Results - Chapter I
Effect of feeding on larval body weight

Feeding bioassays carried out with 4th and 6th instar larvae of Spodoptera litura on the detached leaves of A. cardenasii showed growth retardation when compared with control larvae fed on JL-24 leaves (Figs. 1 & 2). 4th instar larvae were more affected than the 6th instar larvae.

Fig. 1: Effect of feeding of 4th instar S. litura larvae on A. cardenasii leaves. * Each value is mean ± SD of 5 different experiments and for each experiment 10-15 larvae were used.

Fig. 2: Effect of feeding of 6th instar S. litura larvae on A. cardenasii leaves. * Each value is mean ± SD of 5 different experiments and for each experiment 10-15 larvae were used.
Bioassays carried out with the detached leaves of *A. correntina* (Figs. 3 & 4), *A. duranensis* (Figs. 5 & 6) and *A. stenosperma* (Figs. 7 & 8) also showed similar pattern as mentioned above. The 3rd and 4th instar larvae were more affected than late instar larvae (5th or 6th).

Fig. 3: Effect of feeding of 3rd instar *S. litura* larvae on *A. correntina* leaves. * Each value is mean ± SD of 5 different experiments and for each experiment 10-15 larvae were used.

Fig. 4: Effect of feeding of 6th instar *S. litura* larvae on *A. correntina* leaves. * Each value is mean ± SD of 5 different experiments and for each experiment 10-15 larvae were used.
Fig. 5: Effect of feeding of 4th instar *S. litura* larvae on *A. duranensis* leaves. * Each value is mean ± SD of 5 different experiments and for each experiment 10-15 larvae were used.

Fig. 6: Effect of feeding of 5th instar *S. litura* larvae on *A. duranensis* leaves. * Each value is mean ± SD of 5 different experiments and for each experiment 10-15 larvae were used.
Fig. 7: Effect of feeding of 4\textsuperscript{th} instar \textit{S. litura} larvae on \textit{A. stenosperma} leaves. * Each value is mean ± SD of 5 different experiments and for each experiment 10-15 larvae were used.

Fig. 8: Effect of feeding of 5\textsuperscript{th} instar \textit{S. litura} larvae on \textit{A. stenosperma} leaves. * Each value is mean ± SD of 5 different experiments and for each experiment 10-15 larvae were used.
Fig. 9: Effect of feeding of 3rd instar S. litura larvae on leaves of (a) A. correntina, (b) A. duranensis, (c) A. stenosperma, (d) A. cardenasii and (e) JL-24. The larvae were fed for 48 h.
The effect of feeding of *S. litura* larvae on wild species of groundnut leaves is seen as early as after 48 h, when 3\textsuperscript{rd} instar larvae were used. The larvae which fed on wild groundnut leaves (Figs. 9a, 9b, 9c, 9d) did not grow well when compared with the control larvae fed on JL-24 (cultivated groundnut species) leaves (Fig. 9e). Of the four wild species *A. cardenasii* was found to be most effective in inhibiting the larval growth (Fig. 9d).

**Effect of feeding on post-embryonic development and pupal transformation**

There was a marked reduction in the transformation of larvae to pupae when they were fed on wild groundnut leaves as compared to JL-24 fed larvae (Fig. 10). The larvae which fed on *A. cardenasii* leaves were the most affected, where the pupal transformation was only 32\% followed by *A. correntina* (51\%), *A. duranensis* (54\%), and *A. stenosperma* (58\%) as opposed to control (JL-24).

Fig. 10: Effect on larval pupal transformation: 3\textsuperscript{rd} instar larvae of *S. litura* were fed on leaves till they reached pupal stage. Each value is mean ± SD of 5 different experiments and for each experiment 10-15 larvae were used. * After natural mortality.
**Effect of feeding on protein profile and protein content of the haemolymph**

After feeding, when the larvae reached the stage of 6\(^{th}\) instar, they were sacrificed and haemolymph was collected and the protein was quantified as well as analysed by SDS-PAGE.

Table 3: Total haemolymph protein content of 6\(^{th}\) instar *S. litura*. For this experiment 3\(^{rd}\) instar larvae of *S. litura* fed on leaves of different groundnut species and allowed to grow till the 6\(^{th}\) instar stage.

<table>
<thead>
<tr>
<th>Species</th>
<th>Quantity (µg/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JL-24</td>
<td>80 ± 10.09</td>
</tr>
<tr>
<td><em>A. correntina</em></td>
<td>32 ± 2.95</td>
</tr>
<tr>
<td><em>A. cardenasii</em></td>
<td>28 ± 1.39</td>
</tr>
<tr>
<td><em>A. duranensis</em></td>
<td>43 ± 5.28</td>
</tr>
<tr>
<td><em>A. stenosperma</em></td>
<td>39 ± 3.53</td>
</tr>
</tbody>
</table>

As shown in table 3, there was significant decrease in the quantity of total protein present in the haemolymph of the larvae that fed on wild groundnut species when compared with the larvae that fed on cultivated species. This reduction was almost 65\% in *A. cardenasii* while it was 49\% in *A. correntina*, 53.75\% in *A. duranensis* and 48.75\% in *A. stenosperma*. 
Literature survey as well as extensive studies from our laboratory shows that the hexamerins constitute the major component of storage proteins in the haemolymph of lepidopteran insects and play important function as storage proteins which support larva-pupa-adult transformation during post-embryonic development. SDS-PAGE analysis was carried out to check the fate of these hexamerins in the experimental larvae. Results presented in the figure 11 show that the hexamerin content is very much reduced in the case of insects that were fed on leaves of wild groundnut species (lanes 2-5) when compared with the insects that were fed on cultivated groundnut species (lane 1).

**Effect of feeding on larval midgut proteases of S. litura**

The 3rd instar larvae of *S. litura* were fed either with the leaves of JL-24 or *A. corrrentina*. When the larvae reached 6th instar the midgut homogenates were prepared and the midgut proteases were analyzed by activity staining using casein diffusion method. The results did not show a major variation.
between the protease profile of the larvae that were fed with the leaves of JL-24 and *A. correntina*. But there was an increase in the activity of proteases corresponding to 43, 29, 25 and 20 kDa in the case of larvae that were fed on *A. correntina* leaves (Fig. 12, lanes 3-6).

Fig. 12: Zymograph showing digestive proteinase activity of *S. litura* larvae fed on leaves of *A. correntina* and control (JL-24) plants. Lanes 2-6 were loaded with 5 µg protein of larval midgut extract. For this experiment 4\textsuperscript{th} instar larvae were fed for 4 days.

**Characterization of *S. litura* larval midgut proteases**

The gut protease profile from 6\textsuperscript{th} instar larvae which were grown on castor leaves was analyzed by substrate gel electrophoresis using casein diffusion method. For this experiment freshly prepared midgut homogenate was treated with different protease inhibitors and their effect on the proteases was analyzed (Fig. 13). Each lane was loaded with 5 µg of the midgut homogenate protein. The results show the inhibition of the four major
proteases (25, 32, 34 and 43 kDa) by PMSF (lane 3). Furthermore the inhibition was fairly high for 32 and 34 kDa proteases, which were almost completely inactivated. TLCK inhibited completely the protease corresponding to 43 kDa (lane 4) but had no effect on other proteases. None of the proteases were affected by the treatment with TPCK (lane 5).

Fig. 13: Zymogram of *S. litura* midgut proteases showing the effect of different protease inhibitors. 5 µg of gut protein extract was fractionated in 10% SDS-PAGE.
Results- Chapter II
Correlation of leaf surface and feeding inhibition-

As we have seen the retarded growth of the larvae that were fed with the leaves of wild groundnut species, detailed scanning electron microscopic studies of leaf surface was carried out to analyze the physical structures which might be responsible for defenses including the feeding inhibition in the larval forms of *S. litura*.

Parameters relating to physical defenses like trichomes were compared across young and old leaves of wild and cultivated groundnut plants. The results presented in figures 14-17 clearly show a significant difference between adaxial and abaxial surface of the leaves. The trichomes are present abundantly on the abaxial surface of the leaves (Figs. 14 & 15) except for *A. duranensis* which has very few numbers of trichomes (Fig. 14c). The adaxial surface of the leaves show little or no trichomes (Figs. 16 & 17). The density of trichomes was also notably different between the old and young leaves (Figs. 14 & 15). *A. cardenasii* possessed the highest density of trichomes (Figs. 14b & 15b) followed by *A. stenosperma* (Figs. 14d & 15d), *A. correntina* (Figs. 14e & 15e), *A. duranensis* (Figs. 14a & 15a) and JL-24 (Figs. 14c & 15c). The adaxial surface of both young and old leaves of *A. cardenasii* (Figs. 16b & 17b), *A. stenosperma* (Figs. 16c & 17a), *A. duranensis* (Figs. 16c & 17c) and JL-24 (Figs. 16a & 17a) do not contain any trichome.
Fig. 14: Scanning electron micrograph of abaxial surface of old (30 days) leaves of JL-24 (14a), *A. cardenasii* (14b), *A. duranensis* (14c), *A. stenosperma* (14d) and *A. correntina* (e). Each micrograph was taken at 100x magnification. (➡️ Trichome)
Fig. 15: Scanning electron micrograph of abaxial surface of young (3 days) leaves of JL-24 (15a), *A. cardenasii* (15b), *A. duranensis* (15c), *A. stenosperma* (15d) and *A. correntina* (15e). Each micrograph was taken at 100x magnification. (Trichome)
Fig. 16: Scanning electron micrograph of adaxial surface of old (30 days) leaves of JL-24 (16a), *A. cardenasii* (16b), *A. duranensis* (16c), *A. stenosperma* (16d) and *A. correntina* (16e). Each micrograph was taken at 100x magnification.
Fig. 17: Scanning electron micrograph of adaxial surface of young (3 days) leaves of JL-24 (17a), A. cardenasii (17b), A. duranensis (17c), A. stenosperma (17d) and A. correntina (17e). Each micrograph was taken at 100x magnification. (Trichome)
Results - Chapter III
A careful observation of the leaf surface reveals variation in the length of trichomes across the species (Figs. 14 & 15). The results presented in the figure 18a show that trichome is the longest in *A. stenosperma*, followed by *A. cardenasii*, *A. correntina*, *A. duranensis* and JL-24. However, no correlation was found between the trichome length and the percentage of gain in body weight of larvae that fed on the leaves of evaluated groundnut species (Fig. 18b).

![Fig. 18: Correlation between the length (arbitrary units) of trichome and % gain in body weight of *S. litura* larvae. 3rd instar larvae were allowed to feed on different species of groundnut leaves. When the larvae reached 6th instar, their body weight was noted. (a) Length of trichome (b) % gain in body weight. For each experiment 8-10 larvae were used and each value is mean ± SD of four independent experiments.](image-url)
**Purification and characterization of protease inhibitors from A. correntina and A. cardenasii**

In recent years there is a growing interest in the identification of novel PIs because of their potent antiproliferative activity and in prevention of carcinogenesis in a wide range of *in vivo* and *in vitro* systems/models, as well as their use in developing pest resistance in otherwise susceptible plants (Qi *et al.*, 2005). Here we describe the purification and some properties of protease inhibitors from wild *Arachis* species *A. correntina* and *A. cardenasii*.

**Purification and characterization of protease inhibitors from A. correntina**

The crude extract prepared from the thoroughly washed and air dried leaves (5 g) of *A. correntina* was used for the purification of protease inhibitors. All the purification steps were carried out at room temperature unless otherwise specified. The purification of the PIs is summerised in table 4.

### Table 4: Summary of purification of *A. correntina* PIs

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Quantity of protein (mg)</th>
<th>Activity against bovine Trypsin (Inhibitory units, IU)</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract of leaf</td>
<td>40.00</td>
<td>43.50</td>
<td>1.08</td>
</tr>
<tr>
<td>Dialysate after 60% (NH₄)₂SO₄ fractionation</td>
<td>12.80</td>
<td>28.30</td>
<td>2.21</td>
</tr>
<tr>
<td>Affinity purified</td>
<td>0.825</td>
<td>15.88</td>
<td>19.24</td>
</tr>
</tbody>
</table>
PIs from leaf of *A. correntina* were purified to homogeneity using 
(NH₄)₂SO₄ precipitation and trypsin-Sepharose affinity chromatography. The 
specific activity of the crude extract towards bovine trypsin was 1.08 TIU/mg. 
The 60% (NH₄)₂SO₄ fraction was applied to a trypsin-Sepharose column. The 
bound inhibitor eluted as a single peak, on reducing the pH with 10 mM HCl. 
Affinity chromatography proved to be a very convenient step for the isolation 
of PIs from leaves, although the possibility of limited digestion of the inhibitor 
by the immobilized trypsin during purification cannot be excluded. The 
resulting affinity purified PIs have fairly high specific activity (19.19 IU/mg).

**Homogeneity and size of the *A. correntina* PIs**

The homogeneity and apparent molecular mass of the *A. correntina* PIs 
were estimated by SDS-PAGE. When subjected to 12% SDS-PAGE (Schagger 
and Jagow, 1980) under reducing conditions, the purified protease inhibitor 
preparation showed the presence of three polypeptides with molecular weights 
of 16, 18 and 20 kDa (Fig. 19).

![Fig. 19: Purification profile of *A. correntina* PIs. Lanes 1 & 2 were loaded with 15 µg of protein and lanes 3-5 were loaded with 5 µg of protein.](image-url)
Specificity of *A. correntina* PIs

The purified *A. correntina* PIs were tested for their inhibitory activity against trypsin and chymotrypsin (serine proteases), pepsin (aspartic protease) and papain (cysteine protease) using gelatin-embedded 12% SDS-PAGE. These PIs inhibited trypsin and among these three PIs, the 16 kDa PI showed maximum inhibitory activity which is evident by the intensity of the corresponding band with 16 kDa (Fig. 20). *A. correntina* PIs also inhibited chymotrypsin and the 18 kDa inhibitor appeared to have maximum chymotrypsin inhibitory activity which is once again evident by the thickness of the corresponding band (Fig. 21a). However, these PIs failed to inhibit either pepsin (Fig. 21b) or papain (Fig. 21c) as is evident by the clear gel without any inhibitor protein bands.

![Image](image.png)

**Fig. 20: Zymographic study- the gelatin gel containing *A. correntina* PIs was incubated with trypsin (see materials and methods). Lanes 1 & 2 were loaded with 15 µg of protein and lane 3 was loaded with 10 µg of protein. Proteins were stained with Comassie Brilliant blue.**
Thermal stability of *A. correntina* PIs

The thermal stability of *A. correntina* PI activity in 62.5 mM Tris-HCl buffer (pH 7.4) containing 10% glycerol, 1% SDS and 0.001% Bromophenol blue was investigated. Results presented in figure 22 clearly show that the PIs are relatively stable at boiling temperature of the water for 30 min (lane 2).

**Fig. 21:** Zymographic study- The gelatin gel containing *A. correntina* PIs was incubated with chymotrypsin (a), pepsin (b) and papain (c) (see materials and methods). Each lane was loaded with 5 µg of purified protein. Proteins were stained with Comassie Brilliant blue.

**Fig. 22:** Heat stability of inhibitory activity of *A. correntina* PIs at boiling temperature of water for 15 and 30 min. Each lane was loaded with 5 µg of the purified protein.
Inhibitory activity of A. correntina PIs on insect gut proteases

The inhibitory activity of A. correntina PIs was tested using gelatin-embedded SDS-PAGE as well as enzyme assay using synthetic substrates for the proteases.

(i) Inhibitory activity of A. correntina PIs on S. litura proteases

Midgut homogenate as well as midgut luminal enzyme preparation proteins were run in a gelatin SDS-PAGE. The gel was initially incubated with 40 mg of A. correntina crude extract and then incubated in Tris-HCl buffer (pH 7.4) (Fig. 23b). For control, the gel was incubated with buffer only (Fig. 23a). The remaining assay of protease activity was carried out as mentioned in materials and methods. Figure 23b clearly shows that the degree of inhibition of protease activity was much higher towards the midgut luminal enzyme preparation (lane 2) than the midgut homogenate (lane 1).

![Fig 23: Effect of A. correntina leaf protein extract on larval gut proteases of 6th instar S. litura larva. Each lane was loaded with 5 µg of protein. (a) Treated with Tris-HCl buffer (pH 7.4) and (b) treated with plant protein extract.](image)

Lanes 1: Midgut homogenate  
Lanes 2: Midgut luminal enzyme preparation
(ii) Inhibitory activity of A. correntina PIs on S. litura and A. janata gut proteases

A. correntina PIs inhibited around 25% activity of the 6th instar S. litura larval midgut proteases while the inhibition was almost 73% with 6th instar larvae of A. janata (Fig. 24).

![Inhibition of Spodoptera litura (SLMP) and Achaea janata midgut protease (AJMP) activity by purified A. correntina PIs. Inhibition assay was conducted using BApNA as substrate.](image)

**Fig 24:** Inhibition of *Spodoptera litura* (SLMP) and *Achaea janata* midgut protease (AJMP) activity by purified *A. correntina* PIs. Inhibition assay was conducted using BApNA as substrate.

**Purification and characterization of protease inhibitor from A. cardenasi**

The crude extract prepared from the thoroughly washed and air dried leaves (5 g) of *A. cardenasi* was used for the purification of protease inhibitors. All the purification procedures were carried out at room temperature unless otherwise specified. The purification of the PIs is summarized in table 5.
The A. cardenasii protease inhibitor was purified to homogeneity using (NH₄)₂SO₄ precipitation and trypsin-Sepharose affinity chromatography. The specific activity of the crude extract towards bovine trypsin was 0.837 TIU/mg. The 60% (NH₄)₂SO₄ fraction was applied to a trypsin-Sepharose column. The bound inhibitor was eluted as a single major peak on reducing the pH with 10 mM HCl. Although affinity chromatography gave reasonably good result the possibility of limited digestion of the inhibitor by the immobilized trypsin during purification cannot be excluded. Affinity purified PIs have fairly good specific activity (18.9 IU/mg).

**Homogeneity and size of the A. cardenasii PI**

The homogeneity and apparent molecular mass of the A. cardenasii PI was estimated by SDS-PAGE. When subjected to SDS-PAGE under reducing conditions, the gel pattern of the purified protease inhibitor preparation from A.
cardenasii showed the presence of a single polypeptide with a molecular mass of around 18 kDa (Fig. 25).

**Stability of A. cardenasii PI**

Result presented in figure 26 shows that the inhibitory activity of A. cardenasii PI is not affected at a temperature up to 100°C for 30 min.

Fig. 25: SDS-PAGE analysis of purified A. cardenasii PI. Lanes 1 & 2 were loaded with 15 µg of protein and lane 3 was loaded with 5 µg of protein.

Fig. 26: Heat stability of inhibitory activity of A. cardenasii PI at boiling temperature of water for 15 and 30 min. Each lane was loaded with 5 µg of the purified PI.
Immunological similarity between soyabean Bowman-Birk inhibitor (BBI) and A. cardenasii PI

Antibodies were generated against commercially available soyabean Bowman-Birk inhibitor. Soyabean BBI antibodies cross reacted with the A. cardenasii PI (Fig. 27) indicating that the soyabean BBI and A. cardenasii PI have immunological similarity.

Inhibitory activity of A. cardenasii PI on S. litura and A. janata gut proteases

A. cardenasii PI inhibited around 29% activity of the 6th instar S. litura larval gut proteases on the other the same PIs inhibited almost 80% of the total gut protease activity of 6th instar larvae of A. janata (Fig. 28).

Fig. 27: Western blot analysis of purified A. cardenasii PI. The PIs were fractionated by SDS-PAGE, blotted onto nitrocellulose, probed with anti-soyabean BBI antiserum and detected using a secondary alkaline phosphatase-coupled antibody (Materials & methods).
Fig. 28: Inhibition of *Spodoptera litura* and *Achaea janata* midgut protease activity by purified *A. correntina* PIs. Inhibition assays were conducted using BApNA as substrate.