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.

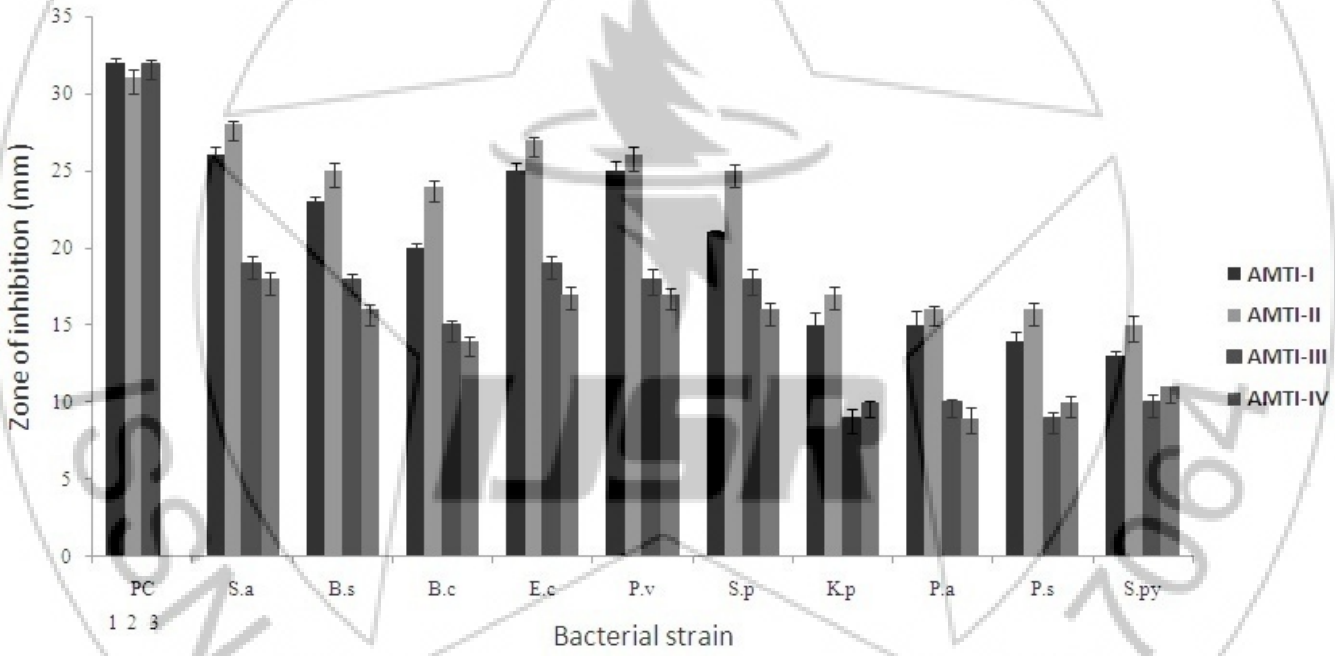

Figure 1: Antibacterial activity of *Abelmoschus moschatus* trypsin inhibitors

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.

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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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