DISCUSSION

In the current study, inhibitors were partially purified from mature dry seeds, which were then assessed for their anti insect potential against the common cutworm, *Spodoptera litura* (Fabricius). Protease inhibitor in partially purified form with maximum inhibitory activity and growth inhibitory potential was further purified to homogeneity as well as characterised.

5.1 Effect of partially purified PIs on digestive physiology of *S. litura*

All partially purified PIs reduced the weight of the *S. litura* larvae when they were weighed in their final larval stage but maximum reduction was observed in the larvae reared on diet incorporated with *C. occidentalis* partially purified PIs. Mean larval weight in *C. occidentalis* PI fed larvae was reduced to 92mg which was much less than larvae reared on *L. leucocephala, C. glauca, A. lebbeck* and *T. stans* partially purified PIs. Even after 10 days of feeding, larvae growing on *C. occidentalis* treated diet were unable to attain the body mass corresponding to that of control larvae which clearly showed that larval growth was affected. These findings are in conformity with the outcome of our earlier work (Vasudev and Sohal, 2013) where a significantly lower mean weight of *S. litura* larvae was observed when they were fed on diet containing partially purified cauliflower PIs as compared to the mean weight of larvae reared on control diet. The decline in weight of *S. litura* larvae could be either due to low consumption of diet or might be the result of post ingestive inhibitory effect of the inhibitors on the digestive enzyme activity of the insect. The gut is the main site of digestive proteases and serine proteases are the predominant enzymes in the gut of Lepidopteran insects (Pauchet *et al.*, 2008). The protease inhibitors can slow down the proteolytic activity by inhibiting the activity of digestive proteases. The inhibition of protease activity restricts the availability of necessary amino acids leading to poor larval growth and development (De Leo *et al.*, 2002; Nanasahe *et al.*, 2008).

Larval period of second instar *S. litura* larvae was significantly prolonged with protease inhibitors partially purified from *L. leucocephala, C. occidentalis, A. lebbeck* and *T. stans* whereas it declined with *C. glauca* PIs. Also pupal period showed
significant increase when larvae were given *C. glauca* and *T. stans* partially purified PIs amended diet whereas it decreased significantly with PIs partially purified from *L. leucocephala, C. occidentalis* and *A. lebbeck*. These findings indicated differential susceptibility of *S. litura* larvae to inhibitors from different plant seeds. Pompermayer *et al.* (2001) had also reported significant affect of partially purified soybean inhibitor on the growth and development of *D. saccharalis* larvae when amended in artificial diet. Also partially purified trypsin inhibitor obtained from *Theobroma cacao* seeds at 0.25% concentration increased the larval and pupal period in *D. saccharalis* and *A. gemmatalis* (Paulillo *et al.*, 2012). The larvae of Lepidoptera insects feed actively on their host plant so as to accumulate efficiently all the nutrients present in the food. If the nutrient accumulation efficiency of the larvae is disturbed, it will directly affect the larval weight and size which could have a negative impact of PI on later life stages of *S. litura* as has been observed in the present study.

It was observed in the current study that lesser number of larvae pupated when they were reared on diet administered with partially purified PIs from *L. leucocephala, C. occidentalis, C. glauca, A. lebbeck* and *T. stans*. Also pupae emerged from treated larvae weighed less than those in the control. Out of these non host plant PIs, maximum reduction in percentage pupation was observed in the larvae treated with different concentrations of *C. occidentalis* PIs where the size of pupae was drastically affected. Small sized pupae were also seen to develop from larvae reared on *L. leucocephala* PI incorporated diet. Small sized pupae clearly accounts for the reduction in weight of the larvae. Paulillo *et al.* (2012) had also observed decrease in percentage pupation in *H. virescens* when given 0.25% partially purified *T. cacao* trypsin inhibitor in the diet. *S. litura* effectively completes its life cycle by adjusting and altering its metabolic process on wide range of host plants. Efficiency of pupation in actively feeding lepidopteran larvae is decided by protein content in diet which is manifested by achieving maximum pupal weight. Any shortage of proteins will lead to less larval growth resulting in reduced pupal weight.

Prepupal and pupal mortality was observed at high doses of partially purified *C. occidentalis* PIs which clearly accounts for the toxic effect of this non host PI on *S. litura* larvae. In holometabolus insects, metamorphosis involves the synthesis of new
adult tissues and enzymes which are principally based on the uptake and utilization of essential proteins restricted in the insect cuticle (Patterson, 1957). These essential proteins are synthesized from free amino acids following a restructuring and remodelling of peptides from larval proteins without total degradation of amino acids. Disturbances in the normal growth and development in the form of malformed adults or mortality of inter molt stages may be attributed to the inhibitory effects of *C. occidentalis* partially purified PI on protein utilization by the larvae.

Percent emergence of females and males emerged from larvae reared on partially purified PIs from *L. leucocephala*, *C. glauca*, *A. lebbeck* and *T. stans* showed interesting findings. Lesser number of females emerged in a concentration dependent manner in comparison to males. No emergence occurred from larvae reared on *C. occidentalis* PI amended diet as there was 100% pupal mortality. More number of males emerged from larvae fed on *T. stans* partially purified PIs amended diet. No female emerged at highest concentration of *T. stans* PI. High rate of morphological abnormalities in emerged adult moths was observed when the second instar *S. litura* larvae were given partially purified *C. occidentalis* PI incorporated diet. These aberrations were more pronounced at higher concentrations. Reduced survival of adults and deformities in them with incomplete emergence in *H. armigera* and *S. litura* was reported by Mittal *et al.* (2014). Also Paulillo *et al.* (2012) observed similar adult deformities in *D. saccharalis* and *A. gemmatalis* when larvae were given partially purified *T. cacao* inhibitor incorporated diet. Adult deformities could be caused by the absence of proteins necessary for metamorphosis. It appears from these results that non host PIs acted as antimetabolites as they targeted the insect’s digestive tract and interfered with assimilation efficiency of insect required for normal growth and development.

Partially purified inhibitors from *L. leucocephala*, *C. glauca*, *A. lebbeck* and *T. stans* caused marked reduction in longevity and fecundity of adults along with percent hatching of eggs laid by adult female moths. The females emerged after being fed on diet containing *L. leucocephala* and *T. stans* failed to lay any eggs. No hatching of eggs occurred at the highest concentration of *A. lebbeck* PI. Detrimental effect on fecundity and fertility has previously been reported on *S. litura* with partially purified PIs from
Brassica oleracea (Vasudev and Sohal, 2013). PIs extracted from a non host plant such as bitter gourd too had detrimental effect on fertility and fecundity of S. litura and H. armigera larvae (Telang et al., 2003). Less fecundity as well as less number of female adults in present study could be accounted for by the reduced bioavailability of protein or amino acids which are required for growth and vitellogenesis. This could also be the reason for the decrease in larval and pupal mass observed in the present study. The decrease in the weight of pupae formed from larvae of S. litura fed on non host PI amended diet could have adversely affected the reproductive capacity of emerged females. Achieving high pupal mass is significant as there exists a strong association between adult body weight and its reproductive potential (Tammaru et al., 1996).

5.2 Effect of partially purified PIs on nutritional indices of S. litura

Partially purified PIs from non host plants changed the metabolic performance of S. litura larvae. Noteworthy decline was observed in relative growth rate of S. litura larvae after ingestion of L. leucocephala, C. glauca, C. occidentalis, A. lebbeck and T. stans PI treated diet. Reduction was dose dependent and highest at higher concentrations. Significant decrease in relative consumption rate was observed with most of the non host PIs except C. glauca where it increased at higher doses in comparison to control. Nutritional indices and its analysis can give some insight into the behavioural and physiological aspects of insect-plant interactions (Lazarevic and Peric-Mataruga, 2003). Low RGR and RCR indicated that less food was utilized by the larvae. Srinivasan and Uthamasamy (2005) had reported that larval weight is based on RCR. Our findings also demonstrated a dose dependent decrease in the larval weight which correlates with the decrease in RCR. Consequently pupation was delayed and the larvae were smaller in size, weighing much less than control which was evident from the low RGR. However, although the RCR decreased in larvae fed on C. glauca treated diet as a result of which the larval period was shortened, the weight of the larvae and other nutritional indices viz. RGR, ECI and ECD declined compared to control. These findings reveal inability of the larvae to utilize the ingested food for its growth thereby suggesting an inhibitory effect of the inhibitor on the larvae of S. litura.

ECI measures an insect’s capacity to utilize the food ingested for growth and development (Koul et al., 2004). It was highest for control and lowest for highest
treatment. This indicated that the insect larva was unable to use the proteins in diet containing partially purified PIs from non host plants. Efficiency of conversion of digested food is another important feeding index which measures the insect’s efficiency to convert digested food into growth & development (Senthil-Nathan et al., 2005). Our findings showed that ECD values decreased in the larvae fed on diet amended with partially purified PIs which suggests that these larvae might not have been able to turn digested food into biomass. It is well known that the quantity of food utilization depends on the digestibility of food and the efficiency with which digested food is changed into biomass (Batista Pereira et al., 2002). Approximate digestibility and metabolic cost increased in a concentration dependent manner in all the treatments. Similar effect of PIs on nutritional indices of several lepidopteran larvae has been reported (da Silva et al., 2012; Mittal et al., 2014; Singh et al., 2014). Insect larvae often compensate for detrimental digestive effects associated with their food by increasing the AD. So increase in the AD in the present investigation, could be an attempt made by the insect to compensate for the inferior nutritive value of food in order to accomplish the desired growth rate. This in turn might have increased the metabolic cost of larvae of *S. litura* as the ability of an organism to convert nutrients especially proteins, positively influences its growth and development (Sogbesan and Ugwumba, 2008). Similar findings were observed in *H. armigera* and *D. saccharalis* when given non host plant protease inhibitors (da Silva et al., 2012; Singh et al., 2014). As utilization, nutrient uptake, body composition and development rate are highly dependent on the balance of nutrients in the food, shortage of these nutrients directly affects the normal physiology of the insect resulting in stage specific mortality as well as abnormality.

5.3 **Effect of partially purified PIs on Digestive proteases of *S. litura***

Most of the partially purified protease inhibitors decreased the activity of trypsin and chymotrypsin in the lumen content and gut tissue of the larvae of *S. litura* at either all feeding intervals or after prolonged feeding of the larvae on treated diet. It was also observed that the higher concentrations of the inhibitors had a greater inhibitory effect on the trypsin and chymotrypsin activity in most of the treatments. Telang *et al.* (2003) had observed a similar effect on *S. litura* and *H. armigera* by using a PI from a non host
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source such as bitter gourd. Other non host plant PIs i.e. peanut, winged bean and E. jambolana showed similar results against Helicoverpa gut proteinases (Harsulkar et al., 1999; Singh et al., 2014). Trypsin and chymotrypsin are important enzymes in the digestive system of lepidopteran insects (Srinivasan et al., 2006). Inhibition of the digestive enzymes leads to strong physiological stress on the insect for the availability of the essential amino acids. This leads to a loss in weight and reduction in reproductive strength of the insect. The decline in the activity of luminal and gut wall proteases of S. litura larvae thus indicate toxic effect of the partially purified inhibitors which might have slowed down larval midgut proteinase activity by blocking the enzymes. However the levels of trypsin were induced in the lumen of the larvae after prolonged exposure to partially purified PIs from A. lebbeck and in the gut tissue at all feeding intervals with partially purified PIs from T. stans. The chymotrypsin activity too increased at most feeding intervals in the lumen and gut content of the larvae fed on diet having A. lebbeck partially purified PIs when compared to control. It has been suggested that some insect larvae respond to PIs in the diet by secreting additional digestive enzymes which are insensitive to inhibition by PIs (Bolter and Jongsma, 1995; Jongsma et al., 1995). However increased secretion of additional proteinases in response to the inhibitors requires the utilization of essential amino acids that could starve the insects (Broadway and Duffey, 1986; Broadway, 1995).

Also, the peritrophic membrane (PM) is a structure imperative for the normal physiology of the insect midgut. Selective pressure during the development of the PM is essential to systematize gut lumen into ecto and endo peritrophic spaces to reduce the evacuation of digestive enzymes with the feces (Terra, 2001). Nevertheless, the present study showed elevated levels of chymotrypsin in fecal matter of the larvae with almost all protease inhibitors and that of trypsin were observed in the larvae fed on diet having partially purified PIs from C. glauca and T. stans. These findings indicated some sort of change in the membrane permeability which might have resulted in disruption of enzyme recycling mechanisms. It could be an alternative explanation for the observed increases in the proteolytic activity in fecal extracts collected from PI fed larvae.
5.4 Effect of partially purified PIs on phytopathogenic fungi

Out of five nonhost plants, only *C. occidentalis* protease inhibitors inhibited the fungal growth in both susceptible (*A. brassicicola, A. alternata*) and resistant (*F. oxysporum*) strains effectively. Plant protease inhibitors were considered to inhibit the mycelia growth of microorganism by an antifeedent mechanism (Vernekar et al., 2001). Trypsin inhibitors isolated from seeds have been reported to interfere in normal fungal cell wall development by preventing proteolytic activation of chitin synthase zymogen (Lopes et al., 2009). This can be better explained by understanding the role of PIs in protecting the seed from pathogens.

5.5 Purification and characterisation of PI from *C. occidentalis*

Purified inhibitor with molecular mass of 14.3kDa was obtained and confirmed with both multistep strategy and three phase partitioning (TPP). Inhibitory activity staining also confirmed the presence of same molecular weight protein. Different molecular mass was reported for PIs from different plant sources, for example, *C. cajan* (14 kDa; Haq et al., 2004), *D. biflorus* (16 kDa; Kuhar et al., 2013), *Glycine max* (17.9 kDa; El-latif, 2015). In both the purification methods, specific activity and purification fold was more or less similar. Sharma and Gupta (2001) used three phase partitioning as a single purification step for protease inhibitors from wheat germ. TPP is a more efficient method to produce purified inhibitor at large scale in millilitre volume (Saxena et al., 2007; Narayan et al., 2008). Our results showed low recovery percentage and purification fold compared to other reported purified proteins from different plant sources. Similar results were obtained with *Terminalia arjuna* and *A. nilotica* (Rai et al., 2008; Babu et al., 2012). This could be because of interferences caused by higher levels of phenols and mucilaginous polysaccharides and high concentration of inhibitor in the seeds (Prabhu and Pattabiraman, 1980; Rai et al., 2008). The purified inhibitor was tested for the type of inhibition and *Ki* determination individually against *S. litura* gut trypsin using BApNA as a substrate. Inhibitor showed non-competitive inhibition and *Ki* value of 0.3μM. There are variable reports regarding type of inhibition and *Ki* value. The inhibitor purified from black gram showed non-competitive inhibition against trypsin with *Ki* value of 309.8nM (Prasad et al., 2010). Also trypsin inhibitor
isolated from *Achyranthes aspera* seeds inhibited bovine trypsin non-competitively with $Ki$ value of $2.9 \times 10^{-10}$M (Geetha *et al*., 2013). The variations may be explained by the fact that different inhibitors have different affinities for trypsin. The pH stability results indicated that inhibitor is active at wide range of pH from 5 to 12 with maximum inhibitory activity recorded at pH 9. In general, all the PIs isolated from plants have a wide pH range of 2–10 (Bijine *et al*., 2011). Singhal (2004) has reported that trypsin inhibitor purified from mungbean seed showed activity between pH 4 and 10. In contrast, the trypsin inhibitor from Gizza 22 soybean variety retained its activity between pH 2 and 12. In the current study, the stability of the inhibitor isolated from non-host plant over a wide range of pH might suggest its efficiency in controlling a variety of phytophagous insects that have variation in their gut environment.

The thermostability of the purified inhibitor was also studied. Protease inhibitors isolated from legumes are quite stable up to 80°C but lose activity above this temperature (Gupta *et al*., 2000; Kuhar *et al*., 2013). In the present study, the trypsin inhibitor extracted from *C. occidentalis* seeds was found to be stable up to 90°C but was completely inactivated above this temperature. Similarly, Babu *et al*., (2012) found that inhibitor extracted from *A. nilotica* lost its activity completely at 100°C. The stability of inhibitor at high temperature may be attributed to its inflexible and compact protein structure which is stabilized by a number of disulfide linkages as suggested for protease inhibitor isolated from pea seeds (Sierra *et al*., 1999).

The inhibitory activity of trypsin inhibitor was measured in the presence of different metal ions. Among the different metal ions, Hg and Cd were found to slightly inhibit the activities whereas rest of the metal ions did not show any significant decrease. Similar study conducted by Kuhar *et al*., (2013) reported interaction of metal ions with inhibitory activity of purified inhibitor from *D. biflorus*, in which Co$^{2+}$, Cu$^{2+}$, Ni$^{2+}$ were found to inhibit the trypsin activity. DMSO at a concentration of 0.6% gave maximum trypsin inhibitory activity whereas at higher concentration of 0.9% it was inhibited. No effect of DMSO was studied with *D. biflorus* PI upto a concentration of 0.5%.

The IC$_{50}$ value of inhibitor purified from *C. occidentalis* seeds for midgut trypsin like proteinases was found to be 4.83µg/ml which was 1.59 times more than that
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for soybean trypsin inhibitor. Jamal et al. (2014) had reported Madhuca indica inhibitor with IC$_{50}$ value of 1.75µg/ml against Helicoverpa gut trypsin like proteinases.

5.6 Effect of purified inhibitor on growth and development of S. litura

Purified inhibitor from C. occidentalis seeds when given in diet caused marked reduction in growth and development of S. litura. Less number of larvae pupated as concentration of trypsin inhibitor from C. occidentalis seeds increased which is clear from the low value of LGI in the treatments as well as compared to control. Larval stage of a lepidopteran insect is a ferocious feeding stage which causes maximum damage to the plants. Less number of nutrients accumulated during this stage can interfere in further developmental stages. Similar finding of less pupation in S. litura has been reported by Arimatsu and Sawangsook (2012) when the insect was given purified Momordica cochinchinensis trypsin inhibitor in diet. Emergence was also reduced as evident from PGI. Also SGI and FI decreased with increase in concentration of purified inhibitor in the diet. Similar effects were observed with positive control (STI) on the larvae of S. litura. A possible reason for the arrested growth and development of larvae of S. litura could be physiological stress caused by the purified inhibitor resulting in a decrease in larval and subsequently pupal weight. Telang et al. (2003) had reported the anti-digestive effect of a non host plant PI i.e. bitter gourd on S. litura. Also, a number of adult deformities were observed in the present study which could be related to the interference in protein metabolism as the same is required for the metamorphosis process.

The reduction in diet utilization suggests that reduction in growth and development of S. litura larvae might have resulted from both behavioural and physiological effects. It is likely that the decrease in RCR could be due to a deterrent effect on the larvae induced by post ingestive toxic effect of the purified inhibitor and accounts for the majority of the decrease in growth rate. The purified inhibitor also altered food utilization indices in S. litura and revealed less conversion of ingested (ECI) and digested (ECD) food to body biomass. A drop in ECI could be due to the reason that more food might have been metabolized for energy purpose and less for conversion to body substance. As a result ECD also decreases. Thus, decreased ECI and
ECD values in the present studies indicate that ingested inhibitor does have some sort of toxicity in gut of insect. The inhibitor also influenced AD and MC of larvae fed on amended diet as they increased with increase in concentration. Nevertheless the growth rate of the larvae was reduced which clearly showed that in spite of an increase in AD, the decrease in ECD could not be compensated clearly revealing the toxicity of the purified inhibitor.

5.7 Effect of purified inhibitor on digestive proteases of S. litura

Insect digestive enzymes can be targeted by plant proteinase inhibitors to disturb the normal food utilization. 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 at almost each treatment interval when second instar larvae of S. litura were given purified C. occidentalis PI incorporated diet. Acute feeding of exogenous inhibitors i.e SBTI and TLCK decreased the trypsin activity in the lumen of S. frugiperda larvae (Lwalaba et al., 2010). Similar results were observed for gut trypsin of S. littoralis larvae when given trypsin inhibitor (Sharma et al., 2012). Also Lomate and Hivrale (2011) noticed that serine proteinase activity was inhibited in H. armigera larvae when given non host plant PIs. Similar pattern of proteolytic activity was observed in the feces of H. armigera larvae fed on non host plant PIs (Patankar et al., 2001). So the inhibitory action of purified inhibitor on S. litura larvae indicated that they downregulated protein digestion as reported by Pandey et al. (2014). The present findings showed that the purified inhibitor had a considerably greater inhibitory effect on S. litura than the partially purified inhibitor from C. occidentalis seeds indicating its potential to confer resistance in plants against S. litura.

5.8 Effect of purified inhibitor from C. occidentalis on phytopathogenic fungi

PIs partially purified from five non host plants were evaluated for their antifungal property against important phytopathogens. Out of these only PIs isolated from C. occidentalis showed maximum antifungal property against both susceptible (A. brassicicola, A. alternata) and resistant (F. oxysporum) strains of fungi. Also purified inhibitor inhibited growth of A. brassicicola, C. acutatum, A. alternata and F.
moniliformae fungi. This can be best explained with the role of PIs which may be related with protection of seeds during early germination in plants when the tissues are mainly exposed to attack by soil borne pathogens (Satheesh and Murugan, 2011). Results of the current study indicated a strong antifungal activity of the purified inhibitor against important phytopathogenic fungi, which was probably related to its inhibitory activity against fungal proteases. This implies that the purified inhibitor of C. occidentalis might have potential as an antifungal agent against broad range of fungal pathogens (Yang et al., 2006; Chandrashekharaiash, 2013).

Our findings thus clearly showed that the larvae of S. litura were susceptible to protease inhibitors from non host plants. Thus protease inhibitors isolated from non host sources can be considered as good candidates for enhancing the resistance of crop plants to the insect pests.