SUMMARY

The insect pest attack on plants triggers the production of a series of secondary metabolites like defensins, lectins, serine protease inhibitors, proteins related to thionines and pathogenesis (Buchanan et al., 2002) which are regulatory in function. Understanding this defensive arsenal along with its mechanism of inhibition will pave possible ways to explore more alternatives to produce pest resistant crops, to reduce the use of synthetic pesticides and also to produce environmental friendly methods to control these insect pests (Xavier- Filho, 1992). Serine protease inhibitors which act on digestive enzymes like trypsin, chymotrypsin, elastase and substilisin with residues of aspartate, serine and histidine in their structure represent a mode of protection with wide range of actions. Thorough studies with serine protease inhibitors of the Fabaceae, Solanaceae and Poaceae plant families have shown that they have affected the growth and development of insects from Lepidoptera, Coleoptera and Orthoptera orders when administered in the artificial diet.

Keeping in mind the significance of plant protease inhibitors, their effect on growth and development of insect pests and interaction with digestive enzymes, in vitro assays, bioassays and nutritional assays of partially purified as well as purified inhibitor were performed against a polyphagous noctuid, S. litura (Fabricius). This lepidopteran insect has attained the status of major pest in India currently as it has been known to infest about 144 plant families and caused 28-100% loss in crop production (Gokulkrishnan et al., 2012). The larvae of S. litura were procured from the vegetable gardens in University campus. After identification, the rearing of S. litura was done in laboratory under controlled conditions on castor leaves.

The results obtained from partial purification of protease inhibitors (PIs) showed 86.77%, 50.71%, 65.56%, 51.12% and 60.03% recovery in L. leucocephala, C. glauca, C. occidentalis, A. lebbeck and T. stans respectively. L. leucocephala seeds showed highest purification fold of 2.79 in partially purified sample followed by T. stans (1.60), A. lebbeck (1.52), C. occidentalis (1.19) and C. glauca (1.10). However, C. occidentalis
showed highest inhibitory activity (82.7%) against bovine trypsin than *L. leucocephala* (80%), *A. lebbeck* (79.35%), *C. glauca* (72.1%) and *T. stans* (70%).

The influence of partially purified inhibitors isolated from *L. leucocephala*, *C. glauca*, *C. occidentalis*, *A. lebbeck* and *T. stans* was studied by conducting bioassays on second instar larvae of *S. litura* after feeding them on artificial diet amended with different concentrations of PIs (25, 50, 100, 200, 400 and 800µg/ml and control). Their effect on growth and development was ascertained by making observations on larval period, larval weight, pupal period, % pupation, pupal weight, % male and female emergence, fecundity, % hatching etc. Nutritional assays were conducted to evaluate toxic effect of the partially as well as purified inhibitor in which RGR, RCR, ECI, ECD, AD and MC were studied. Significant inhibitory influence was observed with the five non host PIs.

Larval weight decreased in a dose dependent manner when *S. litura* larvae were given five non host partially purified PIs in diet. An increase was observed in larval period with increase in concentration of partially purified *L. leucocephala*, *C. occidentalis*, *A. lebbeck* and *T. stans* PIs. However decrease in larval period was noticed with partially purified *C. glauca* PI. Small sized pupae were formed at higher concentrations of *L. leucocephala* and *C. occidentalis* PIs. Prepupal (80.16%) and pupal mortality (100%) was noticed at higher concentrations of *C. occidentalis* partially purified PIs.

Percentage pupation and pupal weight declined in second instar larvae of *S. litura* with increase in concentration of all the five partially purified PIs. Percent female and male emergence decreased in a dose dependent manner when *S. litura* larvae were given partially purified PI amended diet. No adult emerged at 800µg/ml concentration of partially purified *C. occidentalis* PIs when supplemented in artificial diet and given to *S. litura* larvae. Also no female emerged at highest concentration of partially purified *T. stans* PIs. Longevity, fecundity and % hatching declined with increase in concentration of *L. leucocephala*, *C. glauca*, *C. occidentalis*, *A. lebbeck* and *T. stans* partially purified PIs. No egg hatched at 800µg/ml concentration of partially purified *A.*
lebbeck PIs. Aberrations in adults were more pronounced at higher concentrations of L. leucocephala PIs.

Noteworthy decline was observed in RGR of S. litura larvae as well as ECI and ECD after ingestion of partially purified PIs treated diet. AD and MC increased in a dose response manner in all the treatments of L. leucocephala, C. glauca, C. occidentalis, A. lebbeck and T. stans PIs. However RCR decreased with increase in concentration of all the non host PIs except C. glauca where an increase in RCR was noticed.

Analysis made with digestive proteases i.e trypsin and chymotrypsin obtained from lumen content, gut tissue and fecal matter revealed the inhibitory potential of partially purified protease inhibitors against S. litura. Protease inhibitors partially purified from L. leucocephala, C. glauca, C. occidentalis and T. stans suppressed the trypsin activity in the lumen content in a duration dependent manner whereas it was found to increase with A. lebbeck PIs when compared to control. The trypsin activity in gut tissue was inhibited with A. lebbeck PIs when supplemented in artificial diet of S. litura in partially purified form at all treatment durations but with L. leucocephala, C. glauca and C. occidentalis it was inhibited only with prolonged treatment and the inhibition was markedly more at higher concentrations. However increase in trypsin activity was noticed in the larvae fed partially purified T. stans PIs amended diet. Fecal matter analysis revealed induction in enzyme activity when second instar larvae of S. litura were given partially purified C. glauca and T. stans PIs amended diet. On the other hand, suppression in trypsin activity compared to control was observed in the larvae fed on diet containing partially purified L. leucocephala, C. occidentalis and A. lebbeck PIs.

Analysis made with chymotrypsin enzyme in lumen content of S. litura larvae revealed inhibition in its activity at all treatment durations with C. glauca and T. stans partially purified PIs. But in the larvae fed on partially purified PIs from L. leucocephala, the inhibition in chymotrypsin activity was noticed only after 48h treatment. On the other hand in C. occidentalis PIs treated larvae inhibitory effect was noticed at 24 and 72h treatment intervals at all concentrations when compared to control.
whereas in *A. lebbeck* PI treated larvae an induction in enzyme activity was observed at these treatment durations. In the gut tissue the chymotrypsin activity was less when compared to control at all feeding intervals in the larvae treated with partially purified PIs from *L. leucocephala*, *C. glauca* and *C. occidentalis*, but an induction in enzyme activity was observed in the larvae fed on *A. lebbeck* and *T. stans* partially purified PIs with prolonged treatment. However fecal matter analysis revealed lesser chymotrypsin activity in larvae reared on *T. stans* PIs incorporated diet. Induction in enzyme activity was noticed with *C. glauca*, *C. occidentalis* and *A. lebbeck* partially purified PIs at all treatment durations when compared to control. However in larvae fed on partially purified *L. leucocephala* PIs the enzyme activity after showing an increase at 24h treatment duration decreased as compared to control with increase in feeding time.

Also antifungal potential of partially purified protease inhibitors was ascertained. Only protease inhibitors partially purified from *C. occidentalis* inhibited the growth of important phytopathogenic fungi i.e. both susceptible (*A. brassicicola*, *A. alternata*) and resistant (*F. oxysporum*) strains effectively.

Among all the partially purified PIs, those obtained from *C. occidentalis* seeds showed the maximum inhibitory activity against the larvae of *S. litura*, both at developmental and biochemical level. Therefore, it was further purified to homogeneity using gel filtration and anion exchange chromatography techniques. These techniques resulted in a 16.47 purification fold with a 21.67% yield. Beside this it was also confirmed by the use of another method i.e. three phase partitioning (TPP). Purification done by TPP resulted in a 16.61 purification fold and 22.41% recovery of the protein. Electrophoretic analysis of purified *C. occidentalis* PI resolved into a single band with molecular weight of approximately 14.3kDa. The inhibition kinetic studies against *S. litura* gut trypsin were of non-competitive type with *Ki* of 0.3µM. *C. occidentalis* PI had low affinity for *S. litura* gut trypsin when compared with soybean trypsin inhibitor (STI). *C. occidentalis* PI had high stability at different pH values (5.0 to 12.0) and lost its activity at 100°C temperature. Hg²⁺ and Cd²⁺ metal ions slightly inhibited the activity of purified *C. occidentalis* PI whereas it retained its higher activity at 0.6% DMSO. *IC₅₀* was found to be 4.83µg/ml for *C. occidentalis* trypsin inhibitor whereas it was 3.02µg/ml for STI. *LC₅₀* calculated for the purified inhibitor was 137.91µg/ml.
Protease inhibitor purified from *C. occidentalis* was investigated for its effect on the development and enzyme system of second instar larvae of *S. litura*. Soybean trypsin inhibitor was taken as the positive control.

Test diets incorporated with various concentrations (0.375, 1.6, 6, 24, 96µg/ml and control) of purified inhibitor from *C. occidentalis* PI and soybean trypsin inhibitor showed drastic affect on growth and fitness indices of *S. litura*. LGI, PGI and SGI were found to decrease with increase in concentration of purified inhibitor. FI reduction was more with *C. occidentalis* PI than soybean trypsin inhibitor. Nutritional indices revealed decrease in RGR, ECI and ECD in a dose dependent manner whereas AD and MC were increased with increase in concentration. Purified inhibitor when given in diet caused marked reduction in growth and development of *S. litura*. The decrease in LGI clearly showed that less number of larvae pupated as concentration of trypsin inhibitor from *C. occidentalis* seeds was increased. Emergence was also reduced as evident from decrease in PGI. Also SGI and FI decreased with increase in concentration of purified inhibitor in the diet. A number of adult deformities were observed in the present study which could be related to interference in protein metabolism as the same is required for the metamorphosis process.

This was clearly indicated by the observations made on proteolytic enzymes in second instar larvae of *S. litura* where both trypsin and chymotrypsin activities in lumen, gut and feces were inhibited with almost each treatment interval when they were given purified *C. occidentalis* PI incorporated diet.

Purified inhibitor from *C. occidentalis* also inhibited growth of *A. brassicicola*, *C. acutatum*, *A. alternata* and *F. moniliformae* fungi. Maximum zone of inhibition was observed in *C. acutatum* with minimum inhibitory concentration (MIC) of 192µg/ml.

The present findings from the study showed that among the five protease inhibitors partially purified from the seeds of non host plants, those extracted from *C. occidentalis* exhibited the most deleterious effect on the development of *S. litura* larvae. The disturbance in the development process caused by the consumption of these PIs in the partially purified and purified form might be the result of impairment in the
developmental as well as digestive physiology of the larvae which is associated with the inhibition of digestive proteases.

Also the purified inhibitor from *C. occidentalis* inhibited the digestive proteases which clearly demonstrated that these inhibitors impaired insect digestion and could be good candidate proteins to be used in transgenic plants for developing resistance to insect pests. Since serine proteases are the major digestive enzymes in the gut of lepidopteran insects their susceptibility to *C. occidentalis* inhibitor as revealed by the kinetic analysis in the present study indicates that the inhibitor isolated from *C. occidentalis* seeds has immense potential for its use against insect pests as well as phytopathogenic fungi.