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IN VITRO GUT PROTEOLYTIC INHIBITION OF PURIFIED *ADENANTHERA PAVONINA* PROTEINASE INHIBITOR (APPI) IN *SPODOPTERA LITURA* (F.)

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ABSTRACT

The gut proteolytic activity of *Spodoptera litura* (F.) during second, third, fourth and fifth instar was analysed in this study. A higher proteolytic (1.85± 0.402 and 1.91 ± 0.077 µmole tyrosine released/min/ml), trypsin (9.29 ± 0.012 and 19.34 ± 0.342 µmole *p*-nitroaniline released/min/ml) and chymotrypsin activity (14.44 ± 0.081 and 19.95 ± 0.055 µmole *p*- nitroaniline released/min/ml) were observed in the fourth and fifth instars, respectively. The activity of chymotrypsin was predominant during all the instars. Proteinase inhibitor was isolated and purified from the red lucky seed, *Adenanthera pavonia* L. In vitro bioassay was done to evaluate the inhibitory potential of purified *A. pavonina* proteinase inhibitors (ApPI) at 1.25, 2.50, 3.75 and 5.00 µg on the proteolytic, trypsin and chymotrypsin activity during fourth and fifth instars, using specific substrates viz., casein, BA*p*NA and SAAPF*p*NA, respectively, in comparison with standard synthetic inhibitors viz., Soybean Bowman Brik Inhibitor (SBBI) and Soybean Trypsin Inhibitor (SBTI). The results revealed that the gut total proteolytic, trypsin and chymotrypsin enzymes of *S. litura* were more sensitive to the purified ApPI (5 µg), during fourth and fifth instars, which were statistically equivalent to the standard inhibitors (SBBI and SBTI). Hence, it is concluded that purified *A. pavonina* proteinase inhibitor (ApPI) is a potential and promising inhibitor of both chymotrypsin and trypsin in *S. litura* and can be effectively used as bio-insecticidal tool.

Key words: *Spodoptera litura*, trypsin, chymotrypsin, gut proteolytic activity, larval instars, fourth, fifth, SBBI, SBTI, ApPI, potential

Tobacco caterpillar, *Spodoptera litura* (F.) (Noctuidae: Lepidoptera) is the most notorious and polyphagous insect pest. It has wide host range, which feed on 150 species of plants (Rao et al., 1993). All these crops are heavily affected by this insect pest during their growing stages, resulting in heavy yield loss varied from 10 to 30% (Bhagat and Kulkarni, 2012). Proteinase inhibitors (PI) in plants regulates the endogenous proteinase activity and act as a defensive compound against herbivorous insects (Zhu-Salzman and Zeng, 2015). They are chiefly stored in seeds and tubers to an extent of 6 to 10% (Ryan, 1990). The PIs have the ability to bind with insect digestive proteinases and thereby hindering the enzymatic digestion. They are primarily targeting sulphur containing amino acids, which are highly essential for the growth and development of the insects (Broadway, 1986 and Gatehouse et al., 1992). Several works revealed that PIs effectively interfered in the activity of gut proteases both in vitro (Koiwa et al., 1998) and in vivo bioassays (Urwin et al., 1997; Vain et al., 1998). The red lucky seed, *Adenanthera pavonina* L. (Leguminosae) was reported to possess trypsin inhibitory activity (Prabhu and Pattabiraman, 1980; Macedo et al., 2004, Migliolo et al., 2010, Chandrashekharaiah et al., 2017). Sowbhagya et al. (2019) reported that *A. pavonina*, possess trypsin inhibitor activity of 11235.82 TIU g^{-1} seed, 96.06 mg g^{-1} seed protein content and specific activityof116.97 TIU mg^{-1} protein. Present study focuses on deciphering the impact of purified proteinase inhibitor isolated from *A. pavonina* on the gut proteolytic activity of the polyphagous insect pest, *S. litura*.

MATERIALS AND METHODS

The seeds of *A. pavonina* were collected from Agricultural College and Research Institute, Madurai. Trypsin enzyme substrate, BA*p*NA (B4875 – Nα-

Benzoyl-DL-arginine 4-nitroaniline), chymotrypsin enzyme substrate, SAAPF*p*NA (S7388–N-succinyl-alanine-alanine-proline-phenylalanine-p- nitroaniline) and standard inhibitors viz., Soybean Trypsin Inhibitor (SBTI) (T9003) and Soybean Bowman-Brik inhibitor (SBBI) (T9777) were purchased from M/s. Sigma Aldrich, Mumbai, Sephadex G-75 from M/s. MP Biomedicals, France, dialysis tube (LA 393-10 MT having 12,000-14,000 molecular weight cut off) from M/s. Hi-media chemicals and other chemicals used for analysis were analytical grade(AR), purchased through TNAU rate contract.

The target proteinase inhibitor (ApPI) was isolated and purified from the seeds of *A. pavonina* in the NADP sponsored Central Instrumentation Laboratory, Agricultural College and Research Institute, Madurai, as detailed below. ApPI was isolated from the seeds following the procedure of Maggo et al., 1999. Purification of isolated ApPI was done by ammonium sulfate precipitation (Babu et al., 2012) and gel filtration using the Sephadex G-75 (Sasaki et al., 2015).

The initial culture of *S. litura*, egg mass was purchased from National Bureau of Agricultural Insect Resources (NBAIR), Bangalore and the accession number was NBAII-MP-NOC-02. The mass culturing was done using the semi-synthetic diet, as per the NBAIR recommendation, in the Insectary, Department of Agricultural Entomology, Agricultural College and Research Institute, Madurai. The rearing tray and other materials used for rearing purpose were disinfected with 0.1% formaldehyde solution. The diet composed of fraction A. chickpea flour 105g, Methyl para- hydroxyl benzoate 2g, sorbic acid 1g, streptomycin sulphate 0.25g, fraction B. agar-agar 12.75g and fraction C. yeast 40g, ascorbic acid 3.25g, multivitamin capsules 2, vitamin E 1g, 10% formaldehyde 2ml and distilled water 780 ml. Using these products the semi-synthetic diet was prepared and reared by the method followed by the NBAIR, Bangalore.

Midguts of *S. litura* larvae were dissected from second, third, fourth and fifth instar larvae, after 4 hours starvation, in ice cold water containing 0.15M NaCl, for maintaining iso-osmotic condition (Swathi et al., 2016). Gut extract was prepared by macerating the dissected gut (5 guts) in 0.2M Glycine-NaOH buffer (1ml) pH 10.00 containing 2mM dithiothreitol (DTT) and 10% PVP in ice cold pestle and mortar. Then the extract was kept at 10°C for 2 hrs and centrifuged at 12,000 rpm for 15 minutes. The resulting supernatant, called gut enzyme extract, was stored at -20°C one month without loss of acrivity for further assay (Babu et al., 2012). This gut enzyme extract was used as stock solution and its protein content was estimated for each instar separately. Samples were drawn from this enzyme extract stock solution and used for the assay on enzyme activity and the inhibition studies.

The total proteolytic activity in the *S. litura* gut enzyme extract, during second, third, fourth and fifth instar, was estimated as described below. The proteolytic reaction was initiated by incubation of 2µl of gut enzyme extract with 500 µl of 1% casein (substrate), which was prepared using 0.2 M Glycine-NaOH buffer (pH 10.0), for 30 min at 4°C. The gut proteolytic action was stopped by addition of 500 µl of 20% trichloroacetic acid and incubated for 15 min at room temperature. After centrifugation for 15 min at 15,000 × g, absorbance of supernatant was measured at 280 nm against the endogenous tube (without gut extract) (Kembhavi et al., 1993). The liberated tyrosine was measured in the 280 nm using nano spectrometer (Eppendrof BioPhotometer D30). The extinction coefficient for tyrosine is 1.34 µmole^{-1}cm^{-1}. The total proteolytic activity was estimated using the formulae and expressed as µmole tyrosine released per min per ml (Nakanishi et al., 1974).

$$\text{Enzyme activity (total proteolysis)} = \frac{(\text{Abs}_{280}\ \text{nm–blank})/\ \text{min x total volume of reaction mixture}}{1.34\ \mu\text{mole}^{-1}\ \text{cm}^{-1}\ \text{x volume of sample x incubation time}}$$

The activity of trypsin and chymotrypsin in insect midgut of second, third, fourth and fifth instar were assessed using specific substrates. Trypsin enzymatic activity was measured using substrate BA*p*NA (Erlanger, 1961). Gut extract (2 µl) from different instars (2nd, 3rd , 4th and 5th instar) were taken individually and made upto 200µl using 0.2 M Glycine NaoH buffer, pH 10.0 and incubated it under 37°C for 10 minutes. Then the reaction was started by adding 0.01M Tris-HCl containing 0.02 M $CaCl_2$ and 1mM BA*p*NA. The reaction mixture was maintained in 37°C for 10 minutes and later the reaction was stopped by addition of 30% Glacial acetic acid (GAA) (200µl) (Godbole et al., 1994). SAAPF*p*NA was used as a substrate for measuring the chymotrypsin activity (Johnston et al., 1991 and Burgess et al., 1994). The buffer used was 0.1 M Tris-HCl, pH 8.5 containing 0.01 M CaCl2 (Del Mar et al., 1979). The reaction was carried out as described above, except that the incubation temperature

was 30˚C. The liberated *p*-nitroaniline was measured at 410 nm. The extinction coefficient for *p*-nitroaniline is e=8.8 M^{-1} cm^{-1}. The trypsin/ chymotrypsin activity was estimated using the formulae and expressed as µmole *p*-nitroaniline released per min per ml (Srichanun et al., 2012).

Enzyme activity (trypsin/ chymotrypsin)

$$= \frac{(Abs_{410}\ \text{nm–blank})/\ \text{min} \times \text{total volume of reaction mixture}}{8.8\ \mu\text{mole}^{-1}\ \text{cm}^{-1} \times \text{volume of enzyme} \times \text{incubation time}}$$

Based on the above in vitro assays, the larval instars exhibiting peak total proteolytic, trypsin and chymotrypsin activity were selected and the gut enzyme extract collected from those larval instars were used for testing the efficiency of test inhibitor, ApPI.

The inhibitory potential of purified ApPI was tested against *S. litura* larval gut proteolytic activity in comparison with standard synthetic inhibitors viz., SBBI (for total proteolytic and chymotrypsin activity) and SBTI (for trypsin activity) at different concentrations viz., 1.25, 2.50, 3.75 and 5.00 µg under in vitro condition. The enzyme inhibitory assay was done as per the methodology described earlier, but the inhibitors were added before the addition of respective substrates and reaction was continued. Endogenous tube (without gut extract) was maintained. Finally, the specific activity of total proteinases after inhibition was estimated using the formula and expressed as µmole tyrosine released per min per milligram of protein **(**Nakanishi et al., 1974).

Specific activity of total proteases

$$= \frac{(Abs_{280}\ \text{nm–blank})/\ \text{min} \times \text{total volume of reaction mixture}}{1.34\ \mu\text{mole}^{-1}\ \text{cm}^{-1} \times \text{volume of sample} \times \text{incubation time} \times \text{protein content (in mg)}}$$

The specific activity of gut for trypsin and chymotrypsin after inhibition was estimated by the following formula and expressed as µmole *p*-nitroaniline released per min per milligram of protein (Srichanun et al., 2012).

Specific activity (trypsin/ chymotrypsin)

$$= \frac{(Abs_{410}\ \text{nm–blank})/\ \text{min} \times \text{total volume of reaction mixture}}{8.8\ \mu\text{mole}^{-1}\ \text{cm}^{-1} \times \text{volume of enzyme} \times \text{incubation time} \times \text{mg protein in reaction mixture}}$$

The % inhibition over control was estimated based on the residual enzyme activity after addition of inhibitor as follows:

Per cent inhibition over control

$$= \frac{\text{Enzyme activity in control} - \text{Enzyme activity after addition of inhibitor}}{\text{Enzyme activity in control}} \times 100$$

The protein content in gut enzyme extract was estimated by dye-binding method (Bradford et al., 1976), where Bovine serum albumin (BSA) was used as a standard. All in vitro assays were carried out with five replications under Completely Randomized Block Design (CRBD). Standard deviations were calculated from the replicated values and analyzed in MS-Excel. Data on enzyme activity and per cent inhibition over control were analyzed using SPSS (version 17.0) software package to find Analysis of Variance (ANOVA). Grouping was done according to the Duncan's Multiple Range Test (DMRT) (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

The total midgut proteolytic, trypsin and chymotrypsin activity of *S. litura* was estimated using different substrates viz., casein, BA*p*NA and SAAPF*p*NA, respectively, during second, third, fourth and fifth instars. The purified *A. pavonina* proteinase inhibitor (ApPI) was evaluated at different concentration for its inhibitory potential against the midgut total proteolytic, trypsin and chymotrypsin activity of *S. litura* during fourth and fifth instars, as these instars exhibited peak enzyme activity, in comparison with standard synthetic inhibitors (SBBI and SBTI). The results are presented and discussed below.

Midgut proteolytic activity: In *S. litura* larvae, maximum total proteolytic (1.9± 0.077 µmole tyrosine released/min/ml), trypsin (BA*p*NA) (19.34± 0.342 µmole *p*-nitroaniline released/min/ml) and chymotrypsin activity (SAA*p*FNA) (19.95± 0.05 µmole *p*-nitroaniline released/min/ml) were recorded during fifth instar (Table 1). The total proteolytic activity in fourth instar (1.85 ± 0.402 µmole tyrosine released/min/ml) was statistically on par with fifth instar, whereas activity of trypsin and chymotrypsin were significantly different between fourth (9.29± 0.012 and 14.44 ± 0.081 µmole *p*-nitroaniline released per min per ml) and fifth instars. This result reveals that the proteolytic activity was enhanced progressively with larval growth. Ahmed et al. (1976), also stated that fifth instar larva

of *S. litura* showed high proteolytic activity. It may be due to the fact that final instar larvae require high proteinaceous diet, which might influence the larva to secrete more proteinase enzyme. It was supported by the findings of Lee (2010), who revealed that *S. litura* female caterpillars preferred to consume the diet rich in protein during their final instar, which is essential for the vitellogenesis (Boggs, 2009). The present study revealed that the mean chymotrypsin activity (10.17 µmole *p*-nitroaniline released/min/ml) was higher than the mean trypsin activity (5.53 µmole *p*-nitroaniline released/min/ml) (Table 1). This finding was in agreement with the findings of Broadway & Duffey (1986), Johnson et al. (1989) Johnston et al. (1995) and Srinivasan et al. (2006), who reported that *Spodoptera exigua* and *S. frugiperda* (trypsin 7%, chymotrypsin 85%), and *Manduca sexta* (trypsin 10%, chymotrypsin 80%) had high chymotrypsin activity than the trypsin activity. Hence, it is understood that the chymotrypsin activity in the larvae of *S. litura* was higher than the trypsin activity.

Effect of ApPI on larval gut total proteolytic activity: The results disclosed that total proteolytic activity was found to be hindered by the addition of 5µg of purified *A. pavonina* proteinase inhibitor (ApPI) (53.23 ± 1.97 % and 75.85 ± 2.84%) and the standard SBBI inhibitor (51.40 ± 2.71% and 50.19 ± 2.71%) during fourth and fifth instar, respectively (Table 2). This outcome was supported by the findings that proteinase inhibitor from chick pea exhibited better inhibition (69%) of total gut proteolytic activity of *H. armigera* than the standard (41%) (SKTI) soybean Kunitz trypsin inhibitor (Srinivasan et al., 2005). *Acacia nilotica* proteinase inhibitor (AnPI) at 5µg was effective against the total proteolytic activity of *H. armigera* and caused 86.25% inhibition over control (Babu et al.,2012). Da Silva et al. (2014) reported that purified inhibitor of ApPI showed 47 per cent inhibition of total gut proteolysis in *Diatraea saccharalis*. These verdicts corroborated with our findings.

Effect of ApPI on larval gut trypsin activity: The inhibition of trypsin activity was noticed at all levels of purified ApPI and SBTI, when related to control. The extent of inhibition due to purified ApPI ranged from 86.55 to 92.35 per cent during fourth instar and 84.39 to 89.69 per cent during fifth instar, while, the standard synthetic inhibitor (SBTI) inflicted inhibition of trypsin activity to the tune of 88.44 to 92.52 and 84.27 to 89.74 per cent during fourth and fifth instar, respectively (Table 3).

During fourth instar, the addition of purified ApPI and SBTI (5.00 µg) resulted in decline in the trypsin activity 0.71 ± 0.040 and 0.69 ± 0.025 µmole *p*-nitroaniline released per min per ml, respectively (Table 3). The extent of inhibition of the trypsin activity was 92.35 ± 0.439 and 92.52 ± 0.275 per cent, respectively. Similarly, during fifth instar, the ApPI and SBTI (5.00 µg) effectively weakened the trypsin activity (1.99 ± 0.012 and 1.98 ± 0.012 µmole *p*-nitroaniline released per min per ml, respectively). While the control exhibited higher trypsin activity (19.34 ± 0.342 µmole *p*-nitroaniline released per min per ml) and the per cent inhibition over control was 89.69 ± 0.062 and 89.74 ± 0.005, respectively. There was progressive increase in inhibition of trypsin activity with the increase in dose (r=0.97*).

The purified ApPI and synthetic inhibitor (5µg) and Soybean Trypsin inhibitor (SBTI) were statistically on par in exhibiting the inhibitory activity of trypsin. Similarly, SBTI and lima bean trypsin inhibitor (LBTI) showed high inhibition of gut proteases of *S. litura* (Ahmed et al. 1980). In *S. littoralis* aprotinin was potent inhibitor of trypsin as suggested by Lee and Anstee (1995). Araujo et al. (2005) reported the trypsin inhibitory effect of *Tamarindus indica* seeds against the lepidopteran pest *S. frugiperda*. Macedo et al. (2010) testified the effect of *A. pavonina* trypsin inhibitor, which caused inhibition of 91.5% digestive trypsin enzymes in *S. frugiperda*. This statement is in agreement with our results. Katoch et al., (2015) stated that effect of rice bean trypsin inhibitor was very good against the *S. litura.* In addition, our results proved that ApPI is also a potent inhibitor of *S. litura* trypsin enzyme.

Effect of ApPI on larval gut chymotrypsin activity: There was significant difference among per cent inhibition of *S. litura* gut chymotrypsin activity and specific activity due to standard SBBI and purified ApPI at different concentration studied, during the fourth and fifth instar. In control, chymotrypsin activity was high (14.44 ± 0.08 µmole *p*-nitroaniline released/ min/ml), the addition of purified ApPI inhibitor (5.00 µg) resulted in maximum inhibition of chymotrypsin activity (0.04 ± 0.001 µmole *p*-nitroaniline released/ min/ml) than the standard inhibitor (5.00 µg) (0.10 ± 0.003 µmole *p*-nitroaniline released/min/ml). The inhibition of chymotrypsin over control were high due to ApPI at 5 µg, during fourth (99.66 ± 0.01%) and fifth (98.34 ± 0.011) instar.

These findings are supported by the report of

Table 1. In vitro analysis of *S. litura* midgut proteolytic activity

Larval stage	Total proteolytic activity (µmole tyrosine released/ min/ml)*	Trypsin activity (µmole *p*-nitroaniline released/min/ml)*	Chymotrypsin activity (µmole *p*-nitroaniline released/ min/ml)*
2nd instar	0.04 ± 0.036[c]	0.48 ± 0.037[d]	2.00 ± 0.013[d]
3rd instar	0.33 ± 0.068[b]	0.99 ± 0.007[c]	4.29 ± 0.060[c]
4th instar	1.85±0.402[a]	9.29 ± 0.012[b]	14.44 ± 0.081[b]
5th instar	1.91 ± 0.077[a]	19.34 ± 0.342[a]	19.95 ± 0.055[a]
Mean	1.03	5.53	10.17

* Values mean± SD- five replications; In column, means followed by same letters not significantly different (DMRT; p=0.05).

Table 2. Inhibition of *S. litura* larval gut total proteolytic activity by ApPI and synthetic inhibitor (SBBI)

Inhibitor source		Larval stage					
		4th instar			5th instar		
		Total proteolytic activity (µmole tyrosine released/ min/ml)*	Specific activity of total proteolysis (µmole tyrosine released/min/ mg of protein)*	% Inhibition of total proteolytic activity over control*	Total proteolytic activity (µmole tyrosine released/ min/ml)*	Specific activity of total proteolysis (µmole tyrosine released/min/ mg of protein)*	% Inhibition of total proteolytic activity over control*
A.pavonina (ApPI)	1.25 µg	1.38 ± 0.10[cd]	0.79 ± 0.060[cd]	25.04 ± 6.66[d]	1.78 ± 0.04[ef]	0.73 ± 0.020[ef]	6.69 ± 2.60[f]
	2.50 µg	1.15 ± 0.07[bc]	0.66 ± 0.042[bc]	27.36 ± 4.72[c]	1.37 ± 0.09[d]	0.56 ± 0.041[d]	28.22 ± 5.21[d]
	3.75 µg	0.98 ± 0.04[b]	0.56 ± 0.025[b]	38.07 ± 2.82[b]	1.08 ± 0.04[c]	0.44 ±0.018[c]	43.23 ± 2.35[c]
	5.00 µg	0.74 ± 0.03[a]	0.42 ± 0.017[a]	53.23 ± 1.97[a]	0.46 ±0.05[a]	0.19 ± 0.002[a]	75.85 ± 2.84[a]
Standard SBBI (Sigma)	1.25 µg	1.48 ± 0.01[d]	0.85 ± 0.010[d]	6.46 ± 0.90[e]	1.86 ± 0.02[fg]	0.77 ± 0.010[fg]	2.34 ± 1.35[g]
	2.50 µg	1.21 ± 0.07[bc]	0.69 ± 0.040[bc]	23.70 ± 4.97[c]	1.69 ± 0.05[e]	0.70 ± 0.020[e]	11.04 ± 2.63[e]
	3.75 µg	1.09 ± 0.03[b]	0.62 ± 0.021[b]	31.28 ± 2.71[bc]	1.35 ± 0.03[d]	0.56 ± 0.012[d]	29.09 ± 1.64[d]
	5.00 µg	0.77 ± 0.03[a]	0.44 ± 0.021[a]	51.40 ± 2.71[a]	0.95 ± 0.05[b]	0.39 ± 0.021[b]	50.19 ± 2.71[b]
Control	-	1.85 ± 0.40[e]	1.05 ± 0.23[e]	-	1.91 ± 0.07[g]	0.79 ± 0.03[g]	-

*Values mean± SD- 5 replications (p<0.05); In columns, means followed by same letters not significantly different (DMRT; p = 0.05); Protein content for 4th and 5th instar larval gut was 1.747 and 2.415 mg/ml, respectively.

Table 3. Trypsin Inhibitory potential of ApPI vs. synthetic inhibitor (SBTI) in *S. litura*

Inhibitor source		Larval stage					
		4th instar			5th instar		
		Trypsin activity (µmole *p*-nitroaniline released/ min/ml)*	Specific trypsin activity (µmole *p*- nitroaniline released/min/ mg of protein)*	% Inhibition over control*	Trypsin activity (µmole *p*-nitroaniline released/ min/ml)*	Specific trypsin activity (µmole *p*- nitroaniline released/min/ mg of protein)*	% Inhibition over control*
A.pavonina (ApPI)	1.25 µg	1.24 ± 0.023[f]	0.71 ± 0.013[f]	86.55 ± 0.256[f]	3.01 ± 0.066[e]	1.24 ± 0.027[e]	84.39 ± 0.344[e]
	2.50 µg	0.85 ± 0.007[c]	0.49 ± 0.004[c]	90.75 ± 0.085[c]	2.55 ± 0.083[c]	1.05 ± 0.034[c]	86.78 ± 0.432[c]
	3.75 µg	0.81 ± 0.027[b]	0.46 ± 0.015[b]	91.23 ± 0.300[b]	2.39 ± 0.015[b]	0.99 ± 0.006[b]	87.61 ± 0.082[b]
	5.00 µg	0.71 ± 0.04[0]a	0.40 ± 0.023[a]	92.35 ± 0.439[a]	1.99 ± 0.012[a]	0.82 ± 0.005[a]	89.69 ± 0.062[a]
Standard SBTI (Sigma)	1.25 µg	1.07 ± 0.007[e]	0.61 ± 0.004[e]	88.44 ± 0.085[e]	3.04 ± 0.093[e]	1.25 ±0.003[e]	84.27 ± 0.038[e]
	2.50 µg	0.94 ± 0.007[d]	0.54 ± 0.004[d]	89.81 ± 0.085[d]	2.72 ± 0.085[d]	1.12 ± 0.003[d]	85.91 ± 0.035[d]
	3.75 µg	0.81 ± 0.020[b]	0.46 ±0.011[b]	91.23 ± 0.215[b]	2.41 ± 0.021[b]	1.00 ± 0.001[b]	87.49 ± 0.008[b]
	5.00 µg	0.69 ± 0.02[5]a	0.39 ± 0.014[a]	92.52 ± 0.275[a]	1.98 ± 0.012[a]	0.82 ± 0.001[a]	89.74 ± 0.005[a]
Control	-	9.29 ± 0.012[g]	5.31 ± 0.006[g]	-	19.34 ± 0.342[f]	8.00 ± 0.14[f]	-

*Values mean± SD- 5 replications (p<0.05); In column, means followed by same letters not significantly different (DMRT; p= 0.05); protein content for 4th and 5th instar larval gut- 1.747 and 2.415 mg/ml, respectively.

Table 4. Chymotrypsin inhibitory potential of ApPI vs. synthetic inhibitor (SBBI) in *S. litura*

Inhibitor source		Larval stage					
		4th instar			5th instar		
		Chymotrypsin activity (µmole *p*- nitroaniline released/ min/ml)*	Specific chymotrypsin activity (µmole *p*- nitroaniline released/min/ mg of protein)*	% Inhibition over control*	Chymotrypsin activity (µmole *p*- nitroaniline released/ min/ml)*	Specific chymotrypsin activity (µmole *p*- nitroaniline released/min/ mg of protein)*	% Inhibition over control*
A.pavonina (ApPI)	1.25 µg	0.10 ± 0.003^{d}	0.06 ± 0.022^{d}	99.27 ± 0.025^{d}	0.47 ± 0.004^{g}	0.19 ± 0.001^{g}	97.63 ± 0.021^{g}
	2.50 µg	0.09 ± 0.002^{c}	0.05 ± 0.001^{c}	99.36 ± 0.013^{c}	0.41 ± 0.001^{f}	0.17 ± 0.001^{f}	97.93 ± 0.006^{f}
	3.75 µg	0.06 ± 0.002^{b}	0.03 ± 0.001^{b}	99.53 ± 0.016^{b}	0.35 ± 0.014^{cd}	0.14 ± 0.005^{cd}	98.24 ± 0.070^{cd}
	5.00 µg	0.04 ± 0.001^{a}	0.02 ± 0.001^{a}	99.66 ± 0.011^{a}	0.33 ± 0.002^{b}	0.13 ± 0.001^{b}	98.34 ± 0.011^{b}
Standard SBBI (Sigma)	1.25 µg	0.15 ± 0.005^{g}	0.09 ± 0.003^{h}	98.95 ± 0.021^{g}	0.37 ± 0.001^{e}	0.16 ± 0.001^{e}	98.11 ± 0.008^{e}
	2.50 µg	0.13 ± 0.002^{f}	0.08 ± 0.001^{g}	99.05 ± 0.006^{f}	0.36 ± 0.003^{d}	0.15 ± 0.001^{d}	98.19 ± 0.018^{d}
	3.75 µg	0.12 ± 0.004^{e}	0.07 ± 0.002^{f}	99.13 ± 0.070^{e}	0.34 ± 0.002^{c}	0.14 ±0.001^{c}	98.28 ± 0.011^{c}
	5.00 µg	0.10 ± 0.003^{d}	0.06 ± 0.001^{e}	99.29 ± 0.022^{d}	0.32 ± 0.004^{a}	0.13 ± 0.001^{a}	98.39 ± 0.020^{a}
Control		14.44 ± 0.081^{h}	8.27 ± 0.046^{i}	-	19.95 ± 0.055^{h}	8.26 ± 0.023^{h}	-

*Values mean± SD- 5 replications ($p<0.05$); In column,means followed by same letters not significantly different (DMRT; $p = 0.05$); protein content for 4th and 5th instar larval gut 1.747 and 2.415 mg/ml, respectively.

Macedo et al. (2004), who stated that ApPI had high activity towards gut chymotrypsin of *Callosobruchus maculatus*. *Terminalia arjuna* trypsin inhibitor (TaTI) inhibited the both trypsin and chymotrypsin activity of *Antheraea mylitta. Acacia senagal* proteinase inhibitor (ASPI) exhibited 79.44% chymotrypsin inhibition on *Helicoverpa armigera* (Babu et al., 2012). Katoch et al. (2015) reported that *S. litura* chymotrypsin activity was inhibited (78.12±0.7 %) by rice bean inhibitor during fifth instar. The present results established that the purified ApPI also possess very strong inhibitory activity towards chymotrypsin in *S. litura*.

It is concluded that among the total proteases in *S. litura* larval gut, chymotrypsin was contributing maximum share than the trypsin during second, third, fourth and fifth instar. The trypsin and chymotrypsin enzymes of *S. litura* were more sensitive to the purified ApPI, which was displayed by their inhibitory effect under in vitro condition against fourth and fifth instar larvae of *S. litura.* Purified ApPI was the strong inhibitor of chymotrypsin as like standard synthetic inhibitor, SBBI, whereas, with regard to trypsin, the efficacy was comparable to the standard inhibitor, SBTI. But there was a disadvantage, when the larvae were treated at the lower dose of ApPI, there was a possibility for development of adaptations by the production of isoenzymes. Hence, the inhibitor should be used at appropriate concentration, to overcome this disadvantage. Hence, *A. pavonina* proteinase inhibitor can be developed as promising and potential bioinsecticidal tool for the management of *S. litura.*

REFERENCES

Ahmad Z, Saleemuddin M, Siddi M.1980. Purification and characterization of three alkaline proteases from the gut of the larva of army worm, *Spodoptera litura*. Insect Biochemistry 10(6): 667-673.

Ahmad Z, Saleemuddin M, Siddiqi M.1976. Alkaline protease in the larvae of the army worm, *Spodoptera litura*. Insect Biochemistry 6(5): 501-505.

Araujo C L, Bezerra I W, Oliveira A S, Moura F T, Macedo L L, Gomes C E, Barbosa A E, Macedo F P, Souza T M, Franco O L, Bloch-J C.2005. In vivo bioinsecticidal activity toward *Ceratitis capitata* (fruit fly) and *Callosobruchus maculatus* (cowpea weevil) and in vitro bioinsecticidal activity toward different orders of insect pests of a trypsin inhibitor purified from tamarind tree (*Tamarindus indica*) seeds. Journal of agricultural and food chemistry 53(11): 4381-4387.

Babu S R, Subrahmanyam B, Santha, I. 2012. In vivo and in vitro effect of *Acacia nilotica* seed proteinase inhibitors on *Helicoverpa armigera* (Hubner) larvae. Journal of Biosciences 37(2): 269-276.

Bhagat R B, Kulkarni D K. 2012. Evaluation of larvicidal and antifeedant potential of three Jatropha species against *Spodoptera litura* (Lepidoptera: Noctuidae) and two predators (Coleoptera: Coccinellidae). Annals of Biological Research 3(6): 2911-2916.

Boggs C L. 2009. Understanding insect life histories and senescence through a resource allocation lens. Functional Ecology, 23(1): 27-37.

Bradford M M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry 72(1-2): 248-254.

Broadway R M, Duffey S S. 1986. Plant proteinase inhibitors: mechanism of action and effect on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exiqua*. Journal of Insect Physiology 32(10): 827-833.

Burgess E P J, Main C A, Stevens P S, Christeller J T, Gatehouse A M R, Laing W A. 1994. Effects of protease inhibitor concentration and combinations on the survival, growth and gut enzyme activities of the black field cricket, *Teleogtyllus commodes*. Journal of Insect Physiology 40: 803-811.

Chandrashekharaiah K S, Shashank A, Bharadwaj R, Raju N G, Swamy N R. 2017. Characterization of protease inhibitors from the seeds

of *Adenanthera pavonina*. International Journal of Biotechnology and Biochemistry 13(4): 361-370.

Da Silva W, Freire M D G M H, Parra J R P, Marangoni S, Macedo M L R. 2012. Evaluation of the *Adenanthera pavonina* seed proteinase inhibitor (ApTI) as a bioinsecticidal tool with potential for the control of *Diatraea saccharalis*. Process Biochemistry, 47(2), 257-263. doi: 10.1016/j.procbio.2011.11.002

Del Mar E G, Largman C, Brodrick J W, Geokas M C.1979. A sensitive new substrate for chymotrypsin. Analytical biochemistry 99(2): 316-320.

Erlanger B F, Kokowsky N, Cohen W. 1961. The preparation and properties of two new chromogenic substrates of trypsin. Archives of Biochemistry and Biophysics 95 (2): 271-278.

Gatehouse A M, Hilder V A, Powell K, Boulter D, Gatehouse J A. 1992. Potential of plant-derived genes in the genetic manipulation of crops for insect resistance. In Proceedings of the 8th International Symposium on Insect-Plant Relationships. pp. 221-234

Godbole S A, Krishna T G, Bhatia C R. 1994. Purification and characterisation of protease inhibitors from pigeon pea (*Cajanus cajan* (l) millsp) seeds. Journal of the Science of Food and Agriculture 64(1): 87-93.

Gomez K A, Gomez A A. 1984. Statistical procedures for agricultural research. John Wiley and Sons, Singapore 680 pp.

Johnson R, Narvaez J, An G, Ryan C. 1989. Expression of proteinase inhibitors I and II in transgenic tobacco plants: effects on natural defense against *Manduca sexta* larvae. Proceedings of the National Academy of Sciences. 86(24): 9871-9875.

Johnston K A, Lee M J, Brough C, Hilder V A, Gatehouse A M, Gatehouse J A. 1995. Protease activities in the larval midgut of *Heliothis virescens*: evidence for trypsin and chymotrypsin-like enzymes. Insect Biochemistry and Molecular Biology. 25(3): 375-383.

Johnston K A, Lee M J, Gatehouse J A, Anstee J H. 1991. The partial purification and characterisation of serine protease activity in midgut of larval *Helicoverpa armigera*. Insect Biochemistry 21(4): 389-397.

Katoch R, Sharma K, Singh S K, Thakur N. 2015. Evaluation and characterization of trypsin inhibitor from rice bean with inhibitory activity against gut proteases of *Spodoptera litura*. Zeitschrift für Naturforschung C 70(11-12): 287-295

Kembhavi A A, Kulkarni A, Pant A. 1993. Salt-tolerant and thermostable alkaline protease from *Bacillus subtilis* NCIM No. 64. Applied Biochemistry and Biotechnology 38(1-2): 83-92.

Koiwa H, Shade R E, Zhu-Salzman K, Subramanian L, Murdock L L, Nielsen S S, Bressan R A, Hasegawa P M, 1998. Phage display selection can differentiate insecticidal activity of soybean cystatins. Plant Journal 14: 371–379.

Lee K P. 2010. Sex-specific differences in nutrient regulation in a capital breeding caterpillar, *Spodoptera litura* (Fabricius). Journal of Insect Physiology 56(11): 1685-1695.

Lee M J, Anstee J H. 1995. Endoproteases from the midgut of larval *Spodoptera littoralis* include a chymotrypsin-like enzyme with an extended binding site. Insect Biochemistry and Molecular Biology 25(1): 49-61.

Macedo M L R, De Sa C M, Freire M D G M, Parra J R P. 2004. A Kunitz type inhibitor of coleopteran proteases, isolated from *Adenanthera pavonina* L. seeds and its effect on *Callosobruchus maculatus*. Journal of Agricultural and Food Chemistry 52(9): 2533-2540.

Macedo M L, Durigan R A, da Silva D S, Marangoni S, Freire M D, Parra J R. 2010. *Adenanthera pavonina* trypsin inhibitor retard growth of *Anagasta kuehniella* (Lepidoptera: Pyralidae). Archives of Insect Biochemistry and Physiology: Published in Collaboration with the Entomological Society of America 73(4): 213-231

Maggo S, Malhotra S P, Dhawan K, Singh R. 1999. Purification and characterization of protease inhibitor from rice bean (*Vigna umbellata* T.) seeds. Journal of plant biochemistry and biotechnology 8(1): 61- 64.

Migliolo L, Oliveira A S D, Santos E A, Franco O L, Sales M P D. 2010. Journal of Molecular Graphics and Modelling Structural and mechanistic insights into a novel non-competitive Kunitz trypsin inhibitor from *Adenanthera pavonina* L . seeds with double activity toward serine- and cysteine-proteinases. Journal of Molecular Graphics and Modelling 29(2): 148-156.

Nakanishi T, Matsumura Y, Minamiura N, Yamamoto T. 1974. Purification and some properties of an alkalophilic proteinase of a *Streptomyces* species. Agricultural and Biological Chemistry 38(1): 37-44.

Prabhu K S, Pattabiraman T N. 1980. Natural plant enzyme inhibitors: Isolation and characterisation of a trypsin/chymotrypsin inhibitor from indian red wood (*Adenanthera pavonina*) seeds. Journal of the Science of Food and Agriculture 31(10): 967-980.

Rao G R, Wightman J A, Rao D R. 1993. World review of the natural enemies and diseases of Spodoptera litura (F_a)(Lepidoptera: Noctuidae). International Journal of Tropical Insect Science 14(3): 273-284.

Ryan C A. 1990. Protease inhibitors in plants: genes for improving defenses against insects and pathogens. Annual review of phytopathology 28(1): 425-449.

Sasaki D Y, Jacobowski A C, De Souza A P, Cardoso M H, Franco O L, Macedo M L R. 2015. Effects of proteinase inhibitor from *Adenanthera pavonina* seeds on short- and long term larval development of *Aedes aegypti*. Biochimie, 112, 172-186. doi: 10.1016/j.biochi.2015.03.011

Sowbaghya A Y, Shanthi M, Murugan M, Kokiladevi E, Kavitha Pushpum A, Chinniah C. 2019. Proteins and trypsin inhibitors in seeds of various plants. Indian journal of Entomology 81(1): 177-181

Srichanun M, Tantikitti C, Vatanakul V, Musikarune P. 2012. Digestive enzyme activity during ontogenetic development and effect of live feed in green catfish larvae (*Mystus nemurus* Cuv . & Val .) 34(3): 247-254.

Srinivasan A, Giri A P, Gupta V S. 2006. Structural and functional diversities in lepidopteran serine proteases. Cellular & molecular biology letters 11(1): 132.

Srinivasan A, Giri A P, Harsulkar A M, Gatehouse J A, Gupta V S. 2005. A Kunitz trypsin inhibitor from chickpea (*Cicer arietinum* L.) that exerts anti-metabolic effect on podborer (*Helicoverpa armigera*) larvae. Plant Molecular Biology 57: 359-374

Swathi M, Mishra P K, Lokya V, Swaroop V, Mallikarjuna N, Dutta-Gupta A, Padmasree K. 2016. Purification and partial characterization of trypsin-specific proteinase inhibitors from pigeonpea wild relative *Cajanus platycarpus* L.(Fabaceae) active against gut proteases of lepidopteran pest *Helicoverpa armigera*. Frontiers in physiology 7: 388.

Urwin P E, Lilley C J, McPherson M J, Atkinson H J. 1997. Resistance to both cyst and root-knot nematodes conferred by transgenic Arabidopsis expressing a modified plant cystatin. The Plant Journal 12(2): 455-461.

Vain P, Worland B, Clarke M C, Richard G, Beavis M, Liu H, Kohli A, Leech M, Snape J, Christou P, Atkinson H. 1998. Expression of an engineered cysteine proteinase inhibitor (Oryzacystatin-IΔD86) for nematode resistance in transgenic rice plants. Theoretical and Applied Genetics 96(2): 266-271.

Zhu-Salzman K, Zeng R. 2015. Insect response to plant defensive protease inhibitors. Annual Review of Entomology 60: 233-252.

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