RESEARCH ARTICLE

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Negative impacts of protease inhibitors purified from the seeds of *Prunus domestica* and *Annona squamosa* on the developmental physiology of *Helicoverpa armigera*

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Abstract

Using environment-friendly, effective, and stable protein molecules that can prevent or affect the metabolism of the pest *Helicoverpa armigera* could be an effective way to control this pest. Protease inhibitors (PIs) are distributed in the different parts of the plants like seeds and leaves. The protease inhibitors are low molecular weight proteins that can target the larval midgut digestive protease enzyme(s). This current study aims to purify and characterize proteinaceous protease inhibitors from the seeds of *Prunus domestica* and *Annona squamosa* and determine their impacts on the developmental physiology of a notorious pest *H. armigera*. The molecular mass of *Prunus domestica* protease inhibitor (PDPI) and *Annona squamosa* protease inhibitor (ASPI) observed on the silver-stained gel were 20 kDa and 23 kDa respectively. The inhibitor concentration (IC_{50}) values of larval gut proteases in PDPI and ASPI treated are found at a concentration of 4.00µg/ml, which is similar to the inhibition shown by the purified inhibitors against the bovine trypsin. Observations of nutritional parameters indicate the poisonous and opposing effects of PDPI and ASPI on the developmental growth of *H. armigera* larvae. The results show that the affinity-purified PDPI and ASPI are potent insect repellents that can control harmful insects on crop plants.

Major Findings: 1. Two new protease inhibitors known as PDPI and ASPI were isolated and purified from the seeds of *Prunus domestica* and *Annona squamosa* plants respectively, refined to homogeneity, and produced 20 and 23 kDa protein bands on silver-stained gel respectively. **2.** The results show that the IC_{50} of PDPI and ASPI for *H. armigera* midgut proteases and bovine trypsin are 4.0 µg/ml. Both showed adverse effects on the period of larval-pupal development. Under the influence of PDPI and ASPI the fecundity and fertility of adult insects were severely hampered. **3.** Observations of nutritional parameters indicate the poisonous and opposing effects of PDPI and ASPI on the developmental growth of *H. armigera* larvae. **4.** The results show that the affinity-purified PDPI and ASPI are potent insect repellents that can control harmful insects on crop plants.

Keywords: Biopesticide, *Helicoverpa armigera,* Metabolism, Protease Inhibitor

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1. Introduction

Worldwide excessive use of chemical pesticides has a negative impact on agro-economic practices[1]. The use of agrochemicals creates an adverse environment for maintaining sustainability in different parameters[2]. While these chemicals provide quick relief against crop pests but the outcome has hazardous impacts on our economy and agroecosystems[3]. Taking these factors into account, there is a high demand for eco-friendly tactics to control the different harmful pests that affect agricultural practices. For countering these situations, proteinaceous plant protease inhibitors (PIs) have the natural ability to oppose the situation that is developed by the pest[4,5]. As a result of the defence mechanism, PIs are completely endogenic and significantly present in the tubers and seeds in various plant families[1]. Current unsustainable practices have long-lasting impacts on the ecosystem because of their high half-life and create strong selection pressure while PIs are bona fide to crop plants and non-target species[6]. Several plant families like Leguminosae, Solanaceae, and Gramineae are reported for high concentrations of PIs besides that these are ubiquitous in Plant Kingdom[7]. Plants that have economic and nutritional values are vulnerable to *H. armigera.* The PIs target site is midgut protease enzymes of *H. armigera*, disrupting the several developing phases of the pest life cycle and enhancing plant protection against insect-pests in an environment-friendly manner.

Annona squamosa is a garden plant of the family Annonaceae commonly known as custard apple[8]. *A. squamosa* has a high proteinaceous proportion, therefore it can be utilized as a value-rich food source among humans as well as animals[9]. The Rosaceae family consists of hundreds of species in the *Prunus* genus, but only some are documented in the genetic resources information system[10]. *P. domestica* is commonly known as red plum or European plum. *P. domestica* fruits contain protein, dietary fibre, and carbohydrates[11]. The inhibitory activity of *P. Domestica* against α-glucosidase, α-amylase, lipoxygenase, and pancreatic lipase enzymes is well reported and the inhibition values are expressed as IC_{50} values.[12]

The protease inhibitory attributes in the seed of this plant have not been largely investigated therefore, it is essential to screen and assess its protease inhibitor potential and detrimental effects on the physiological parameters of *H. armigera*[13]. *H. armigera* can express differential midgut proteases, therefore studying novel and effective PIs from diverse non-host plants could serve as a promising tool to dwindle the adverse effect of insect-pests naturally[14]. A continuous search for novel PIs from the non-host plants will strengthen the future effective approach of insect-pest control programs. To a large extent, the inhibitory role against the protease enzymes in the seeds of both plants is not much explored therefore it is essential to evaluate the inhibitory effect of these inhibitors on the different pathophysiological parameters of *H. armigera.*

In this study, the repressive effects of PDPI and ASPI on 4th instar larvae of *H. armigera* were examined by both *in vitro* and *in vivo* methodologies. Regardless it is very complicated to underline the impact of PDPI and ASPI on *H. armigera*, therefore a series of experiments have to be performed to highlight the physiological and biochemical effects of PDPI and ASPI on the physiological development of *H. armigera*.

2. Materials and Methods

2.1 Materials

Plant seeds *of P. domestica* and *A. squamosa* were collected from fields of CSIR-National Botanical Research Institute (NBRI), Lucknow, U.P., India. Insect culture experiments of *H. armigera* larvae were procured from the insectary of Acharya Narendra-Deva University of Agriculture and Technology, Ayodhya, UP, India. The insects were kept approximately at 28±2°C, 60% relative humidity, 14 hours of light, and a darkness period of 10 hours. The reagents and chemicals used in the entire experiment were purchased from Sigma Chemical Co. (Sigma-Aldrich). The supplier of the Sephadex G-75 matrix was Amersham Biosciences Uppsala, Sweden. Markers, BApNA, and trypsin-Sepharose CL-4B were obtained from Sisco Research Laboratories Pvt. Ltd. Mumbai. Only analytical-grade chemicals were used in the course of the experiments.

2.2 Isolation and Purification of Trypsin Inhibitors from the Seeds of *P. domestica* and *A. squamosa*

The protein assay was done followed by Lowry's method[15]. The dried seeds were converted into powder form and then fixed several washes with chilled acetone and hexane to make them colourless and defatted. The supernatant

was discarded and subsequently treated with (NH4)2SO4 to fractionate into three distinct precipitation cuts at 4°C for 20 minutes and centrifuge at 12000 rpm. The protein fractions pellets were dialyzed with 0.1 M phosphate buffer at a pH of 7.6 and then lyophilized.

2.3 Chromatography

During the gel-filtration chromatography procedure, the pooled fractions identified as $F_{30–60}$ underwent purification through a column (Sephadex G-75, 100cm×02cm), and multiple bed volumes of 0.01M phosphate buffer at a pH of 7.6 were used for stabilization. A BIORAD Fraction collector (Model 2110) was employed to collect fractions of 4.5 milliliters, starting with a flow rate of 0.30 mL/min[16]. Spectrophotometric analysis of the protein was conducted at an absorbance of 280 nm, and the void volume of the column was evaluated by blue-dextran dye. Collected fractions with high inhibitory activity against the trypsin enzyme were pooled and lyophilized. Pooled and lyophilized fractions eluted from the gel filtration columns having trypsin inhibition activity were further loaded onto a column of DEAE- Sephadex A-25 for the next level purification with an ion-exchange column. The matrix column was balanced using numerous bed capacities of 50mM Tris-hydrochloric acid buffer at a pH value of 7.6. The boundless proteins were first washed out by using the buffers. The attached proteins with the matrix were removed by smearing a linear salt gradient of sodium chloride at different ranges in a 50mM Tris-HCl buffer at a pH of 7.6. The peaks showing the trypsin inhibition activities were collected and then subjected to lyophilization. Active fractions isolated via ion-exchange chromatography were applied to column that had been pre-conditioned with 0.10M Tris-hydrochloride buffer at a pH of 7.6, along with 0.10M NaCl and 05.0mM $CaCl_2$. Proteins were eluted using a 100 mM HCl solution.

2.4 Protease Inhibitory Activity

Using BApNA as a specific substrate for the enzymatic activity of trypsin, the trypsin inhibitory activity assay of *P. domestica* and *A. squamosa* seeds was carried out. Trypsin was mixed with various volumes of PDPI and ASPI and the mixture was reserved for continuous addition with 0.10 M Tris-HCl at a pH of 7.6 and kept in a water bath for 10 min. incubation at 37°C. To evaluate the residual activity of trypsin, 1.0 mL of 1.0 mM BApNA was mixed with 0.10 M Tris-HCl at a pH 7.6, previously warmed to 37°C, and the mixture was incubated for 10 minutes[17]. The enzymatic reactions were terminated by the addition of 2.0 ml of 10% C2HCL3O2. Followed by the centrifugation process, the released C6H6N2O2 in the transparent solution was measured against the control by spectrophotometer. The percent inhibition was observed at the absorbance of 410 nm. A protease individual inhibitor unit is characterized as the amount of inhibitor necessary to decrease the enzymatic activity by one unit. The fractions were assessed for their inhibitory effects, leading to the selection of the most promising fraction for further investigation. Each assay was conducted in triplicate. The potential of the protease inhibitors to affect the enzymatic activity[18] and the protein concentration in each fraction were quantified for all samples.

2.5 Polyacrylamide Gel Electrophoresis (PAGE)

PAGE was run by following the methodology of Laemmli[19]. A buffer of stacking gel 4% and resolving gel 12% was involved, both were kept at room temperature. Purified sample 25µl and marker 15µl were loaded into the well. The gel underwent silver staining, and the molecular weight of the unidentified proteins was assessed using the Genei gel-doc fire reader program.

2.6 Temperature and pH Tolerance

The thermostability of purified PDPI and ASPI was evaluated using a 0.1 M Tris-HCl solution over a temperature spectrum of 10 to 120°C. The samples underwent centrifugation and were incubated for 30 minutes. The activity of the protease inhibitor was recorded. Furthermore, the adverse effects of PDPI and ASPI were investigated at different pH levels by incubating the purified inhibitors with buffers ranging from pH 2 to 12 at a temperature of 25°C, with the final volume adjusted using various buffers. For pH levels 2 and 3, glycine hydrochloride, sodium acetate, and acetic acid buffers were utilized. Phosphate buffers were applied for pH levels 6.0 to 7.0, while Tris-hydrochloric acid was used for pH 8. Glycine sodium hydroxide was employed for pH levels 9.0 to 10.0, and sodium phosphate buffers were used for pH levels 11 to 12. After a 24-hour incubation period, the impact of the trypsin inhibitor was assessed as previously conducted.

2.7 Rearing of Insect

The arrangement of the artificial diet was similar to the methodology of Giri and Kachole[20]. Whole the experiment was completed at room temperature.

2.8 Preparation of Larval Midgut

The *H. armigera* larvae, which were in their 4th instar and actively feeding were frozen to achieve immobility and subsequently beheaded. As previously indicated, the midguts were collected along with their contents[21]. To make the gut enzyme extract, midguts were blended in a freezing 0.1 M glycine-sodium hydroxide buffer with a pH of 10.0, at a concentration of 5 guts/mL of buffer. Following a period of two to three hours at 10°C, the homogenate was centrifuged for fifteen minutes at 4°C at 12,000 rpm. The supernatant obtained was preserved in a deep freezer (-20°C) and later utilized as an extract of gut protease enzymes.

2.9 Assessment of the Protease Inhibitory Effects of PDPI and ASPI on Bovine Trypsin and Protease Extracts Obtained from *H. armigera* Larvae

To assess the trypsin inhibition ability of PDPI and ASPI, it is necessary to mix them with an appropriate concentration of crude gut extract and bovine trypsin across various replicate sets. Bovine trypsin and extract of larval midgut should be pre-incubated with PDPI and ASPI for 10 minutes at 37°C in a Tris-hydrochloride buffer, conducted separately. The crude gut extract serves as the reference in this analysis. The parameter for protease enzyme activity is determined by measuring the release of p-nitroaniline/minute for each mg of protein. The results are presented as IC_{50} values or percentage inhibition relative to the control.

2.10 Protease Stability of PDPI and ASPI Against *H. armigera* Gut Proteases

The retention time of food within the gut of *H. armigera* larvae is estimated to be around three hours. An investigation into the stability of PDPI and ASPI against intestinal proteases involved the addition of trypsin inhibitors in defined amounts, followed by the extraction of HGP after 45 minutes. The protease inhibitor activity was determined by using BApNA as a substrate.

2.11 Impact of PDPI and ASPI Protease on the Growth and Development of Larvae

The activity of PDPI and ASPI was calculated *in vivo* by feeding different doses of diet to 4th instar larvae of *H. armigera*[22]. Larvae of *H. armigera* were individually placed in petri- dishes, either control or 3 different concentrations of an inhibitor: 0.05% w/w, 0.50% w/w and 1% w/w for a period of 3hr. After this treatment, the larvae were allowed to progress to the next pupal stage. The experiments were carried out at a relative humidity of 62.0% and a temperature of 27°C. After the study, several factors were analysed, including the weight of the hatchlings, growth of the larvae, percentage of successful hatching, emergence of adults, and mortality rates of the hatchlings. The assessment of resistance effectiveness was based on the number of larvae that progressed to the pupal stage and the comparison of body weight between the resistant and control groups[23,24].

2.12 Antifeedant Activity

Different combinations of PDPI and ASPI were utilized to evaluate their antifeedant effects on *H. armigera*, with SKTI acting as the positive control. Following a three-hour pre-starvation phase, 4th instar larvae of *H. armigera* were assigned to various dietary treatments. The monitoring of the larvae's food area utilization took place after a one-day feeding period, allowing for a comparison between the treated and control diets. Antifeedant Index (AI) was determined by the formula[25], $AI= [(C - T)/(C + T)] \times 100$ antifeedant index were observed in percentage, where C represents the diet consumed by the larvae on control and T represents the diet that has been treated. On which concentration of PDPI and ASPI insect mass were reduced is referred to as Effective Dose (ED50).

2.13 Determination of Fecundity

After several days, PDPI and ASPI were treated at 0.05%w/w, 0.50% w/w, and 1% w/w, the pupae of *H. armigera* were reserved individually. Moths were applied in a petri dish for oviposition and mating. The normal diet against PDPI and ASPI was used as control, and eggs were calculated. Oviposition preference (in percent) = [(eggs number of TI plant seeds treated larvae)/(calculated eggs in control group larvae)×100]. Larval eggs are collected from treated foods and controls, isolated, and newly

developed larvae are laid upon host leaves. The egg-hatching percentage was calculated and reported.

3. Result

3.1 Isolation, Purification, and Electrophoretic Analysis

The purification folds of PDPI and ASPI were estimated as 7.58 and 7.02, and the yield percent recovery of both PDPI and ASPI was calculated as 5.27 and 4.85 respectively (Table 1). In silver-stained gel, molecular weights of PDPI and ASPI are 20 kDa and 23 kDa, respectively.

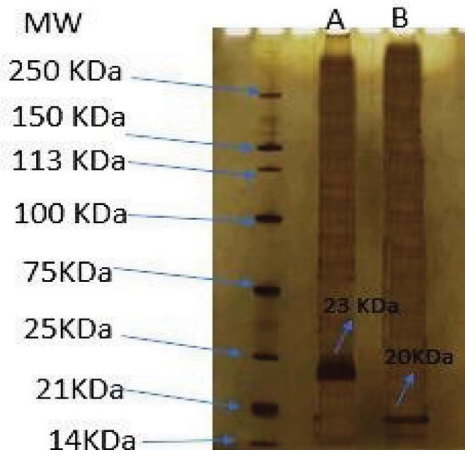


Figure 1. Silver-stained gel electrophoresis with a molecular mass of 23 kDa and 20 kDa. (**A**) and (**B**) Lanes bands indicate the molecular masses of ASPI and PDPI respectively.

3.2 Stabilities- pH and Temperature

The inhibitor at pH 5.0 to 8.0 in PDPI and pH 4.0 to 7.0 in ASPI showed trypsin inhibitory activity. The average activity of 63% was seen between pH 5.0 and 8.0 in PDPI and 58% in ASPI approximately (Figure 2). At pH 2.0 and above 12.0, PDPI and ASPI lost 90% trypsin inhibitory activity approximately (Figure 2). At different temperature ranges between 10 and 120°C, it was detected that inhibition activity of PDPI and ASPI was stable above at 72°C and 68°C for 30 min, but a total loss of around 70% and 65% trypsin inhibition activity was observed during, the sample was incubated for 15 min. at 100°C. The inhibitor activity was affected at the temperature of 120°C in both PDPI and ASPI (Figure 3).

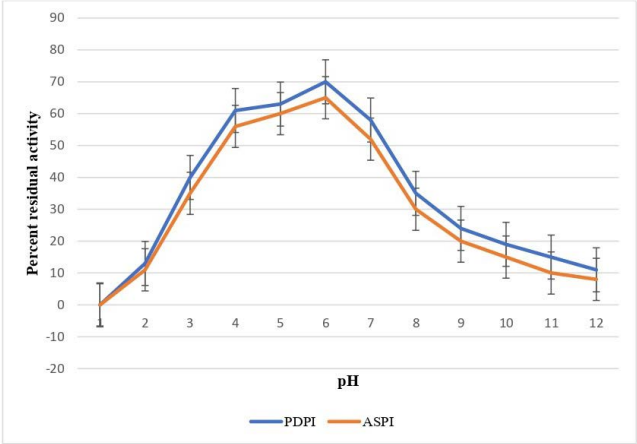


Figure 2. pH stability profile of purified PDPI and ASPI.

Table 1. Purification and percent recovery of PDPI and ASPI from the seeds of *P. domestica* and *A. squamosa*

Purification	Total Volume (ml)		Total Protein (mg)		Total Trypsin Inhibitory Units (TIU)		Specific Activity (TIU/ mg protein)		Purification Factor		Yield (% Recovery)	
	PD	AS	PD	AS	PD	AS	PD	AS	P D	A S	PD	A S
Crude inhibitor extract from seeds (250gm)	465	450	3952.7	3747.08	187627.37	175958.89	47.46	46.95	1.00	1.00	100	100
Heat treatment (Heat stable protein) at 60°C; 30 min	381	369	2763.35	2642.30	180042.32	169936.44	65.15	64.31	1.37	1.36	95.95	96.57
Gel-filtration (Sephadex G-75) chromatography	2.75	2.50	68.87	64.53	12271.21	11264.23	180.45	174.55	3.80	3.71	6.54	6.40
Trypsin-Sepharose CL-4B affinity chromatography	1	0.75	27.52	25.89	9901.96	8544.65	359.80	330.03	7.58	7.02	5.27	4.85

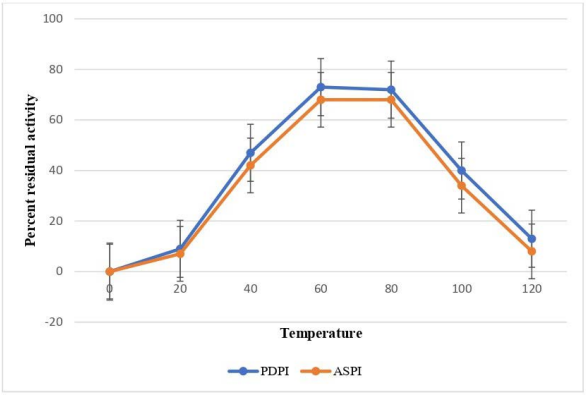


Figure 3. Thermal stability profile of purified PDPI and ASPI.

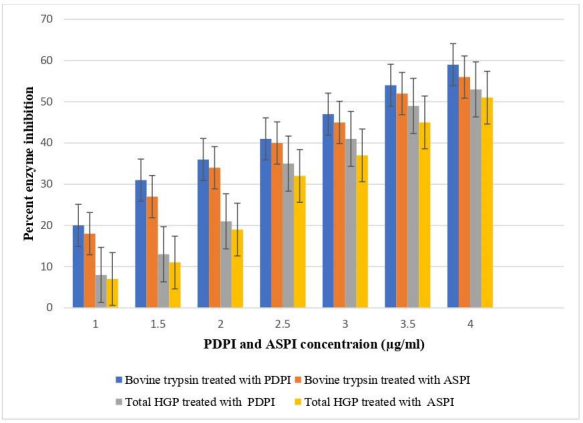


Figure 4. Inhibition of total bovine and midgut proteases of *H. armigera* by PDPI and ASPI:The assay was performed using BApNA as substrates.

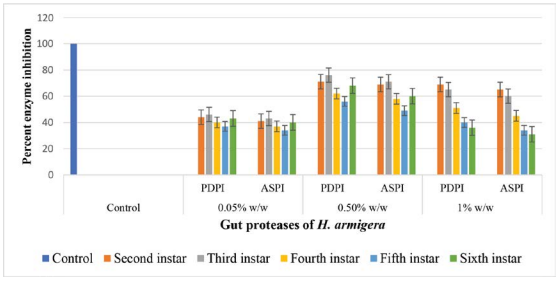


Figure 5. Long-term treatment of 2nd to 6th instar larvae with variations of PDPI and ASPI. SKTI was used as a positive control (data not shown).

3.3 Bioassay of PDPI and ASPI Against *H. armigera*

The proteolytic activity of trypsin-like serine proteases responsible for digestion was well-known in the digestive systems of *H. armigera* larvae during their 1st instar to 5th

Table 2. Impact of PDPI and ASPI on mean larval weight, mean pupal weight, mean pupal duration, pupicidal activity, pupation, and adult etamergence

Dosage of PDPI and ASPI (% w/w)	Mean Larval Weight (mg)		No. of Larva		Larvicidal Activity (%)		Mean Pupal Weight (mg)		Mean Pupal duration		Pupicidal activity (%)		Pupation (%)		Adult emergence (%)	
	PD	AS	PD	AS	PD	AS	PD	AS	PD	AS	PD	AS	PD	AS	PD	AS
Control	343±1.32	337±2.19	56	56	2±1.25	1±8.95	274±2.59	264±1.65	8.38	7.56	0	0	88±2.89	85±2.89	89±1.45	87±1.2
0.05	301±1.50	305±1.91	49	51	11±1.14	08±1.51	259±0.68	251±1.25	09.21	8.27	30±1.98	29±0.89	79±1.54	75±3.79	70±2.57	70±2.24
0.50	152±2.63	143±1.58	24	23	42±2.32	39±2.62	211±2.58	200±3.21	12.38	11.59	70±0.98	66±2.45	68±4.14	64±2.74	58±2.65	55±2.35
1.00	52±0.98	48±2.36	17	16	14±1.38	16±2.84	192±2.78	179±3.21	14.98	12.54	88±2.35	85±1.24	61±2.46	56±3.56	54±2.35	52±0.32
SKTI (0.50% w/w)	174±1.95	174±1.95	26	24	48±2.35	49±3.14	241±0.98	237±4.98	12.59	11.09	68±4.25	65±3.21	67±2.35	65±0.32	59±0.45	54±3.14

Table 3. Influence of PDPI and ASPI at different concentrations on antifeedant activity,deformities of pupae and adult, and fertility and fecundity

Dosage of PDPI and ASPI (% w/w)	Antifeedant Activity (%)		Deformities (%)				Fertility and Fecundity (*N*=2)					
			Pupae		Adult Insect		Eggs		Reduction in Egg Laying/Female (%)		Egg Hatching (%)	
	PD	AS	PD	AS	PD	AS	PD	AS	PD	AS	PD	AS
Control	0	0	2.42 ±1.98	1±2.35	2±4.23	2±2.30	369	364	00	00	66.00	65.10
0.05	0	0	28 ±2.31	26±0.89	32±2.31	30±0.98	141	140	61.78	61.53	25.23	25.01
0.50	59± 3.56	57±1.38	70 ±0.98	65±2.56	74±1.59	71±2.79	78	74	78.89	79.67	13.98	13.23
1.00	55±1.49	52±2.79	80±3.54	74±2.73	98±3.27	97±3.89	21	16	94.30	95.60	03.87	02.93
SKTI (0.50 % w/w)	-	-	69±0.89	65±1.26	72±3.29	69±2.57	56	51	84.82	85.98	10.09	09.27

instar stages of development (Figure 5). The optimum level of activity was noted through the fourth instar larval stage. The changes in gut protein content accorded with the differences in digestive proteolytic activity, particularly up to the 4th instar larval stage (Figure 5). However, during the 5th instar larval stage, a minor reduction in trypsin activity is linked to the concentration of proteins in the gut.

3.4 *In vitro* Inhibition Assay with PDPI and ASPI

PDPI and ASPI significantly inhibited the *H. armigera* midgut proteases at the different dosages of 1 to 4µg/ml. 54% in PDPI and 52% in ASPI suppression of bovine trypsin displayed at the concentration of 3.5µg/ml (Figure 4). The IC_{50} values of larval gut proteases in both PDPI and ASPI were found at the concentration of 4.00µg/ml, which is steady with the suppression shown by bovine trypsin (Figure 5).

3.5 Analysing Different Effects of PDPI and ASPI on the Developmental Stages of *H. armigera* from Larvae to Mature Insects

The findings of this study reveal that when *H. armigera* larvae were elevated on a diet enhanced with several types of amounts of PDPI and ASPI, a reduction in the average weight of larvae was observed. The larval weight of PDPI and ASPI was decreased by 152 mg and 143 mg at a concentration of 0.50% w/w, whereas at 1% w/w PDPI and ASPI, the weight declined to 52 mg and 48 mg respectively (Table 2). In comparison, the average weight of larvae raised on a regular diet was 343 mg and 337 mg respectively (Table 2). The average weight decrease that has been seen in the investigation, which reached half of the normal weight shows the ED50 value which was recorded at 0.50% w/w concentration of ASPI and PDPI. The impression of PDPI and ASPI at minor doses on weight loss was more visible. After 24 hours of pupation, the pupae's average weight was also affected. Pupae that received a regular diet weighed an average of 274 mg, while those raised on a diet containing 0.50% w/w ASPI and 0.50% w/w PDPI weighed 211 mg and 200 mg, respectively (Table 2). Conversely, pupae weighed 192 mg and 179 mg on average when raised on a diet with 01.0% w/w of PDPI and 01% w/w of ASPI (Table 2).

The antifeedant activity of PDPI and ASPI was investigated around 59% and 57% at 0.50% w/w whereas 55% and 52% at the concentration of 1% w/w (Table 3). The larval diet did not change, even when the doses were reduced. It is worth noting that PDPI and ASPI toxins have a significant effect on *H. armigera* larvae, increasing mortality at low doses. Pupation in PDPI and ASPI larvae was affected at around 68% and 64% pupation at 0.50% w/w whereas approximately 61% and 56% at 1% w/w in both PDPI and ASPI respectively (Table 3). In the control group, pupation of PDPI and ASPI were 88% and 85% respectively (Table 3). According to the research data, the value of LD50 of PDPI and ASPI were

around 0.50%w/w. The pupation rate of PDPI and ASPI was decreased by approximately 30.68% and 34% whereas the adult emergence of both PDPI and ASPI was reduced to 39.32% and 40.22% when compared with the control groups (Table 3). When three different types of PDPI and ASPI were included in the diet, there were many cases of abnormal pupae and malformed adults reported.

After further analysis, the deformities in pupae of PDPI and ASPI were calculated approximately 80% and 74% at 1% w/w concentration (Table 3), whereas in the adult insects treated with PDPI and ASPI were found approximately 98% and 97% respectively at the same concentration. The egg laying of PDPI and ASPI from control to 1% w/w was reduced from 369 to 21 and 364 to 16 (Table 3). Compared to the control and sample 1% w/w the egg hatching of PDPI and ASPI was reduced from 66% to 3.87% and 65.10% to 2.93% respectively. At a concentration of 0.50% w/w the egg hatching value of both PDPI and ASPI were 13.98 and 13.23 respectively and additionally declined to 3.87 and 2.93 at 1.00% w/w of PDPI and ASPI (Table 3).

4. Discussion

Different research groups purified and characterized the protease inhibitors from the different parts of the host as well as non-host plants[26-30]. Trypsin inhibitor extraction and purification of legume seeds have been studied extensively[31-33]. Additionally, method of gel filtration, thermal treatment, and purification was also used, which increased the purification up to 186 folds[34]. In this investigation, the 20 kDa and 23 kDa proteins in plant seeds of *P. domestica* and *A. squamosa* pointed to the likelihood of trypsin inhibitor of the Kunitz category. There have been documented Kunitz-type protease inhibitors like 19 kDa in soybeans, 20 kDa in mustard seeds, and 14 kDa in *Cajanus cajan* seeds, all of which have different molecular weights than the *P. domestica* and *A. squamosa* trypsin inhibitor[35,36]. Kunitz protease inhibitors are well-studied serine complexes that suppress the digestive enzymes of insect pests to protect plants. Kunitz receptors have one or two polypeptide chains of 18-34 kDa[37-40]. At 72 and 68°C the stability of PDPI and ASPI was displayed maximum. Thermal stability of PDPI and ASPI were presented at the temperature range between 10 to 120°C. PDPI and ASPI were stable at 72°C and 68°C but thereafter, the thermal stability reduced regularly retentive stability when heated at 100°C for 30 min. In simple, legume PIs are very stable above 80°C, although stability is generally lost above this temperature[41,42]. The average activity of 63% between pH 5 and pH 8 in PDPI and 58% in ASPI was observed. At pH 2 and above 12, PDPI and ASPI lost approximately 90% trypsin inhibitory activity. Trypsin inhibitor from jackfruit seeds was stable up to a pH range between 3 and 12[43]. However, the trypsin inhibitor from pigeon pea seeds maintained its activity within the pH range of 7 to 10; however, it experienced a 20% reduction in activity when subjected to acidic pH levels between 3 and 5[44]. 4th instar larvae showed almost complete inhibition of the activities compared to all the instars of the HGPs against PDPI and ASPI. These explanations further support the idea that the 4th instar is the worst developmental stage of *H. armigera*. Furthermore, it is in the fourth instar larval period that the maximum protein activity and the maximum number of protein isoforms are stated[45,46]. PIs from bitter plant gourd inhibited 80% of the protease activity of *H. armigera*[47]. In addition, PIs from tomatoes are very resistant to insect-pests, and the protease activity of *H. armigera* larvae consuming both host and non-host plants was inhibited by 50-80%. Notably, TTI seems to be a more effective inhibitor than the extracts derived from bitter gourd and tomato. The IC50 values of PDPI and ASPI against bovine trypsin and *H. armigera* midgut proteases were reported 3.5µg/ml and 4.0µg/ml respectively. This value of PDPI and ASPI to total HGPs was justified to AsPI (*Acacia senegal* protease inhibitor), CaKPI (*Cicer arentinum* protease inhibitor) resistance to trypsin enzyme of *H. armigera*[48]. *H. armigera* reared on feeds enriched with 0.2% or 0.5% (w/v) soybean trypsin inhibitor showed a decrease in mean larval weight[49]. In our investigation, we documented that the PDPI and ASPI are dependable with these reports, which recommends that weight reduction can be attributed to the inability of the body to use the necessary amino acids for growth. Protease inhibitors perform a significant function in the breakdown of proteins, affecting the accessibility of amino acids. PDPI and ASPI interact with *H. armigera* in intestinal enzymes and simply digest the material, producing substances that reduce protein degradation and amino acid availability. The antifeedant activity of PDPI and ASPI at 0.50% w/w was investigated at 59% and 57% and at 1% w/w around 80% and 74% were documented. The antifeedant activity of the plant can be used to defend crops up to the level of biological control[50]. The protease inhibitor isolated in

this investigation presented significant anti-host activity counter to *H. armigera*, indicating a higher effectiveness[51].

Although *H. armigera* larvae consumed more food, the antifeedant effect remained the same[52]. This recommends that the antifeedant effect only show at maximum doses. Molecules responsible for the antifeedant effect may have a significant impact on plant defences until their adverse effects are detected by slow-acting biopesticides. In addition, the PDPI and purified ASPI have shown a strong prohibition effect on *H. armigera* food behaviour, which makes insects more sensitive to natural pests.

5. Conclusion

PDPI and ASPI have the potential to disrupt the developmental physiology of *H. armigera* larvae by modifying their physiological and biological processes. This phenomenon can be explored by plant breeders in their efforts to cultivate resistant plant varieties. Anti-tryptic properties of *Prunus domestica* and *Annona squamosa* have yet not been explored. The findings of this research could be useful for the discovery of novel competitive trypsin inhibitors. It is also significant to cite that reducing or interfering with the life cycle of insect

H. armigera. This is an important approach to affecting their population without killing them directly, thus helping to reduce economic losses caused by plant insect pests.

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