in their antibacterial activity. Results obtained in the present study suggest that trypsin inhibitors AMTI-I and AMTI-II from *Abelmoschus moschatus* may serve as good candidates for the development of novel antimicrobial agents.

7. Acknowledgement

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8. Future scope

Protease inhibitors with antimicrobial activity will find more applications in medical, pharmaceutical and agricultural fronts compared to trypsin inhibitors alone. Results obtained in the present study support that the trypsin inhibitors from *Abelmoschus moschatus* can find application in the medical front as therapeutic agents for infections caused by specific bacterial strains and comparative studies help in identifying a powerful one among the inhibitors. They can also be explored in the agricultural front for developing transgenics after carrying out extensive *in vitro* studies against midgut proteases of insect pests. It is worth identifying the portion (domain) of the protein exhibiting both the activities for its applications.

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