SCREENING, ISOLATION AND CHARACTERIZATION OF PLANT PROTEASE INHIBITORS AGAINST LARVAL GUT PROTEASES OF *SPODOPTERA LITURA* (FABRICIUS) (LEPIDOPTERA: NOCTUIDAE)

Thesis submitted to
the University of Calicut in partial fulfilment of the
requirements for the Degree of

DOCTOR OF PHILOSOPHY IN ZOOLOGY

by

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DEPARTMENT OF ZOOLOGY
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2021

CERTIFICATE

This is to certify that the thesis entitled "**SCREENING, ISOLATION AND CHARACTERIZATION OF PLANT PROTEASE INHIBITORS AGAINST LARVAL GUT PROTEASES OF *SPODOPTERA LITURA* (FABRICIUS) (LEPIDOPTERA: NOCTUIDAE)**", is an authentic record of research work carried out by **Mrs. Sajitha, R.**, under my guidance and supervision as a full time research scholar in partial fulfillment of the requirement of the **Degree of Doctor of Philosophy in Zoology** under the Faculty of Science, University of Calicut. This is an original work and no part of this thesis has been presented previously for any other Degree.

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DECLARATION

I, **Sajitha, R.**, do hereby declare that the present work in this thesis entitled "**SCREENING, ISOLATION AND CHARACTERIZATION OF PLANT PROTEASE INHIBITORS AGAINST LARVAL GUT PROTEASES OF *SPODOPTERA LITURA* (FABRICIUS) (LEPIDOPTERA: NOCTUIDAE)**" submitted to the University of Calicut, as partial fulfilment of Ph.D. programme for the award of the degree of Doctor of Philosophy in Zoology is original and carried out by me under the supervision of Dr. Kannan V.M., Professor, Department of Zoology, University of Calicut. I further declare that no part of this thesis has been submitted previously for any other Degree of any university.

Sajitha, R

Calicut University,
Date: 04/10/2021

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Sajitha R

Dedicated

To My Beloved Parents, Husband and Kids

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ABBREVIATIONS

°C	:	Degree Celsius
2-ME	:	2-mercarptoethanol
Alkaline PAGE	:	Alkaline Polyacrylamide Gel Electrophoresis
APS	:	Ammonium persulfate
BAPNA	:	Nα-benzoyl-DL-arginine-4-nitroanilide
BSA	:	Bovine Serum Albumin
CNBr	:	Cyanogen Bromide
Da	:	Dalton
DEAE	:	Diethylaminoethyl
DMSO	:	Dimethyl sulfoxide
g	:	Gram
HAPI	:	*Hibiscus acetossella* Protease Inhibitor
kDa	:	Kilo Dalton
M	:	Molar
mg	:	Milligram
ml	:	Millilitre
mM	:	Milli Molar
Na2CO3	:	Sodium carbonate
NaHCO3	:	Sodium bicarbonate
nM	:	Nano Molar

PI	:	Protease inhibitor
PPI	:	Plant Protease Inhibitor
SDS	:	Sodium dodecyl sulphate
SE	:	Standard Error
TCA	:	Trichloro acetic acid
TEMED	:	N,N,N',N'- tetramethyl ethylene diamine
Tris	:	Tris(hydroxymethyl)aminomethane
µg	:	Microgram
µL	:	Microlitre
µM	:	Micromolar

INTRODUCTION

1. INTRODUCTION

Agriculture sector plays a strategic role in the economic development of a country. One of the major threats that the agriculture sector faces today is profitability, as the costs of farming continues to rise. Second one is ruining of soil fertility and third is the threat posed by the pests, weeds and diseases. Pests, an age-old enemy of agriculture, continue to impede this sector by damaging crops and therby food production.Pests are harmful species whose population or density goes beyond the damage threshold level either throughout the year or during specific season (Dent, 2000). Insect pests though tiny, appear in large numbers and are capable of large-scale destruction. On average the pests are known to cause about 37% loss in agriculture worldwide (Haq, 2004). Thus pest control has become an inevitable part of any successful agricultural practices. Advancement in the pest control strategies is necessary for meeting the demands of the booming population. At present, crop protection relies predominantly on the use of environmentally toxic agrochemicals, that are also deleterious to human health (Haq et al., 2004)

By the middle of the 19th century, the golden age of pesticides, the pest control strategies began to incorporate the systematic use of chemicals. With the discovery of the insecticidal properties of dichloro-diphenyl-trichloro-ethane (DDT) and many other chlorinated hydrocarbons, pesticides became the only tools to check the pest menace both in agricultural and public health applications (Ambuj,

1991). The extensive use of conventional insecticides has thus resulted in several major concerns in both sectors. The development of resistance to a given pesticide by both target and pest resurgence and replacement, loss of natural enemies and effects on non-target species were the major concerns in the agricultural sector (Ripper, 1955). This is particularly relevant for broad spectrum insecticides, whose toxic action is not limited to the target species. Insecticide resistance is characterized by rapid evolution under strong selection of gene(s) that confers survival from insecticides (Ahmad et al., 2008). The consequences are increased crop injury and potential loss in crop production (Tapa, 1998) and also disruption of biological control as their natural enemies are wiped out (Dutcher, 2007). On the other hand the insecticides have deleterious effects on the beneficial insects too. There are clear evidences for the significant decline in the abundance and distribution of many pollinators, with some extinction both in local and global level (Goulson et al., 2015). Although first generation pesticides have considerable environmental damage, there was a perception that modern pesticides are much safer (Dudley et al., 2017). But recent studies reveal that the intensity of environmental damage caused by these insecticides is significant. Environmental contamination with neonicotinoid insecticides have led to a decline in wild bees (Woodcock et al., 2016), butterflies (Forister et al., 2016), aquatic insects (Dijk, 2013), and insect-eating birds (Wood and Goulson, 2017). Since the regulatory tests largely focus on short-term studies in which test organisms are exposed to a single chemical, the risks posed by the pesticides are not correctly captured (Milner and Boyd, 2017). Chemical methods alone have negative impacts on the

environment and public health. Thus Integrated Pest Management (IPM) was introduced.

Integrated Pest Management relies on a combination of chemical control, biological control, cultural control, mechanical control, and genetic control or expressing foreign insecticidal genes in crops. Current control also relies on the use of transgenic crops with *Bacillus thuringiensis* (Bt) expressing genes (Haq, 2004) at least in some parts of the world, though conventional pesticides have not been completely replaced. Transgenic Bt crops expressing the Cry protein genes (crystal proteins or endotoxins) target key pests. Compared to the conventional Bt sprays, which could only protect the plant surface, the toxin in Bt crops is expressed throughout the tissues. Thus the transgenic plants can effectively control sucking insects, root pests, stem and fruit borers (Benedict, 2003) However, there are limitations to the use of transgenic Bt plants as well because of issues related to public acceptance. Bt toxins are capable of controlling relatively a narrow range of insects. Persistence of the Bt toxin in excessive amount within the plant throughout the growing season paves way for insect resistance (Moar et al*.,* 1995).With long term use of Bt crops, pests develop resistance to the crop as in the case of *Spodoptera frugiperda, Helicoverpa zea, Busseola fusca*, *Diabrotica virgifera virgifera* and *Pectinophora gossypiella* (Tabashnik, 2013). In spite of significant crop loss caused by aphids, no Bt toxin with adequate aphid toxicity has been described. Hence, it is necessary and desirable to develop safer and more effective transgenic alternatives to reduce crop losses. One such alternative is the plant defence proteins that are more

specific against insect pests (Maqbool et al., 2001). In most plants, they offer horizontal resistance against insect attack (Fabrick et al., 2002). Plant protease inhibitors are one of such plant defensive molecules, which are exploited nowadays for their ability to control insect pests.

The protease inhibitors (PIs) are small molecules that play vital role in many biological processes related to metabolism and cell physiology (Rawlings, 2004). In plants, they have been related to the regulation of endogenous enzymatic activities, mobilization of storage proteins, modulation of apoptosis and programmed cell death and stabilization of defence proteins/compounds against insect, microbial and animal attack (Mosolov and Valueva, 2005).

The role of protease inhibitors in plant defence against insect pests was unveiled when Mickel and Standish observed the inability of the larvae of *Tribolium confusum* to grow on soybean products (Mickel and Standish, 1947). Later on the presence of trypsin inhibitors in soybean and its toxic effect on the larvae was proved (Lipke et al., 1954). These findings suggest the ability of plant PIs to block protein digestion and retard the insect growth and development (Ryan, 1990). Apart from in vitro assays with insect gut proteases (Pannetier et al., 1997) expression of protease inhibitor gene in transgenic plants (Vain et al., 1998) were also carried out. Generally transgenic crops with protease inhibitors against insect digestive enzymes are designed not to kill the insects that feed, but rather to retard their development. Presumably, this is the basic difference in the chemical based pest control methods which aim at complete control

through immediate pest mortality (Koundal and Rajendran, 2003). There are several successful examples of transgenic plants expressing protease inhibitors of plant origin conferring insect resistance such as in tobacco, rice, cotton, strawberry, poplar and peas (Ussuf, 2001). In addition, combinations of Bt with protease inhibitor genes have been used to create pest-resistant plants such as poplar trees (Zhang et al., 2005). All such multi-gene clones were more toxic to both larvae and adults of target insects than single gene clones (only Bt).Thus the role of plant protease inhibitors in pest control has promising effects compared to the existing pest control strategies. Moreover many PIs of plant origin have antibacterial and antifungal properties (Kim et al., 2009), which would protect plants from fungal and microbial diseases too. In this scenario identification and purification of potent plant protease inhibitors against insect pests are of utmost importance.

OBJECTIVES OF THE STUDY

1. **Screening of plant extracts to identify protease inhibitors against the larval gut proteases of *Spodoptera litura*.**

2. **To isolate potent plant protease inhibitor most effective against larval gut proteases of *Spodoptera litura*.**

3. **To characterize the isolated plant protease inhibitor against larval gut proteases of *Spodoptera litura*.**

4. **To study the *in vivo* effects of the plant protease inhibitor on the *Spodoptera litura* larvae.**

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 AGRICULTURE

Agriculture sector plays a vital role in the economic development of every country. For many developing countries like India, agriculture is the important source of livelihood for over 65% of its population. Apart from the major cereal crops, vegetables, oil seeds and fibre crops are important constituents of Indian agriculture (DOR, 2013). India stands second in the world vegetable production contributing 14% of world's total vegetable yield (Vegetable statistics. Technical Bulletin Indian Institute of Vegetable Research 2000-2011).India is the 4th largest producer of edible oil seed, 3rd largest consumer of the same and 2nd largest importer of edible oil seeds in the world (DVVOF, 2017). Groundnut accounts for about 45% of oilseed production in India and is mainly cultivated in the southern and north western states of India. Pest attack is the major cause of yield loss in yield of most of the vegetable, oil seed and fibre crops in India. Insect pests cause 16% loss in the groundnut yield (Ahir, 2018). Among the fibre crops, cotton is the most important crop grown in India. Major yield loss in cotton cultivation is also due to insect pests. Rathee and Dalal reported that the insect pests on an average cause 15%-25% yield loss in the cash crops (Rathee and Dalal, 2018). Advances in the agriculture after green revolution and extensive monoculture has resulted in the surplus growth of many insect pests (Pingali et al.,1997)

2.2 INSECT PLANT INTERACTIONS

Plants and insects are highly diverse groups owing to their ability to cope up with wide range of niches. Each plant interacts differently with different insects. Plants usually serve as an energy source or nesting place for insects. Insects mainly act as protectors, dispersers or fertilizers for plants. Plant-insect interactions can be mutualistic as in the case of interactions with plant and pollinators/ seed dispersers/plant guarding ants. Commensalism is exhibited to a certain extent, where larvae of monarch butterfly store cardiac glycoside of certain milk weeds for defensive purposes. Antagonistic interaction is a pronounced one, where both phytophagy by insects and insectivory by plants are included. Plants respond to insectivory by making changes in their morphology, phenology, physiology, allelochemistry or in cell structure and growth (Ohgushi, 2008). Majority of insect herbivores are specific to the host and the plant part on which they feed on. But polyphagous insects have a wide host range as they do not show any host specificity. Apart from the direct effects of herbivory, insects act as vectors of pathological microorganisms, which get transmitted when the insects feed on the plants.

2.3 INSECTS AS PESTS

Pests are harmful species whose population or density goes beyond the damage threshold level either throughout the year or during specific season (Dent, 2000). According to Debacli insects are pests when they are sufficiently numerous in numbers and conflict with the profit percent in the agriculture (Debacli, 1964). Pests are organisms

that cause detrimental loss in any sector for man and his properties (Kenten and Woods, 1976). Pest attack in farming sector adversely affects both crop and livestock health, thus resulting in poor yield. Insect pest is an important biotic challenge to plants (Giri et al., 2006).The pests are reported to cause 20-30% total loss in the agriculture sector (Fuchs and Mackey, 2003). Most of the insect pests that attack the crops come under the order lepidoptera. *Spodoptera litura* and *Helicoverpa armigera* are devasting polyphagous pests which cause serious damage to many important crops worldwide (Aparna et al., 2000; Venette et al., 2003). The diamond black moth, *Plutella xylostella*, distributed all over India is a serious pest of cruciferous vegetables (Neha et al*.,* 2019). Apart from lepidopterans, locusts, bugs and many beetles pose crop loss. The asian pentatomid *Halyomorpha halys*, a polyphagous pest cause severe damage to various plants including ornamentals (Hoebeke and Carter, 2003).The papya mealy bug, *Paracoccus marginatus* which attained a major pest status in India during 2009 caused 90% yield loss to many flowers, fruits, plantation crops and cotton (Neha et al., 2019). *Phenacoccus solenopis*which was initially a threat to cotton, have attained a polyphagous pest status by infesting on most of the cultivated crops belonging to malvaceae, solanaceae, leguminoceae and cucurbitaceae (Vennila et al., 2010). Thus pest control by any means has become an inevitable process in any farming practice.

2.4 POLYPHAGOUS PESTS

Polyphagous insect pests are the most destructive group of pests in the agriculture. They can destroy all the vegetation in a certain

area when the climatic conditions are favourable. *S.litura* which has a wide host *range* of up to 120 species (Venette et al., 2003) and stands second to *Helicoverpa armigera* which can infest on 181 cultivated and uncultivated crops worldwide (Manjunath et al., 1989). In India both *S.litura* and *H.armigera* attack resultsin significant yield loss. Apart from lepidopteran pests, locuts, grasshoppers and termites are also known to cause damages in the agriculture sector.

2.4.1 *SPODOPTERA LITURA* AS A PEST

Spodoptera litura or tobacco cut worm or army worm is a polyphagous defoliator of many crops and pose deleterious damage to them by their voracious feeding behaviour. Since they are polypshagous pests, their host range is large and includes the crops grown for food, fibre, plantation and forestry crops. Late harvested crops are mostly affected where severe infestation can lead to even 100% root damage resulting in considerable yield loss. They are also found in a complex with other defoliating pests, but may also damage tubers and roots. Defoliation up to 29% and 48.7% were reported from *Colocasia esculenta* and *Glycine max* respectively (Pillai et al., 1993).

2.4.2 PEST STATUS OF *SPODOPTERA LITURA*

Spodoptera litura is widely distributed throughout tropical and temperate regions of South and East Asia, Europe, Africa and Oceania (Shu et al., 2017). In India, the pest is widely distributed in almost all states and cause significant losses to economically important crops. *S.litura* outbreaks in soybean fields in Kota, Marathwada and Vidharbha regions of India was reported and caused monetary loss

upto 4.5 crores (Dhaliwal and Koul, 2010). The pest remains active from the mid of August to October in the soybean fields resulting in 26-29% yield loss (Punithavalli, 2013). It is also a serious pest of groundnut, cotton, tobacco, vegetables and pulses. Severe attack by *S.litura* is marked by complete sceletonization of the leaves, shoot wilt due to the mining activity of larvae, which eventually leads to complete destruction of the plant (Hill, 1975).

2.4.3 PEST CONTROL STRATEGIES FOR *S.LITURA*

S.litura larvae are polyphagous defoliators. Severe damage by this pest occur mainly during dry climatic conditions with high humidity. Adults are strong fliers and they migrate a long distance (Tu et al., 2010). Low winter temperatures are the major limiting factor affecting their distribution (Bale, 1991).They cannot survive winters (Fu et al., 2015). Approximately 12- 23% damage to tomatoes in the monsoon season, and 9-24% damage in the winter (Patnaik,1998) are caused by *S.litura.* Regular field monitoring and cropping the alternate host plants and combination of the biocontrol and chemical control methods are important components of pest control.

2.4.3.1 Regular monitoring

Effective monitoring of *S.litura* for many years was made possible by the use of its male sex pheromone, (ZE) 9,11-tetradecadienyl acetate and (ZE) 9,12-tetradecadienyl by Tamaki (Tamaki, 1973). The presence of newly hatched larvae can be detected by the scratch mark on the leaf surfaces due to larval feeding.

2.4.3.2 Cultural and Mechanical control

Cultural control methods include deep ploughing of fields during summer to expose the pupae. Sowing optimal seed rate recommended for particular crops and avoiding pre-monsoon sowing will help to reduce the pest attack. Mechanical control consisits of collection and destruction of egg masses and actively feeding larval stages, using castor as trap crop, erecting bird perches and light traps (Punithavalli, 2014).

2.4.3.3 Chemical method

Pest control in the past years mainly depended on the extensive and improper use of chemical pesticides. *S.litura* have developed resistance to many commercially available pesticides (Ramakrishnan et al., 1984; Naeem Abbas et al., 2014) like cypermethrin, fenvalerate, organophosphate (Profenofos), pyrethriods (deltamethrin), carbamate and quinalphos (Armes et al., 1997; Kranthi et al., 2001, 2002). Eventhough many new molecules such as chlorantraniliprole, spinosad and emamectin benzoate have shown promising results against *S. litura* (Gadhiya et al., 2014) spinosad resistance is becoming widespread in several insect pests such as *Spodoptera exigua* (Ishtiaq and Saleem, 2011). Laboratory studies showed that *S.litura* developed resisitance against spinosad and fold of resisitance increase to 3921 after 11 generations (Rehan and Freed, 2014), which indicates the chance of resistance development in *S.litura* field population too. Narayanmma et al., reported that flubendiamide 480 SC and chlorantraniliprole 18.5 SC, the newer insecticides were found to equally reduce *S.litura* larval

populations (Narayanamma et al., 2013) Treatment of *S.litura* eggs with thiodicarb 75 WP was reported to cause 88.43% mortality of eggs, followed by flubendiamide 480 SC (69.95%) and emamectin benzoate 5 SG, which has posed 63.98% mortality of *S.litura* eggs (Natikar and Balikai, 2015c). Plant oils with synthetic pyrethroid mixtures gave a higher mortality rate on 8-day-old larvae of *S. litura* than the synthethic pyrethroids alone (Anju and Srivastava, 2012).

2.4.3.4 Biological control

The polyphagous nature of *S.litura*, development of insecticide resistance and destruction of natural enemies has made it difficult for mananging this pest. Thus the need for considering the role of natural enemies for controlling it became important. But initially mass release of egg and larval parasitoids for the control of *S.litura* achieved only partial success (Patel et al., 1971; Michael et al., 1984). Parasitoids and pathogens attacking different stages of *S.litura* are being identified recently but their field trials are not reported.

a) Egg parasitoids

Four species of trichogrammatids, one braconid and one scelinoid has been reported as egg parasitoids (Rao et al., 1992). Mass release of egg-larval parasite *Chelonus heliopae* in 1971-1973 in Gujarat, India, against *S. litura* proved to be ineffective in controlling the pest in cauliflower fields (Rao et al., 1992). Later in 1974, weekly release of another egg parasite *Telenomus remus* in tobacco nursery showed no parasitism (Patel et al., 1971). Braune reported that complete parasitism by *Telenomus remus* on *S.litura* was observed

only in small egg masses and the percentage of parasitization decreased with an increase in the size of egg mass (Braune, 1982).

b) Larval parasitoids

The larval stages of *S.litura* are more prone to parasitism. In a survey conducted by Battu (Battu, 1977), in the castor and cauliflower fields of Punjab, found that *Parasarcophaga misera* and *Campolitis* sp. attacked *S.litura* larvae. Barrion and Litsinger reported the presence of *Peribaea orbata* as a gregarious larval parasitoid on *S. Litura* (Barrion and Litsinger, 1987). Laboratory studies conduted by Yan et al., showed that *Microplitis prodeniae* were engaged in high levels of parasitism against *S.litura* larvae in China (Yan et al., 2014). But the field studies are not yet evaluated. Bhatnagar et al., reported that the mermithid nematodes, *Ovomermis albicans*, *Pentatomermis* sp. and *Hexamermis* sp. parasitized the larval *S.litura* (Bhatnagar et al., 1985).

c) Pupal parasitoids

Only few parasitoids were reported to infest on the pupal stage of *S.litura*. Rao and Satyanarayana reported a pupal parasitoid of *S.litura*, *Lasiochalcida erythropodus* from Andra Pradesh, India (Rao and Satyanarayana, 1984).

d) Predators

The predators of *S.litura* include both predatory insects and spiders. Deng and Jim reported that *Conocephalus* sp. predates on the egg masses of *S. litura* in Guanxi, China (Deng and Jim, 1985). Both the nymphal and adult stages of the pentatomid *Andrallus spinidens*

were observed to feed on larval *S.litura* in the rice fields of Himachal Pradesh (Pawar, 1976).

e) Pathogens

The pathogens of *S.litura* include bacteria, fungi, protozoans, virus and nematodes. Several bacteria were reported to infect *S.litua* and result in several disorders in their growth and development. The laboratory tests conducted by Ansari et al., revealed that *Serratia marcescens* were pathogenic to larval *S.litura* both when ingested through artificial diet and natural plant food (Ansari et al., 1987). Narayanan and Jayaraj reported that a protozoan *Nosema carpocapse* was found to infect *S.litura* larvae in India (Narayanan and Jayaraj, 1979). Among the fungal pathogens, *Beauveria bassiana was* found to infect *S.litura* in cauliflower crops (Zaz and Kushwaha, 1983).Viral infections on *S.litura* has been reported from countries like India, China, New Zealand and Japan. Nuclear polyhedrosis viruses (NPV) are the most potent among them all. Field trials with NPV in black gram fields in Andhra Pradesh gave effective control against *S.litura*. The effects were similar to the chemical control with insecticides (Krishnaiah et al., 1985). A novel NPV reported from Pakistan, named as *Spodoptera litura* nucleopolyhedronvirus (SpIt) has shown promising results in controlling the 2^{nd} and 3^{rd} instars of *S.litura* (Ghulam Ali et al., 2018). The entomopathogenic nematodes Steinernematids and Heterorhabditids carry symbiotic bacteria *Xenorhabdus* spp and *Photorhabdus* spp, in their gut respectively (Boemare, 2002), which when released into the inset gut causes septicaemia and kills the host within 24-48 hours. Innoculates from

Steinernema abbasi and *Heterorhabditis indica* showed 50% (within 24 hours) and 75.6% (after 72 hour) mortality of larval *S.litura* respectively (Babita et al., 2017)

2.5 PLANT DEFENCE MECHANISMS

Plants exhibit both physical and chemical protective mechanisms to deter herbivory. Physical barriers like hard seed coat, toughened leaves, spines, trichomes, thorns and bark play an important role in direct plant protection. Apart from this, numerous plant species produce certain chemicals, both primary and secondary metabolites that are toxic to pests and pathogens and can be used as alternatives to chemical pesticides (Sahayaraj et al., 2008). Primary metabolites for plant defence mainly include plant protease inhibitors (PPIs), lectins etc and secondary metabolites include phenolic compounds, alkaloids, tannins, terpenoides and rotenoides (Macedo et al., 2003; Hanley, 2007). Normally in most of the plants the defensive molecules are produced under natural conditions itself (Boulter, 1993). But in certain plants they get expressed only under herbivory (Jamal et al., 2013). Plant protease inhibitors play a significant role as anti-metabolic proteins by inhibiting the digestive proteases of the insects and pests and prove their insecticidal activity against them (Jouanin et al., 1998; Arimura, 2009). In tomato, the protease inhibitor initiation factor (PIIF) get initiated due to wounding. Protease inhibitor initiation factor then lead to a cascade of events resulting in the synthesis of PIs which are mainly cytosolic (Meige et al*.,* 1976).

2.6 PROTEASES

Proteases are the single class of enzymes that have crucial roles in both in insect physiology and commercial applications. Proteases are essential for the existence of all kinds of living organisms. (Barrett, 2000) and are widely found in plants, animals and microorganisms (Kenny 1999).One of the most important biological catalytic reactions which has been attributed to proteases is proteolysis. Proteolysis is the hydrolysis of peptide bond by attacking the carbonyl group of the peptide (Eatemadi et al., 2017). Thus proteases are enzymes which catalyse the hydrolytic cleavage of peptide bond in the target proteins. In the case of plants, proteases are involved in almost all aspects of their growth and development. They play inevitable role in the mobilization of seed storage proteins during germination, initiation of senescence and programmed cell death (Schaller, 2004), photo-morphogenesis during seedling development and circadian rhythms (Estelle, 2001).

2.6.1 CLASSIFICATION OF PLANT PROTEASES AND THEIR FUNCTIONS

On the basis of the site of action on protein substrates, proteases are broadly classified into endo or exo enzymes. They are further classified into serine, cysteine, aspartate and metalloproteases (Bode and Huber, 1992). This classification is on the basis of the nature of the key amino acid in the active site and the mechanism of peptide bond cleavage, (Yang et al., 2009; Turk et al., 2012 ; Verbovšek et al., 2015). Cysteine, serine and threonine proteases

respectively utilizes Cys, Ser and Thr amino acid residue for the nucleophilic attack of the peptide bond. Since the active site of these proteases is occupied with specicific amino acids, their names are coined accordingly. On the other hand an activated water molecule acts as a nucleophile to attack the peptide bond in the case of aspartyl, glutamic and metallo proteases. Moreover the metalloproteases have a metal ion in their active site (López-Otín and Bond, 2008).

2.6.1.1 Serine protease

The active site of serine proteases are characterized by the presence of the amino acid serine. They are widespread among viruses, bacteria, and eukaryotes, which reveals their vital role in the organisms. In general serine proteases are active at neutral and alkaline pH, with an optimum activity between pH 7 and 11. Their isoelectric points generally range between pH 4 and 6. (Rao et al., 1998). In gerneral the molecular masses of serine proteases range between 18 and 35 kDa, with the exception of serine protease from *Blakeslea trispora,* which has a molecular mass of 126 kDa (Govind Mehta et al., 1981). The substrate specificities of serine proteases are very broad that includes esterolytic and amidase activity. Serine alkaline proteases that are active at highly alkaline pH represent the largest subgroup of serine proteases. Serine proteases are broadly classified into two families, namely the chymotrypsin family and the subtilisin family (Fan and Wu. 2005). One of the well characterized serine proteases is trypsin, which plays a critical role in a wide spectrum of pathological processes like, inflammation, atherosclerosis and cancer (De Clerck et al., 2004). Serine proteinases were identified from the gut extracts of

many insect families, particularly those from lepidoptera (Houseman et al., 1989) The order lepidoptera, which includes a number of crop pests, have the pH optima of their guts in the alkaline range of 9-11 (Applebaum, 1985)

Mechanism of Cleavage

Serine proteases mainly have two subdomains which form a cleft within which the active site is present. The active site has either three (triad) or rarely two (diad) residues which are responsible for catalysis (Blay and Pei, 2019). Serine, Histidine and Aspartate are referred to as the catalytic triads. Apart from this, the active site is surrounded by certain amino acid residues which aids recognition of specific amino acid residues in the substrate and form substrate binding sites of the enzyme (Rawlings, 2013). The residues on the N-terminal side of the bond which is to be broken in the substrate is named as P1, P2, P3 and those on its C- terminal is named as P1', P2'and P3'. In the enzyme the protein pockets interacting with P1, P2, P3, P1', P2', and P3' were designated as S1, S2, S3, S1', S2' and S3' respectively. In the case of chymotrypsin, the S1 site has small side chains: one serine and two glycines, which facilitates the accommodation of bulky P1 residues such as Phenylalanine, Tryptophan and Tyrosine.

The catalysis of amide bond cleavage by serine proteases comprise of two steps: acylation and deacylation.

In acylation, the histidine deprotonates the catalytic serine and the aspartate residue increase the basisity of histidine by temporarily accepting the proton from histidine. The serine hydroxyl group

nucleophilically attacks the carbonyl carbon of the substrate resulting in the formation of a temporary tetrahedral intermediate. The proton on the histidine facilitates the decomposition of this temporary tetrahedral intermediate and release of an amine resulting in the formation of a relatively stable acyl- enzyme intermediate (Hedstrom, 2002).

In deacylation, histidine residue activates a water molecule resulting in the formation of another tetrahedral intermediate which displaces the enzyme and reverts it to its original configuration (Blay and Pei, 2019).

2.6.1.2 Cysteine proteases

The activity of cysteine proteases depend on a catalytic dyad consisting of cysteine and histidine. Generally, cysteine proteases are active only in the presence of reducing agents such as HCN or cysteine. They occur in both prokaryotes and eukaryotes. Cysteine proteases have neutral pH optima, with the exception of lysosomal proteases, which are maximally active at acidic pH. This class of protease mediate general functions such as catabolism of intracellular protein and specialized functions such as selective activation of extracellular protein degradation, macrophage function, bone resorption or signaling molecules (e.g. interleukin, protein kinase C, encephalin) (Zucker et al., 2000). Plant proteases such as papain and bromelanin are the best known cysteine proteases (Fan and Wu 2005). Clostripain, produced by the anaerobic bacterium *Clostridium histolyticum*, exhibits a stringent specificity for arginyl residues at the carboxyl side of the splitting bond and differs from papain in its

obligate requirement for calcium. Streptopain, the cysteine protease produced by *Streptococcus* spp., shows a broader specificity, including oxidized insulin B chain and other synthetic substrates. Clostripain has an isoelectric point of pH 4.9 and a molecular mass of 50 kDa, whereas the isoelectric point and molecular mass of streptopain are pH 8.4 and 32 kDa, respectively.

2.6.1.3 Metalloproteases

Metalloproteases comprises of proteases which require a divalent metal ion for their activity (Barett, 1995). Majority of the metalloproteases have a catalytically active zinc atom, while in some cases it may be replaced by cobalt or nickel. They include enzymes from a variety of sources such as collagenases from higher organisms, thermolysin from bacteria and hemorrhagic toxins from snake venoms, (Ohta et al., 1996; Bjarnason and Fox, 1988)

2.6.1.4 Aspartyl proteases

Aspartic acid proteases (acidic proteases), are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Most of the aspartic proteases exhibit optimum activity at low pH (pH 3 to 4) and their isoelectric point falls between pH 3 to 4.5. The molecular masses of the acidic proteases range from 30 to 45 kDa. Majority of the aspartic proteases fall under the pepsin family, which includes the digestive enzymes like pepsin and chymosin, few fungal proteases and lysosomal cathepsins D. The retropepsin family includes the viral proteases.

2.7 ROLE OF PROTEASES IN INSECTS

Insects rely on a complex proteinase system and produce ample amount of proteases both digestive and non-digestive in function. The digestive proteinases are present in the gut of the insects and helps to digests the dietary proteins obtained from their food source .(Jongsma and Bolter, 1997; Harsulkar et al., 1999; Applebaum, 1985; Terra et al., 1996; Reeck et al., 1999). Essential amino acids obtained from this proteolytic digestion are utilized for its growth and development. Serine and cysteine proteinases are the two major proteinase classes in the insect digestive systems (Haq et al., 2004). In lepidopteran larvae serine proteinases dominate and contribute to about 95% of their total digestive activity (Srinivasan et al., 2006). Based on the studies carried out on the midgut enzymes of different coleopteran pests it was found that the coleopteran larval gut have a wider range of dominant proteinases (Murdock et al., 1987).Apart from the digestive proteinases non-digestive proteases also have diverse roles in insect biology. Several serine and cysteine proteases play major role in the insect egg and embryo development. These proteinases are synthesized as zymogens in the ovaries and later get activated during embryogenesis Vitellin and a few other egg-specific yolk granule proteins in the insect eggs are digested by proteases to release amino acids which are used in embryonic development (Raikhel and Dhadialla, 1992). Serine proteases in the insect hemolymph play several physiological functions in defense and immune responses against infection and wounding, which include melanotic encapsulation, antimicrobial peptide synthesis activation (Barillas-Mury, 2007; Kanost and Gorman, 2008; Cerenius

et al., 2010; Jiang et al., 2010). Cocoonase a serine protease from silk moths digests sericin, the silk protein that cements fibroin threads together in the cocoon, facilitating the emergence of adult moth. (Kafatos et al., 1967a, b)

2.8 PROTEASE INHIBITORS (PIs)

Protease inhibitors, the natural antagonists of proteases, are in general small proteins or peptides which inhibit the proteolysis process either fully or partially (Majumdar, 2014). They are present in diverse forms in numerous animal and plant tissues and also in microorganisms. Corresponding PIs of majority of the proteases occur in nature. In general they play a significant role in the regulation of many physiological responses, such as digestion, fibrinolysis, blood coagulation, complements cascade, phenoloxidase cascade, cell migration, release of hormones and peptides (Macedo et al., 2011; Tang et al., 2008; Zou et al., 2005). In plants the main function of protease inhibitors are the regulation of endogenous proteases and plant defence against herbivory (Mosolov et al., 2001; Brik, 2003; Shewry, 2003). The defensive role of protease inhibitors is well established. The discovery of the role of protease inhibitors in plant defense dates back to 1947 with the observations made by Michel and Standish, where they found the inability of certain insect larvae to develop normally on soybean products (Mickel and Standish, 1947). The defensive role of PIs are based on their ability to inhibit the insect gut enzymes which results in shortage of essential amino acids (Hilder et al., 1993; Jongsma and Bolter, 1997) required for their normal growth and development. PIs can also attribute their defensive role by

interfering with the important biochemical or physiological processes such as moulting of insects and proteolytic activation of enzymes (Gutierrez-Campos et al., 1999). Serine protease inhibitors are the most studied class of protease inhibitors and are widespread in plant kingdom (Mello et al., 2002; Haq and Khan, 2003).

2.8.1 CLASSIFICATION OF PLANT PROTEASE INHIBITORS

Plant protease inhibitors can be grouped primarily into serine, cysteine, aspartic and metallo protease inhibitors (Abbenante and Fairlie, 2005). Among these, serine protease inhibitors are the largest and most widely distributed superfamily of PIs (Di Cera, 2009; Hedstrom 2002; Krowarsch et al., 2003).

2.8.1.1 Cysteine protease inhibitors

Cysteine protease inhibitors inhibit the activity of cysteine proteases. They are widely distributed in plants, animals and microorganisms (Oliveira et al., 2003). These inhibitors are involved in plant defence as they have insecticidal activity. The members of this family are subdivided into three classes namely stefins, cystatins and kininogens (Barrett et al., 1986). Another family of cysteine protease inhibitor reported was phytocystatins (Zhao et al., 1996; Habib and Fazili, 2007), which includes most of the cysteine protease inhibitors reported from plants (Bolter, 1993). Oryzacystatin present in rice (Abe et al., 1987a,b), inhibitors isolated from maize (Abe et al., 1992), soybean (Botella et al., 1996) and apple fruit (Ryan et al., 1998) are examples of phytocystatin.

2.8.1.2 Metallo protease inhibitors

The metalloprotease inhibitors in plants fall under two families the metallocarboxypeptidase family and a cathespin D inhibitor family. The former is found in potato and tomato plants (Rancour and Ryan, 1968; Graham and Ryan, 1997) while the later in potatoes only (Keilova and Tomasek, 1976). These inhibitors are found to accumulate in potato tubers and leaves during development and as a response to wounding along with the potato inhibitor I and II (Ryan, 1990). Thus the wounded leaf parts have the capacity to inhibit the five major digestive enzymes trypsin, chymotrypsin, carboxypeptidase A, carboxypeptidase B and elastase of higher animals and in many insects (Hollander-Czytko et al., 1985).

2.8.1.3 Aspartyl protease inhibitors

Only a limited knowledge is there on the role of aspartic proteinases in insect digestion**.** The low pH (pH optima ~ 3 - 5) of midguts of many coleopterans and hemipterans provide favourable environment for aspartic proteinases to act upon (Houseman et al., 1987). Though no aspartyl proteases were isolated from coleopteran gut enzymes, pepstatin a strong aspartyl protease inhibitor was reported to inhibit the proteolysis of the midgut enzymes in *Leptinotarsa decemlineata* (Colorado beetle) indicating the presence of aspartic proteinase in their midgut extracts (Wolfson and Murdock, 1987). Cathepsin D, an aspartyl PI from Potato tubers (Mares et al., 1989) shows considerable amino acid sequence analogy with Soybean trypsin inhibitor (SBTI). Aspartyl PIs are also reported from

sunflower, barley and in potato tubers (Park et al., 2000; Lawrence and Koundal 2002).

2.8.1.4 Serine protease inhibitors

Serine protease inhibitors are widely distributed in the living organisms, right from the bacteria to the human beings. The major function of serine protease inhibitors in plants are reserve protein mobilization, regulation of endogenous proteinases during seed dormancy and protection against the proteolytic enzymes of insects and parasites. They can also act as storage proteins (Haq et al., 2004). Plant serine protease inhibitors represent between 1% - 10% of total protein in the seeds and tubers (Volpicella et al., 2011; Rustgi et al., 2018). These protease inhibitors are classified into Bowman-Birk serine protease inhibitors, cereal trypsin/α- amylase inhibitors, mustard trypsin inhibitors and squash serine inhibitors (Laskowski and Qasim, 2000; De Leo et al., 2002; Bateman and James, 2011). Serine proteinase inhibitors have anti-nutritional effects against several lepidopteran insect species (Shulke and Murdock, 1983; Applebaum, 1985). Recent results showed that the pathogen / insect secreted proteases activate the plant immune pathway (Cheng et al., 2015).It was observed that herbivory induced jasmonic acid accumulation activates the synthesis of serine protease inhibitors (Halitschke and Baldwin, 2003; Boex- Fontvieille et al., 2016), one of the main anti nutrirtional components secreted by plants. These serine protease inhibitors interfere with the insect digestive system and retard the growth and development of the insects. Serine protease inhibitors from rice, barley, cowpea, soybean maize and sweet potato were

overexpressed in several plant species which resulted in resistance against several insect pests (Vila et al., 2005; Dunse et al., 2010; Gatehouse, 2011; Luo et al., 2012). These results show the high potential of serine protease inhibitors in pest control.

2.8.2 Sources of protease inhibtitors

Proteases inhibitors are widely distributed in plants, animals and microorganisms and shows versatile functions and characteristics.

2.8.2.1 Protease inhibitors from prokaryotes

Though the unicellular organisms, both prokaryotes and eukaryotes have different classes of catalytic proteases which constitute upto 6% of their total gene count, they contain only very few genes coding for protease inhibitors (Kantyka et al., 2010). Out of the 67 families of PIs listed in the MEROPS database (www.merops.ac.uk) only 18 families were reported from prokaryotes. The major families of the prokaryotic PIs are Ovomucoid, Aprotinin Alpha-1-peptidase inhibitor, Peptidase B inhibitor, Marinostatin, Ecotin, *Streptomyces* subtilisin inhibitor, Equstatin, *Streptomyces* metallopeptidases inhibitor, Metallopeptidases inhibitor, Alpha-2-macroglobulin, Chagasin, Serine carboxy peptidase Y inhibitor, Staphostatin, Pro eosinophil major basic protein, Bacteriophage lamda CII protein and *Aspergillus* elastase inhibitor. With the exception of ecotins and staphostatins many of these gene products show trypsin inhibitory activity with unknown biological functions.(Kantya et al., 2010).

2.8.2.2 Protease inhibitors from fungi

PIs of fungal origin remain the least explored. Only a few PIs from fungi are well characterized. The fungal PIs mainly fall into two categories small molecule protease inhibitors and protein protease inhibitors. The protein protease inhibitors from fungi belong to serine, cysteine, metallo and aspartic protease inhibitors. These PIs exhibit either endogenous or exogenous protease inhibition. Exogenous PIs act as defensive molecules while endogenous regulates the acivity of internal proteases. Most widely used small molecule irreversible cysteine protease inhibitor E-64 isolated from *Aspergillus japonicas* is used in protease inhibitor cocktails (Sabotic and Kos, 2012). Kojistatin A isolated from *A. oryzae* (Yamada et al., 1998), thysanone from *Thysanophora penicilloides* (Christopher et al., 1999) are examples of other cysteine protease inhibitors from fungi. Serine protease inhibitors like vibralactone and polyozelin were isolated from the basidiomycetes *Boreostereum vibrans* and *Polyozelllus multiplex* respectively (Wang et al., 2014; Yang et al., 2015). Apart from this, proteasome inhibitors were also isolated from fungi. Gliotoxin isolated from *A. fumigatus* exhibited antimicrobial, antiviral, antiparasitic effects and induces apoptosis. It is reported to inactivate enzymes such as creatine kinase, alcohol dehydrogenase, farnesyl transferase and it inhibits the chymotrypsin like activity of 20S proteasome noncompetitively. (Kroll et al., 1999; Hurne et al., 2000). Fungal PIs have several applications. Many of them are used in protease inhibitor cocktails. E-64 and its synthetic derivatives were found to be protective agents in diabetes,

cardiovascular diseases, cancer, osteoporosis, muscular dystrophy etc. (Sabotic and Kos, 2012).

2.8.2.3 Protease inhibitors from animals

Protease inhibitors are distributed in different animal species right from the lower animals to mammals including human beings. In the case of cniderians, trypsin/chymotrypsin inhibitors were isolated from the sea anemons like *Radianthus macrodactylus* (Sokotun et al., 2007a), *Actinia equina Anthopleura aff, xanthogrammica* and *Anthopleura fuscovirids* (Minagawa et al., 2008). Most of these inhibitors are highly active against trypsin and show moderate activity against other serine proteases (Tzi Bun et al., 2012). An antibacterial protease inhibitor has been repoted from the epithelial and gland cells of Hydra, which reveals the metazoan defense mechanism (Augustin et al., 2009). About 13 protease inhibitors with sequence similarities to serine proteases were reported from the medicinal leech *Hirudo medicinalis*. The thoracic extract of horn fly *Haematobia irritans irritans* contains serine protease inhibitor HiTi, which inhibits its own trypsin –like enzyme and an endoprotease of *E.coli*, which reveals the its role in regulating both endogenic and pathogenic proteases (Azzolini et al., 2005). A 7kDa trypsin inhibitor from the salivary glands of female *Aedes aegypti* was found to exhibit anticoagulant activity during their blood feeding time (Watanabe et al., 2010). Protease inhibitors isolated from other insects like *Bhoophilus microplus* (Macedo- Ribeiro *et al*., 2008), *Antheraea mylitta* (Rai et al., 2010) also proved the role of protease inhibitors in regulating endogenous proteases. Majority of the protease inhibitors isolated from

crustaceans were found to regulate the antibacterial activity in them. Protease inhibitors from *Procambarus clarkii,* red swamp cray fish showed antibacterial activity towards *Bacillus subtilis* and *B.thuringiensis* (Li et al., 2010) and that from the black tiger shrimp *Penaeus monodon* also exhibited antibacterial activity (Somprasong et al., 2006). Protease inhibitors from the eastern oyster Crassostre*a virginica*, was found to inhibit trypsin subtilisin and perkinsin, a protease secreted by *Perkinsus marinus* a parasitic protozoan on the Oyster (Xue et al., 2006). Protease inhibitors with bacteriostatic activity was also isolated from ova of *Rana grahami* (odor frog).In the case of mammals serine protease inhibitors with antiviral activity was reported from the Madine Darby canine kidney (MDCK) cells. Thus the protease inhibitors isolated from different organs in different classes of animals exhibited different functions in accordance to the peculiarity of the tissues from which they are isolated and also in their mode of life.

2.8.2.4 Protease inhibitors from plants

Protease inhibitors are widely distributed in plant kingdom (Ryan, 1973). It is estimated that upto 10% of the total water soluble proteins in the seeds of both monocots and dicots comprises of protease inhibitors (Mutulu and Gal, 1999). Most well studied PIs from plants belong to three main families namely, Fabaceae, Solanaceae and Poaceae (Richardson, 1991). Later, PIs from other families like Malvaceae, Rutaceae and Moringaceae (Bijina et al., 2011) were reported. Plant protease inhibitors were synthesized normally during plant development and are also expressed in response to the attack by

insects and pathogenic microorganisms (Ryan, 1990). PIs induced by herbivory inhibits the respective proteases present in the insect gut (Lawrence and Koundal, 2002).

Protease inhibitors from different sources, including plants have been purified and characterized (Christeller, 2005; Mosolov and Valueva, 2005). From plants the protease inhibitors were mainly isolated from seeds, leaves, fruits and tubers (Xavier-Filho and Campos, 1989; Richardson, 1991; Kendall, 1951; Wingate et al., 1989). A 20 kDa trypsin protease inhibitor was isolated from *Peltophorum dubium* (Richardson, 1991). Silveria et al, isolated and purified a 19kDa Kunitz protease inhibitor from the seeds of *Plathymenia foliolosa*, *Plathymenia foliolosa* Trypsin Inhibitor (PFTI), which inhibits the larval midgut proteases of *Anagasta kuehniella* and *Diatraea saccharalis* significantly (Silveria et al., 2008). Another Kunitz type protease inhibitor from the seeds of *Crotallaria pallida, Crotallaria pallida* Trypsin Inhibitor (CpaTI) was recorded by Gomes et al. The CpaTI was reported to inhibit the gut enzymes of *Callasobruchus maculatus* and *Ceratitis capitata* strongly (Gomes et al., 2005). Battachryya et al., purified a 20kDa protease inhibitor from the seeds of *Caesalpinia bonduc* which inhibited the gut protease activity of *Spodoptera litura* (Battachryya et al., 2007). A 15 kDa and 3 kDa inhibitors was purified from the seed of Sapindus saponaria by Macedo et al, and it inhibited the gut enzyme activity in Corcyra cephalonica, Diatreae saccharalis and Anticarisa gemmatalis (Macedo et al., 2011).

2.8.3 ROLE OF PLANT PROTEASE INHIBITORS IN PLANTS

Initial studies on SPIs revealed that in plants they participate in several functions like the mobilization of storage proteins as in the case of seeds, flower development, morphogenesis and regulation of cell death during senescence, plant development and plant defence mechanisms against insects and other pathogens (Birk, 2003). During senescence the SPI activity decreases and thus the activity of serine proteases responsible for senescence get augmented. Thus delay in senescence depends on the balance between the activity of SPIs and their respective serine proteases. (Pak and Van Doorn, 2005). Under water deficit conditions, the hydrolytic protein degradation in the leaves were prevented by the accumulation respective SPIs (Downing et al., 1992). The PIs were found to be active against many insect pests (Pannetier et al., 1997; Koiwa et al., 1998), nematodes (Williamson and Hussey, 1996), spore germination and mycelium growth in fungus *Alternaria alternata* (Dunaevskii et al., 1997). Thus the plant PIs may be synthesized during their normal course of development as well as in response to various stress conditions like insect attack, pathogens, wounding or environmental stresses such as high salinity (Solomon et al., 1999, Koiwa et al., 1997).

2.8.4 APPLICATIONS OF PLANT PROTEASE INHIBITORS

Protease inhibitors of plant origin, apart from their protective and maintenance role in plants also have several applications in the biotechnological and pharmaceutical fields. Protease inhibitors were

expressed in plants to increase their resistance against pathogenic organisms.

2.8.4.1 Use of plant protease inhibitors for production of commercially important proteins in plants

Plants can be a platform for the production of recombinant proteins such as drugs, vaccines, antibodies, hormones, enzymes, cytokines, secondary metabolites and industrial proteins (Hood and Christou, 2014). The ability of plants to perform post translational modification made it possible for being a platform for recombinant protein production (Yao et al., 2015). The first commercialized recombinant protein produced in plants was the avidin protein in the chicken egg (Hood et al., 1997). A biggest challenge faced for this application is the degradation of recombinant proteins by the plant proteases (Pillay et al., 2014) which are abundantly present in the plant vacuole, chloroplast and apoplast (Van der Hoon, 2008). Co-expressing appropriate plant PIs along with recombinant proteins in plants would minimise the proteolytic degradation of recombinant proteins (Pillay et al., 2014). Thus by knowing the proteases present in the secretory pathway of plants help to decide which inhibitor/s have to be co-expressed. Since serine proteases are the active proteases in plant cells most works focus on SPIs. It was reported that a synthetic chymotrypsin and trypsin inhibitor from *Nepenthes alata* co-expressed in transgenic rice cell suspension enhanced the accumulation of the recombinant human granulocyte-macrophage (hGM-CSF) in it, without affecting the plant growth and development (Kim et al., 2007). Serine and aspartic proteases are abundantly found and the main

protease activity modulators in the leaf apoplast of *Nicotiana benthamiana* (Goulet et al., 2012). Transient co-expression of inhibitors of both the proteases increased the accumulation of recombinant murine antibody by 70-80% (Goulet et al., 2012).Thus it is found that protease inhibitor expression have a positive impact on protein levels with negligible effects on plant growth and development (Castilho et al., 2014).

2.8.4.2 Applications in medicine

Another field of interest is the application of PIs in the therapeutics. Investigations on the role of PIs to combat several clinical disorders dates back to 1950's (Vogel et al., 1968). The *Glycine max* (Soybean) BBI isolated from its seed was found to have potent vanquishing effect on colon and anal gland inflammation (Billings et al., 1990). It also reduced the initiation and regularity of colorectal tumors even at low concentrations as 10mg/100g of diet in rodents (Kennedy et al., 2002). Moreover soybean BBI was found to disrupt the cell cycle distribution pattern by blocking the G0- G1 phase in HT29 colon cancer cells (Clemente et al., 2010). A 14 kDa PI purified from ragi (RBI), exhibited cytotoxicity against chronic myeloid leukemia cell, K 562 (Sen and Dutta, 2012). Bowman-Birk inhibitor (BBI) isolated from *Cicer arietinum* (Chickpea) was found to inhibit the viability of both breast cancer (MDAMB-231) and prostate cancer (PC-3 and LNCP) cells at all tested concentrations (Magee et al., 2012). Many such plant protease inhibitors were reported to exhibit anti- proliferative effect against many cancer cell lines paving way for the application of PIs in cancer therapeutics. PI isolated from *Moringa*

oleifera belonging to Moringaceae family is the potential source for new drug development against thrombin, trypsin, chymotrypsin, cathespin, elastase and papin in pharmaceutical industries (Binjina et al., 2011)

2.8.4.3 Applications in pest control

Pests are age old enemies of farmers, which pose upto 25% yield loss in agriculture. Plants in general have natural defensive mechanisms against herbivory, both physical and chemical control. A broad range of herbivory induced defensive molecules mediate plant protection. Among them, plant protease inhibitors are the most studied. The role of PPIs in plant protection against insect pest attack was known to science since the observations made by Mickel and Standish (Mickel and Standish, 1947), where the larvae of *Tribolium confusum* showed inability to grow on soybean products. Later on Shulke and Mudrock proved the anti-nutritional effect of the protease inhibitors against the lepidopteran insect pests (Shulke and Mudrock, 1983).

2.8.4.3.1 Genetically modified crops expressing plant protease inhibitors

The first successful transfer of protease inhibitor gene of plant origin was that from cowpea, encoding a double-headed trypsin inhibitor (CpTI) into tobacco plants. Followed by the successful trials of this transgenic crop against *Manduca sexta* (Ussuf et al., 2001) the CpTI gene was engineered to many crops including potato, oil seed, rape, rice, and soft fruits such as strawberry. The CpTI transgenic strawberry plants showed high resistance to the vine weevil (Graham et

al., 1995). Furthermore the transgenic rice with CpTI genes conferred enhanced resistance level against rice stem borers (Xu et al., 1996). Trypsin inhibitor from sweet potato imparted high insect resistance when expressed in cauliflower (Ding et al., 1998) and severely retarded larval *S.litura* growth and development in transgenic tobacco plants (Yeh et al., 1997)**.** Transgenic crops with the potato trypsin inhibitors P-I and P-II also showed increased resistance to lepidopteran and orthopteran insects (Gatehouse, 1999). The efficacy of P-II gene introduced transgenic japonican rice varieties also conferred high insect resistance in greenhouse trials. A cysteine PI from rice expressed in poplar plants showed high toxicity against poplar feeding beetles (Leple et al., 1995).

Protease inhibitor genes from different sources were incorporated into different rice varieties and their effects have been studied. Transgenic rice with soybean trypsin inhibitor gene has shown marked resistance to *Nilaparvatha lugens* and *Cnaphalocrosis medinalis* (Li et al., 2005). Corn cystatin (CC) cDNA expressed rice varieties were reported to impart highest inhibitory activity against the gut enzymes of *Sitophilus zeamais*. (Duan et al., 1996). Transgenic wheat with barley trypsin inhibitor (BTI) gene was found to effectively control the agroumoius grain moth *Sitotroga cerealetta* (Altpeter et al., 1996)Thus the genetically modified crops with the plant protease inhibitor gens confer resistance to the pests of interest.

Apart from the pest control effect, some of the plant protease inhibitors protect plants from certain viral and fungal infections too. Transgenic crops with antiviral protease inhibitors have been raised

and their effects were well studied. Cystatin, the cysteine protease inhibitor from rice incorporated in tobacco plants have shown resistance against potyviruses. It is found to be effective mainly against tobacco etch poty virus (PEV) and potato virus Y (PVY) (Gutierrez-Campos et al., 1999). Recombinant corn trypsin inhibitor was raised by the over expression of corn trypsin inhibitor in *E.coli*. It was found that this recombinant inhibitor can inhibit the hyphal growth and conidium germination in the plant pathogenic fungi like *Aspergillus parasiticus*, *A.flavus* and *Fusarium moniliforme* (Chen et al., 1999).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 CHEMICALS USED

Azocasein, trypsin, Cyanogen bromide (CNBr) activated Sepharose -4B and ammonium persulphate were purchased from Sigma Aldrich, USA. Ammonium sulphate, sodium bicarbonate, sodium carbonate, sodium chloride, sodium hydroxide, Tris, Acrylamide, Bis-acrylamide, Glycine, dialysis membrane, coomassie brilliant blue R-250, coomassie brilliant blue G- 250, bovine serum albumin (BSA) fraction V and Beta- mercaptoethanol were from Himedia Laboratories Private Limited, Mumbai, India. Sodium dodecyl sulphate (SDS), glycerol, methanol, glacial acetic acid and Amicon 3 kDa protein concentrator were purchased from Merk, Germany. Trichloro acetic acid (TCA), Tetramethylenediamine (TEMED), Schiff's reagent, N α-Benzoyl- D, L-arginine 4- nitroanilide hydrochloride (BAPNA), protein molecular weight marker and formaldehyde were from Sisco Research Laboratories Limited, Mumbai, India. Proteinase K was purchased from Quiagen, Holden, Germany. All other chemicals used in the experiment were of analytical grade.

3.2 Methods

3.2.1 About *Spodoptera litura*

Experimental Organism: *Spodoptera litura*

Kingdom : Animalia
Phylum : Arthropoda
Class : Insecta
Order : Lepidoptera
Family : Noctuidae
Genus : *Spodoptera*
Species : *litura*

Spodoptera litura Fabricius, commonly known as tobacco cut worm, cotton leaf worm or tropical army worm is a polyphagous pest distributed throughout India. Its natural distribution ranges from the Oriental and Australian regions to some parts of Palearctic region. The countries with major widespread distribution of *S.litura* are India, China, Indonesia, Japan and Malaysia.

The adult is a stout built dark brown moth with white lines on its wings. They live for 10 to 24 days. Eggs are laid in batches covered with hairs on the underside of the leaves. The eggs are initially greenish in colour which later on turns to black before hatching. The larvae (caterpillars) are about 3.7 cm long, pale greenish brown coloured with bright yellow lines on the back and sides of the body. A black ring encircles the body at both the ends. The egg, larval and pupal stage lasts for 3 to 4, 18 to 20 and 4 to 5 days, respectively (Ballal, 2007)

The larval stages of *S.litura* are voracious feeders, which feed on leaves, stems and bolls (in the case of cotton). During severe infestation the whole leaves of the plants will appear skeletonized. They attack crops in patches and move from field to field in large groups, this gives them the name tropical army worm. Tropical army worm is a big threat to intensive agricultural systems (Malarvannan *et al.,* 2008). It has a wide host range of more than 150 host species. It is a serious pest of plants like cotton, jute, tobacco, soyabean, rice, maize, tea and vegetables (Rao *et al*., 1993). Its wide dissemination and pest status has contributed to its ability for both seasonal migration and facultative diapause (Devanand and Rani,2008).Loss in yield caused by larval infestation of *Spodoptera litura* ranges from 10 to 20% (Talukder and House, 1994).

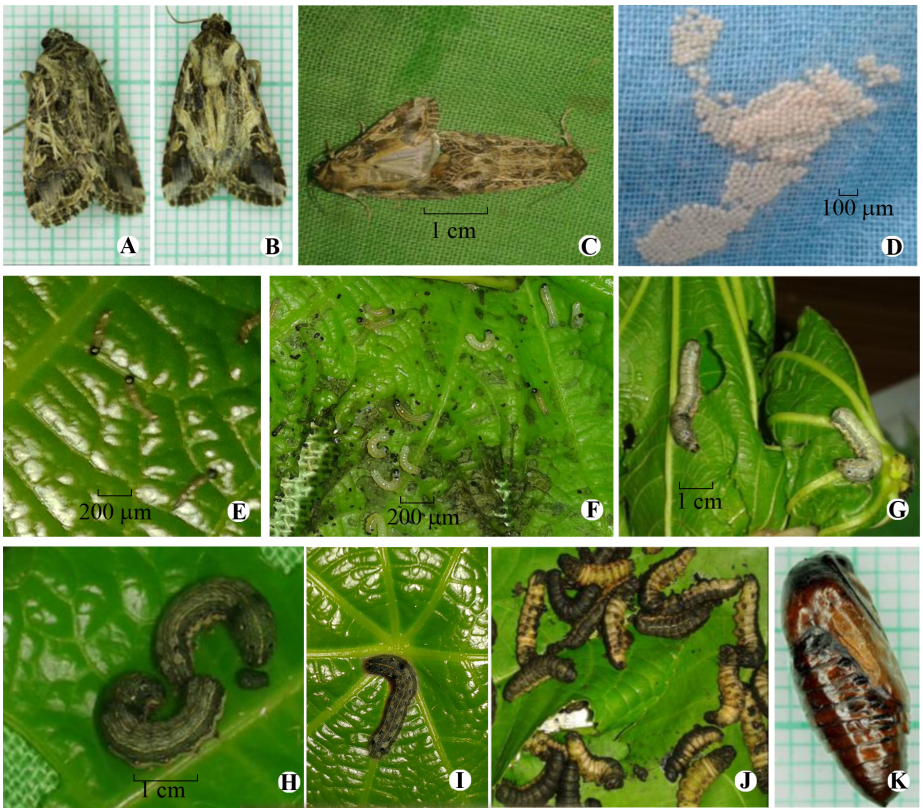
3.2.1.1 Culture and maintenance of *Spodoptera litura* in the laboratory

The pupae of *S.litura* were purchased from NBAIR Live Insect Repository **(**National Accession No: NBAII-MP-NOC-02), Bangalore, India. The emerged adult moths were kept in glass beakers covered with muslin cloth and fed with diluted honey (30%). The moths were allowed to mate and lay eggs. Larvae hatched out within 4-5 days and were reared in glass beakers with tender leaves of *Ricinus communis* initially and later transferred to plastic troughs with somewhat mature leaves as they grew in size. The culture was maintained at 25±2ºC and 75±3% relative humidity at 12 hour day and night cycle mimicking the natural condition. The total larval period was found to range from 19-21 days and consisted of 6 larval instars. Actively feeding fifth-instar larvae of *S.litura* were used for experiments. Few larvae were kept aside to continue their growth till pupal stage to maintain the laboratory culture.

3.2.1.2 Life cycle of *Spodoptera litura*

Spodoptera litura is a polyphagous pest with holometabolous life cycle consisting egg, larval, pupal and adult stages. *S. litura* have six larval instars before pupation (Cardona *et al*., 2007). The larvae feed on leaves of host plants leading to skeletonisation and leaf defoliation in the advanced stages.

Figure 3.1 Different stages of *Spodoptera litura* life cycle



A. *S.litura* adult Female; B. *S.litura* adult Male; C. *S.litura* adult mating (Male: left, Female: right); D. *S.litura* eggs; E. 1st instar larvae; F. 2nd instar larvae; G. 3rd instar larvae ; H. 4th instar larvae; I. 5th instar larvae; J. Prepupal stage; K. Pupal stage

Eggs

The eggs are spherical, yellowish creamy in colour and covered with yellowish brown hairs. They hatch within 4 days and prior to hatching, colour of the egg mass change to dark black.

Larvae

Spodoptera litura passes through six larval instars in 25 days on *Ricinus communis* leaves. Newly hatched larvae are tiny with blackish green head and translucent body with tiny hairs. The first instar larval stage lasts for about 3 days. Second instar larval stage also last for 3 days with pale green colour and are hairless. Third instar larvae were characterized with dark green colour and three yellowish colour bands on the dorsal surface from mouth to the posterior end and a dark ring on the first abdominal segment. This instar lasts for 3 to 4 days and moults to fourth instar. In the fourth instar larvae, the dorsal lines become more prominent. The dorsal central line changes to bright orange and the yellow lateral lines become brighter. Black intermittent spots appear along both the lateral yellow bands and this stage and lasts for 3 days. Next is the fifth instar in which the larval colour changes to dark brownish black with the lateral markings same as that of fourth instar larvae. In this stage the black spots along the yellow lateral lines appear triangular and brighter than the prior stage. This stage lasts for 3 days. The fifth instar larva moults to sixth instar which is stout built and dark brown in colour. The body markings were little faded compared to the last instar and this instar lasts for 2 to 3 days, which later moves to prepupal stage. The prepupal stage is marked with C- shaped body with

dark black coloured dorsal and creamy white ventral side with no appendages. This stage lasts for 1 day and the next day it gets transformed to pupa.

Pupae

Initially the pupa appears greenish yellow in colour which later becomes dark reddish brown. The adult emerges within 5 to 6 days.

Adults

The adults are greyish brown in colour. The forewings are grey to reddish-brown with creamy white patterns (in males, the wing base and tip have bluish shade); the hind wings are silvery white with grey margins. The males are bright coloured than females. Mating occurs the next day after adult emergence.

3.2.2 Preparation of larval gut extract of *Spodoptera litura*

Actively feeding fifth-instar larvae of *S.litura* were chilled on ice and midgut was dissected out. The midgut was homogenized in sodium bicarbonate buffer, pH 9.0 (1ml/g of tissue).The homogenates were centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant containing soluble protein was used for the protease/ protease inhibition assay.

3.2.3 Collection of plants and preparation of plant extract

The plants parts for the study were collected from Palakkad, Malappuram and Kannur districts of Kerala and from Tiruchirapalli district, Tamil Nadu and stored in zip lock covers at -20°C until use. The

plant specimens were identified by Dr. Pradeep, Assistant Professor, Department of Botany, University of Calicut. The plant parts were washed thoroughly with running water, distilled water and then with bicarbonate buffer prior to overnight soaking in bicarbonate buffer pH 9.0.

The soaked plant parts were homogenized and centrifuged at 10000 x g for 10 minutes and the soluble proteins recovered were used for the protease inhibition assay or stored at -20°C until use.

3.2.4 Protease assay

A. Reagents

1. Bicarbonate buffer

100mM sodium carbonate was prepared and pH was adjusted to 9.0 with 100 mM sodium bicarbonate.

2. Azocasein

Stock solution of azocasein was prepared by dissolving 44.8mg of azocasein in 250µl bicarbonate buffer. Working standard was prepared by diluting the stock four times with 100 mM bicarbonate buffer pH 9.0.

3. Trypsin

It was prepared by dissolving 1mg trypsin in 1 ml 10 mM HCl.

4. Trichloro acetic acid (TCA)

5% TCA was prepared by dissolving 5g TCA in distilled water and made up to 100 ml.

5. NaOH

50mM NaOH was prepared by dissolving 2 g NaOH in distilled water and made up to 100 ml.

B. Procedure

In this assay azocasein was used as the substrate, which is a azo-dye conjugated to casein. Protease assay was done in a total volume of 20.2µl, containing 10µl bicarbonate buffer pH 9.0 and 5µl gut extract/trypsin (as enzyme source).This reaction mixture was incubated at 37°C for 30 minutes after adding 5.2µl working standard of azocasein. After 30 minutes 80µl of 5% Trichloro acetic acid (TCA) was added to stop the reaction and centrifuged at 10,000 x g for 10 minutes. The supernatant (50µl) from each tube was mixed with 150 µl of 50mM NaOH. Proteolytic degradation of azocasein liberates azo dye into the supernatant which can be measured quantitatively. Proteolytic activity was recorded by measuring the absorbance at 440nm in a microplate reader. All assays were done in duplicate and repeated three times. One unit of enzyme is defined as the amount of enzyme which converts 1nMole of substrate per minute per millilitre

3.2.5 Screening of plant extracts for protease inhibitor

This was done by protease inhibition assay using gut extract of *S.litura* as the enzyme source.

3.2.6 Protease inhibition assay

Proteasse inhibition assay was done in a total volume of 20.2µl. Five microlitre of gut extract was pre-incubated with 10 µl of plant extract (inhibitor) for 10 minutes. Later 5.2µl azocasein (substrate) was added and assay proceeded as in protease assay. All assays were done in duplicate and repeated three times.

Specific inhibition activity of the inhibitor is calculated by using the formula:

$$\textit{Specific inhibition activity} = \frac{\text{Total activity in the presence of inhibitor}}{\text{Total amount of the inhibitor protein}}$$

Calculation of percentage inhibition:

The absorbance of the control was taken as 100% enzyme activity. Absorbance of the released dye in the control (azocasein alone) was subtracted from the absorbance of plant extract (inhibitor) alone and the value thus obtained represents the protease activity present in the plant extract. This value was subtracted from the absorbance of the test (in presence of the inhibitor) to get the actual absorbance in the absence of any protease activity from the plant extract. The absorbance thus obtained is converted in to the enzyme activity taking the absorbance of control as 100% activity. This value was subtracted from 100 to get percentage inhibition.

$$\%\ \text{INHIBITION} = \frac{100 - (\text{Abs of inhibitor} + \text{gut extract} - \text{Abs of inhibitor alone})}{\text{Abs of gut extract alone}}$$

3.2.7 Proteinase K treatment of plant extracts

To check the proteinaceous nature of the inhibitor, 90µl of plant extract was incubated with 2.3µg of Proteinase K at 56ºC overnight. Proteinase K inactivation was done by heating the mixture at 96ºC for 5 minutes. The mixture was centrifuged at 10,000 x g for 10 minutes at 4°C. The soluble protein in the supernatant was used for protease inhibition assay. Appropriate controls were also done.

3.2.8 Estimation of protein

Protein concentration of the plant extracts were determined by the method of Lowery et. al.,. Bovine serum albumin was used as the standard (Lowry et al., 1951).

3.2.9 Purification of the inhibitor from selected plant

The protease inhibitor was purified from *Hibiscus acetosella* seed extracts by ammonium sulfate fractionation, ion exchange chromatography and trypsin affinity chromatography.

3.2.9.1 Selection of plant for purification of the protease inhibitor

From the list of plant extracts screened to check the presence of protease inhibitors, the plant extract with highest inhibition, availability and no report of protease inhibitor was selected for the purification of protease inhibitor against *S.litura*. *Hibiscus acetosella* seed extract which inhibited the gut protease activity of 5^{th} instar larvae of *S.litura* to the extent of 88.61±0.40% was selected for the purification of protease inhibitor.

Hibiscus acetosella

Kingdom: Plantae

Order :Malvales

Family :Malvaceae

Genus : ***Hibiscus***

Species : ***acetosella***

Figure 3.2 ***Hibiscus acetosella***

H.acetosella is commonly known as African rosemallow. It is a perennial short lived shrub. The leaves are dark red in colour, flowers and fruits are reddish- pink.

3.2.9.2 Preparation of extract from the seeds of *Hibiscus acetosella*

H.acetosella seeds were crushed to powder by grinding it in liquid nitrogen. One gram of the powdered seed was soaked with 2ml of bicarbonate buffer pH 9.0 by continuous stirring for 4 hours and the filtered using sterilized muslin cloth. The resulting extract was centrifuged at 10,000x g for 10 minutes at 4°C and the supernatant with soluble proteins were used for further purification of the inhibitor or stored at -20 ºC until use.

3.2.9.3 Ammonium sulfate fractionation

Differential precipitation of proteins in the crude extract of *Hibiscus acetosella* was achieved by adding different percentages (0-30%, 30- 50% and50- 70%) of ammonium sulfate to the crude extract (Englard and Seifter, 1990). The precipitation was done at 4 ºC. The supernatant recovered after 30% ammonium sulfate precipitation was used for precipitating proteins at 50% concentration of ammonium sulfate and the supernatant from which was subjected for 70% ammonium sulfate precipitation of proteins. The precipitates obtained from each precipitation step were redissolved minimum volume of 100 mM bicarbonate buffer pH 9.0. The redissolved precipitates were then transferred to separate dialysis bags and dialysed against 25mM sodium bicarbonate buffer pH 9.0 at 4º C. The dialysis was done for 24 hours with 3 changes of buffer. After dialysis the fractions were

centrifuged at 10,000 x g for 10 minutes at 4 ºC and used for protease inhibition assay. Protein concentration of the dialysed fractions was estimated using Bradford's dye binding method.

Ammonium sulfate fraction with highest inhibition was further purified by ion exchange chromatography.

3.2.9.4 Ion Exchange Chromatography

The ammonium sulfate precipitated fraction with highest inhibition (50% fraction) was dialysed against 25mM Tris buffer pH 8.1(equilibriation buffer) and further purified by ion exchange chromatography using Source Q (anion exchanger) 5ml column in a Biorad NGC Quest Plus FPLC system .

Ion exchange chromatography consists of the following steps

- Equilibriation of column: The column was equilibrated with 25mM Tris buffer pH 8.1 by passing 5 Column volume (CV) of buffer.
- Sample application : This was done with a flow rate of 0.2 ml min^{-1}
- Column wash: In this step the unbound proteins were washed off from the column with 25 mM Tris buffer pH 8.1 with a flow rate of 1 ml min^{-1} using 10 CV buffer.
- Elution: The fractions were eluted in a continuous gradient from 0 to 500 mM NaCl in 25mM Tris buffer (pH 8.1) with a flow rate of 0.5 ml min^{-1}.

- Column wash : Column wash after elution was done with 1M NaCl in 25 mM Tris buffer pH 8.1, followed by Tris buffer pH 8.1 in an isocratic manner with 5 and 10 CV respectively.

Fractions from the column were checked for the protease inhibition, pooled and concentrated using amicon UF-3kDa membrane. The purity of the inhibitor was checked by running on SDS PAGE.

3.2.9.5 Trypsin Affinity Chromatography

The ion exchange purified and concentrated fraction was subjected to final purification using trypsin affinity chromatography. The trypsin affinity resin was prepared by coupling 10 mg of trypsin to 250 mg of Cyanogen bromide (CNBr) activated Sepharose 4B.

3.2.9.5.1 Coupling of trypsin to the CNBr activated Sepharose 4B gel

1. CNBr activated Sepharose 4B (250 mg) was swelled with double distilled water for 8 hours and washed with 0.1M $NaHCO_3$ containing 500 mM NaCl pH 8.3. (coupling buffer) prior to coupling with trypsin.
2. Trypsin (10 mg) was dissolved in 1 ml coupling buffer
3. Trypsin solution was added to the swelled resin and rotated end over end for 1 hour at room temperature.
4. Excess trypsin was washed away with 5 column volume (CV) of coupling buffer.

5. The medium was then transferred to 0.1M Tris buffer pH 8.00 and allowed to stand for 2 hours. This is to block any remaining trypsin unbound active sites.

6. Finally the medium was washed with 4 cycles of alternating pH buffers. Each cycle consists of a wash with 100 mM acetic acid/ sodium acetate buffer, pH4.00 containing 500 mM NaCl followed by a wash with 100 mM Tris buffer, pH 8.00 containing 500mM NaCl. Each buffer wash consists of 5 CV.

3.2.9.5.2 Loading of ion exchange fraction onto Trypsin-Sepharose column

The concentrated fraction from ion exchange chromatography was loaded on to trypsin affinity column and after 20 minutes of incubation, column was washed with 100 mM Tris buffer pH 8.0 until protein free and elution was done with 0.1M HCl and the fractions obtained were neutralized with 0.1N NaOH.

3.2.10 Characterisation of the purified inhibitor

3.2.10.1 Determination of subunit molecular weight

Subunit molecular weight of the inhibitor was determined by running the inhibitor on Sodium Dodecyl Sulphate –Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.2.10.1.1 SDS –PAGE

It is an analytical method used to separate charged molecules according to their molecular mass in an applied electric field. Sodium

Dodecyl Sulphate –Polyacrylamide Gel Electrophoresis (SDS–PAGE) was done as per Leammli's method (Leammil, 1970) with 10% acrylamide. For determining the subunit molecular weight of the protein, the mobility of protein was calculated based on the mobility of the molecular weight standards in polyacrylamide gel electrophoresis. Purity of the protein in each stage of the purification is also checked by SDS- PAGE.

Reagents

1. Acrylamide solution (30%)

Acrylamide 15grams and bis acrylamide 0.4 grams were dissolved in water and made up to 50 ml with distilled water.

2. Preparation of Buffers

i) Buffer I pH 8.8

0.614M Tris buffer pH 8.8: 7.43 g of Tris dissolved in water and pH adjusted to 8.8 with HCl and 164mg SDS added and made upto 100ml with distilled water.

ii) Buffer II pH6.8

0.147 M Tris buffer pH 6.8: 1.78g of Tris dissolved in water and pH adjusted to 6.8 and added 108mg of SDS made upto 100ml with distilled water.

iii) Chamber buffer pH 8.3

0.025 M Tris, 0.192 M glycine containing 1% of SDS in distilled water pH 8.3

3. Fixative

Fixative was made by mixing fifty percentage of methanol and 75µl of formaldehyde in water.

4. Staining solution

60 mg Co-omassie Brilliant blue R-250 dissolved in 44ml methanol and 44ml distilled water and added 12ml glacial acetic acid

5. Destaining solution

Mixed 50 ml methanol, 75ml glacial acetic acid and 875ml distilled water.

6. 6X loading dye

To make 10ml 6X loading dye, added 0.591 g Tris HCl and 0.6 g SDS, 4.8 ml glycerol, 0.9 ml mercaptoethanol and 3mg Bromophenol blue mixed together and made upto 10ml.

7. Ammonium per sulfate

15mg Ammonium per sulfate dissolved in 1ml distilled water.

Gel preparation

A Mini gel of 10% acrylamide was prepared and run as follows:

i) Separating Gel

For making separating gel added 3 ml acrylamide solution and 5.5 ml buffer I and 450µl Ammonium persulfate (APS) solution and

10µl TEMED and mixed well in a beaker and poured it into the Gel cassete and allowed to polymerize.

ii) Spacer gel

For making spacer gel, added 500µl of acrylamide solution and 4.25 ml buffer II and 250µl APS and 5µl TEMED and mixed well in a beaker. It is poured over the separating gel and the comb was placed.

Protein samples mixed with 6X loading dye and placed in boiling water bath for 5 minutes. The protein sample was loaded on to the gel. After loading the protein sample electrodes were connected after filling the chamber buffer in the chamber. Applied an electric field of 25 mV and run was continued until the bromophenol blue dye reaches the bottom of the gel. Disconnected the power supply and the gel was transferred to the fixative solution. After fixing for 45minutes the gel was stained in the staining solution for 45 minutes and destained in destaining solution and photographed.

3.2.10.3 Non-denaturing electrophoresis (Alkaline PAGE)

In Native Alkaline PAGE the gel was casted and run at alkaline conditions without any reducing agents and detergents.

A. Reagents

1. Solution A: pH 8.8-9.0

1N HCl	-	24 ml
Tris	-	18.1g
TEMED	-	0.12ml

Distilled water to make up to 100ml

2. Solution B: pH 6.6-6.8

1N HCl - 48 ml

Tris - 5.98 g

TEMED - 0.46 ml

Distilled water to make up to 100ml

3. Solution C:

Acrylamide - 28 g

Bis - acrylamide- 0.735 g

Distilled water to make up to 100ml

4. Solution D:

Acrylamide - 20 g

Bis - acrylamide- 5.0 g

Distilled water to make up to 100ml

5. Solution G:

Ammonium persulfate - 14 mg/ 10 ml

6. Solution E:

0.005% Bromophenol blue solution

7. Running buffer: pH 8.3

Dissolve 6 g Tris and 28.8 g glycine in distilled water. Make up to 1000 ml.

8. Fixative

Fifty percentage methanol, containing 75µl of formaldehyde in water.

9. Staining solution

60 mg Co-omassie Brilliant blue R-250 dissolved in 44ml methanol and 44ml distilled water and added 12ml glacial acetic acid

10. Destaining solution

Mixed 50 ml methanol, 75ml glacial acetic acid and 875ml distilled water.

B. Gel preparation

Separating gel

Mix equal volumes of solution A and solution C. Add solution G to this mixture in 1:1 ratio.

Stacking gel

Mix 1 part solution B, 1 part solution D, 4 part solution G and 2 part distilled water. Non-denaturing electrophoresis was done to separate native proteins.

For this 30µl of sample was mixed with 3µl of glycerol and co-omassie brilliant blue dye and loaded on to the gel and run with a current of 25 mA until the dye reached the bottom of the gel. Disconnected the power supply and the gel was transferred to the fixative solution. After fixing for 45minutes the gel was stained in the staining solution for 45 minutes and destained in destaining solution and photographed.

3.2.10.3 Identification of the inhibitor protein by Mass Spectrometry

The inhibitor purified from *Hibiscus acetosella* was identified by MALDI TOF. For this SDS-PAGE was done and stained with colloidal Coomassie brilliant blue stain and protein band was excised from the gel and subjected to mass spectrometry at Rajeev Gandhi Center for Biotechnology, Trivandrum in BDALDE FLEX-PC ultraflexTOF/TOF instrument. The excised gel bands were cut and trypsin digestion was performed by incubating the samples overnight at 37 °C. The supernatant with digested peptides was collected and further used for MALDI TOF MS analysis.

The acquired spectral data were analyzed using BIOTOOLS (version3.2) and search engine used was MASCOT (version2.3, Matrix Science London U.K) for protein identification. The protein identifications were obtained by searching against the plant protease inhibitor database from the Uniprot repository. During database search, Oxidation of methionine was selected as variable modification and cysteine carbamidomethylation was selected as a fixed modification.

Trypsin was chosen as the enzyme used with specificity of one missed cleavage.

3.2.10.3.1 Preparation of Colloidal Co- omassie brilliant blue staining

To prepare 100ml of Colloidal co-omasie stain following compounds were added in the order and were made up to100 ml.

a. Ammonium sulfate - 5 grams

b. Coomasie G- 250 - 0.02g

c. Phosphoric acid - 2.35ml

d. Alcohol - 10ml

e. Distilled water to make up to 100ml

Mix a, b, c well until the dye dissolves (a reddish colour appears).Then add reagent d as soon as the dye dissolves and make up to 100 ml with distilled water.

3.2.10.4 Checking the glycosylation status of the inhibitor

The samples were subjected to SDS –PAGE using 10% acrylamide in a Mini slab gel. Gels after running were soaked in 7.5% (v/v) acetic acid for 30 minutes and then in 0.2% (w/v) periodic acid for 2 hours. The periodic acid solution was removed and Schiff;s reagent was added and incubated for about 30 minutes, until a reddish pink bands of glycoprotein was visible. Ovalbumin was loaded as a positive control .The Schiffs reagent was removed and the gel was soaked in

7.5% acetic acid for 1 hour and subsequently stored in water (Dubray and Bezard, 1982).

3.2.10.5 Study of thermal stability of the purified inhibitor

Thermal stability of the inhibitor was determined according to Chaudhary *et al* (Chaudhary *et al*., 2008). The purified inhibitor (100µl) was incubated in a water bath at different temperatures (30, 40, 50, 60, 70, 80, 90 and 100^{o} C) for 30 minutes. The samples were cooled to room temperature, centrifuged at 10,000 x g for 10 minutes at 4^{o} C. The supernatant with soluble proteins were used to test the residual protease inhibition activity by protease inhibition assay.

3.2.10.6 Determination of pH optimum of the purified inhibitor

The stability of the inhibitor at different pH was evaluated according to Klomklao *et al* (Klomklao et al., 2011) with slight modification. The buffers used were: 0.05M glycine HCl, pH 2.00; 0.05 M sodium acetate, pH 5.00; 0.1 M sodium phosphate, pH 7.00; 0.1M Tris-HCl, pH 8.00; sodium bicarbonate buffer, pH 9.00 and pH10.00. After 30 minutes incubation of the inhibitor at different pH in room temperature the residual protease inhibitory activity was tested.

3.2.10.7 Effect of detergents, reducing and oxidizing agents on the protease inhibition by the purified inhibitor

3.2.10.7.1 Effect of Triton X 100

The inhibitor was incubated with 0.5 and 1% w/v Triton X-100 for 30 minutes, later it was dialyzed against bicarbonate buffer pH 9.0.

The residual protease inhibition activity was assessed using protease inhibition assay. Two controls were maintained in one, the inhibitor was mixed with bicarbonate buffer pH 9.0 equal to the volume of Triton X-100 added in the test. In the second control , 0.5 and 1% w/v of Triton X-100 was mixed with bicarbonate buffer pH 9.0 equal to the volume of inhibitor used in the test, and after 30 minutes incubation both the controls were dialyzed against bicarbonate buffer pH 9.0 and checked the residual protease inhibition. The residual protease inhibition was checked by protease inhibition assay using trypsin as the enzyme source.

3.2.10.7.2 Effect of SDS

The inhibitor was incubated for 30 minutes with 0.5 and 1% w/v SDS, later it was dialyzed against bicarbonate buffer pH 9.0. The residual protease inhibition activity was assessed using protease inhibition assay. Two controls were maintained in one, the inhibitor was mixed with bicarbonate buffer pH 9.0 equal to the volume of Triton X-100 added in the test. In the second control, 0.5 and 1% w/v of SDS was incubated with bicarbonate buffer pH 9.0 and after 30 minutes incubation both the controls were dialyzed against bicarbonate buffer pH 9.0 and checked the residual protease inhibition. The residual protease inhibition was checked by protease inhibition assay using trypsin as the enzyme source

3.2.10.7.3 Effect of β-mercaptoethanol

The effect of reducing agent β-mercaptoethanol was analyzed by incubating inhibitor with 3 and 5 mM of β-mercaptoethanol for 30 minutes. Bicarbonate buffer mixed with 3 and 5mM of β-

mercaptoethanol was used as control. The residual protease inhibition activity was measured using protease inhibition assay using trypsin as the enzyme source.

3.2.10.7.4 Effect of Dimethyl sulphoxide (DMSO)

To check the effect of oxidizing agent on the activity of inhibitor, it was incubated with 0.5 and 1% (v/v) Dimethyl sulphoxide (DMSO) for 30 minutes and for control same volume of DMSO was mixed with bicarbonate buffer. The residual inhibitory activity was measured using proteqase inhibition assay in which trypsin was used as an enzyme source.

3.2.10.8 Effect of metal ions on the activity of the purified inhibitor

The effect of metal ions on the inhibition was done according to Sayem *et al* (Sayem *et al.,* 2006) with slight modifications. The metal ions selected for this are Zn^{2+} and Hg^{2+}. The inhibitor was incubated with 10mM of each metal ions for 30 minutes. For control the metal ions were included in bicarbonate buffer and the residual protease inhibition was checked using protease inhibition assay.

3.2.10.9 Determination of Ki value and nature of inhibition for Trypsin

For the determination of Ki value different inhibitor concentrations (72.5nM, 145nM, 217.5 nM and 290nM) was tested with two different substrate (BAPNA) concentrations (0.38mM and 0.5mM) using trypsin as the enzyme. The reciprocal of velocity (1/v) *versus* concentration of protease inhibitor for each substrate concentration was

plotted and Ki was calculated from the interception of the two lines and the mechanism of inhibition was also inferred from the plot.

3.2.10.10 *In vivo* effect of the inhibitor on 4th instar larvae of *S.litura*

The fourth instar larvae were fed with 1.16 µg of the purified inhibitor twice in a day by applying on to the leaves of *Ricinus communis* and dried before feeding. In the control the larvae were fed with leaves applied with equal volume of bicarbonate buffer instead of the inhibitor. Both the larval weight and mortality was noted. The experiment was carried out till the larvae entered into prepupal stage/ until all the test larvae were dead/ adult emergence, whichever is the earlier. The experiment was carried out at 25±2^{o}C and 75±3% relative humidity at 12 hour day and night cycle mimicking the natural condition.

3.2.11. Statistical analysis

Statistical analysis was done using R program.

RESULTS

4. RESULTS

4.1 SCREENING OF PLANT EXTRACTS FOR IDENTIFYING PROTEASE INHIBITORS AGAINST LARVAL GUT PROTEASE OF *SPODOPTERA LITURA*

Plant protease inhibitors are the natural defensive molecules present in plants which provide protection against herbivory. Thus screening and identification of plant's inbuilt defensive molecules will be helpful for formulating effective pest control strategies.

In this study 123 plants were screened for checking the presence of protease inhibitors against the 5th instar larval gut proteases of *Spodoptera litura*. Plant parts from different families used for screening were collected from Palakkad, Malappuram and Kannur districs of Kerala and Tiruchirapalli district of Tamilnadu. Out of 123 plant extracts screened to identify extracts containing protease sinhibitors, 52 plants showed inhibition above 40% against 5th instar larval gut proteases of *Spodoptera litura* (Table 4.1).

Table 4.1: List of plants screened against larval gut proteases of *Spodoptera litura*

Sl. No	Plant species (family)	Common name (Vernacular name)	Plant part used	%Inhibition (Mean±S.E)
1	*Ardisia solanaceae* Roxb. (Primulaceae)	Shoebutton ardisia, Duck's eye (Kaka-njara)	Seed	88.6±1.19
2	*Hibiscus acetosella* Welw. Ex Hiern (Malvaceae)	False roselle, African rosemallow (Pulivenda)	Seed	88.61±0.40

Sl. No	Plant species (family)	Common name (Vernacular name)	Plant part used	%Inhibition (Mean±S.E)
3	*Calopogoniu m mucunoides* Desv. (Fabaceae)	Wild groundnut, Calapo	Seed	85.55±0.13
4	*Eleusine coracana* (L.) Gaertn. (Poaceae)	Finger millet,Ragi (Kora)	Seed	76.5±0.24
5	*Carica papaya* L. (Caricaceae)	Papaya (Omakkaya)	Seed	76.35±0.58
6	*Accacia concina* DC (Fabaceae).	Shikakai (Sikakkai)	Seed	73.84±0.17
7	*Coccinia grandis* (L.) Voigt. (Cucurbitacea e)	Ivy gourd, scarlet gourd	Seed	69.91±1.06
8	*Murraya koenigii* (L.) Sprengel. (Rutaceae)	Curry tree (Kariveppila)	Seed	70.80±0.73
9	*Abelmoschus tuberosus* Medik. (Malvaceae)	Pink swamp mallow	Seed	70.72±0.48
10	*Abelmoschus moschatus* Medik. (Malvaceae)	Musk mallow (Kasthurivenda)	Seed	70.18±0.81
11	*Abelmoschus manihot* (L.) Medik. (Malvaceae)	Sunset muskmallow	Seed	69.53±0.28
12	*Syzygium samarangenes e* (Blume) Merr. & L.M. Perry (Myrtaceae)	Wax apple (Paninir champa)	Seed	67.03±1.02

Sl. No	Plant species (family)	Common name (Vernacular name)	Plant part used	%Inhibition (Mean±S.E)
13	*Litchi chinensis* Sonn. (Sapindaceae)	Lychee	Seed	66.1±0.98
14	*Solanum xanthocarpum* Schard and Wendl. (Solanaceae)	Yellow berried nightshade	Seed	65.61±1.32
15	*Solanum nigrum* L. (Solanaceae)	Black nightshade	Seed	63.12±1.45
16	*Tinospora cordifolia* (Willd.) Miers (Menispermaceae)	Heart- leaved moonseed	Seed	62.84±1.00
17	*Myristica fragrans* Houtt. (Myristicaceae)	Nutmeg (Jadikka)	Seed	65.12±1.01
18	*Hyptis suaveolens* (L.) Kuntze (Lamiaceae)	Bush mint (Nattapoochedi)	Seed	64.0±1.25
19	*Mimosa diplotrica* C. Wright ex Sauvalle (Fabaceae)	Giant sensitive plant (Aanthottawadi)	Seed	62.83±1.06
20	*Piper nigrum* L. (Piperaceae)	Black pepper (Kurumulaghu)	Seed	60.42±1.24
21	*Mucuna pruriens*(L.) DC. (Fabaceae)	Velvet bean (naykkurana)	Seed	60.00±0.84

Sl. No	Plant species (family)	Common name (Vernacular name)	Plant part used	%Inhibition (Mean±S.E)
22	*Senna occidentalis* (L.) Link. (Fabaceae)	Coffee senna (Ponnionthakara)	Seed	60.0±1.00
23	*Prunus domestica* L. (Rosaceae)	Plum	Seed	59.6±0.76
24	*Mimusops elengi* L. (Sapotaceae)	Spanish cherry (Ilanni)	Leaf	69.91±0.49
25	*Hibiscus hispidissimus* Griff. (Malvaceae)	Hill hemp bendy (Njaranpuli)	Seed	56.32±0.11
26	*Catharanthus roseus* (L.) G.Don (Apocynaceae)	Periwinkle (Shavamnari)	Seed	56.20±0.26
27	*Asparagus racemosus* Willd (Asparagaceae)	Buttermilk root (Sathavari)	Seed	55.12±1.60
28	*Sterculia quadrifida* R.Br (Malvaceae)	Peanut tree (Pavizhathondi)	Seed	54.5±0.19
29	*Senna obtusifolia* (L.) H.S. Irwin and Barneby (Fabaceae)	Sicklepod (Sakramardakam)	Seed	52.8±0.72
30	*Strychnos nux-vomica* L. (Loganiaceae)	Poison nut (Kanjiram)	Seed	49.86±0.54

Sl. No	Plant species (family)	Common name (Vernacular name)	Plant part used	%Inhibition (Mean±S.E)
31	*Illicium verum* Hook.F (Schisandraceae)	Star arnise (Thakkolam)	Seed	51.8±0.37
32	*Phyllanthus amarus* Schumach and Thonn. (Phyllanthaceae)	Carry me seed (Kizharnelli)	Seed	51.2±0.29
33	*Cucurbita pepo* L. (Cucurbitaceae)	Pumkin(Mathan)	Seed	49.51±1.27
34	*Persea americana* Mill (Lauraceae)	Avocado (Vennapazham)	Seed	47.10±1.20
35	*Amaranthus spinosus* L (Amaranthaceae).	Prickly amaranth (Mullancheera)	Seed	47.25±1.20
36	*Celosia cristata* L. (Amaranthaceae)	Cocks comb (Kozhipoo)	Seed	47.59±.55
37	*Amaranthus dubius* Mart ex Thell (Amaranthaceae)	Red spinach (Chumappu cheera)	Seed	47.96±0.31
38	*Pouteria campechiana* Baehni. (Sapotaceae)	Canistel (Muttapazham)	Seed	47.32±0.86
39	*Coleus aromaticus* Benth. (Lamiaceae)	Cuban oregano (Panikoorkka)	Leaf	46.65±1.1

Sl. No	Plant species (family)	Common name (Vernacular name)	Plant part used	%Inhibition (Mean±S.E)
40	*Prunus cerasus* L. (Rosaceae)	Sour cherry (Elavaluka)	Seed	46.78±0.33
41	*Nyctanthes arbor-tristis* L. (Oleaceae)	Coral jasmine (Parijatham)	Seed	47.23±1.68
42	*Capsicum frutescens* L. (Solanaceae)	Chilli (Pachamulagu)	Seed	44.40±0.20
43	*Linum usilatissimum* (Linaceae)	Flax (Cheruchanavithu)	Seed	46.35±1.54
44	*Ziziphus jujuba* Mill. (Rhamnaceae)	Jujuba (Elanthapazham)	Seed	43.68±0.37
45	*Abutilon indicum* L. (Malvaceae)	Indian mallow (Velluram)	Seed	43.16±0.28
46	*Ipomea pes caprae* (L.)R.Br. (Convolvulaceae)	Railroad vine(Naripadam)	Seed	42.14±1.03
47	*Ipomea obscura* (L.)Ker Gawl (Convolvulaceae)	Obscure morning glory(Tirutali)	Seed	43.92±1.42
48	*Citrus limon* (L.) (rutaceae)	Lemon (Cherunaragam)	Seed	40.77±0.58
49	*Calycopteris floribunda* Lam (Combretaceae)	Pullani	Seed	40.68±0.35
50	*Vitex negundo* L. (Lamiaceae)	Chaste tree (Vennochi)	Seed	40.22±0.39
51	*Capsicum annum* L. (Solanaceae)	Capsicum, Sweet pepper	Seed	41.24±0.55

Sl. No	Plant species (family)	Common name (Vernacular name)	Plant part used	%Inhibition (Mean±S.E)
52	*Helicterus isora* L. (Malvaceae)	East Indian screw tree (Eswaramooli)	Seed	40.28±0.12
53	*Saracca asoka* (Roxb.) Willd (Fabaceae)	Sita ashok (Hemapushpam)	Seed	39.25±0.37
54	*Ipomea tricolor* Cav. (Convolvulaceae)	Morning glory (Taliyari)	Seed	37.34±4.70
55	*Garcinia cambogia* L. (Clusiaceae)	Malabar tamarind (Kudampuli)	Seed	35.90±0.15
56	*Nicotina tobaccum* L. (Solanaceae)	Tobacco (Vettila)	Leaf	31.80±0.45
57	*Cucumis sativus* L. (Cucurbitaceae)	Cucumber (Vellari)	Seed	30.36±0.90
58	*Coriandrum sativum* L. (Apiaceae)	Corriander (Malli)	Leaf	29.00±0.95
59	*Canavalia maritima* (Fabaceae)	Bay bean (Manal amara)	Seed	31.71±1.42
60	*Lawsonia inermis* L. (Lythraceae)	Mehandi (Maylanji)	Seed	31.08±1.07
61	*Citrulus lanatus* (Thunb) (Cucurbitaceae)	Watermelon (Chakkaramathan)	Seed	30.01±0.51
62	*Anogeissus latifolia* (Robx. Ex DC.) (Combretaceae)	Axle wood tree (Mazhakanjiram)	Leaf	30.78±1.55

Sl. No	Plant species (family)	Common name (Vernacular name)	Plant part used	%Inhibition (Mean±S.E)
63	*Mavalviscus arboreus* Cav. (Malvaceae)	Sleeping hibiscus (Mottu chembarathi)	Flower	30.71±0.35
64	*Curculigo orchioides* Gaertn. (Hypoxidaceae)	Golden eye grass (Nilappana)	Leaf	29.72±0.94
65	*Tribulus terestrius* L. (Zygophyllaceae)	Puncture vine (Cheriya neringilu)	Seed	29.21±0.30
66	*Senna allata* (L.) Robx. (Fabaceae)	Candle bush (Chakrathakara)	Seed	29.0 ±0.54
67	*Mangifera indica* L. (Anacardiaceae)	Mango (Manga)	Seed	28.60±0.32
68	*Cardiospermum halicacabum* L. (Sapindaceae)	Balloon vine (Jyothish mati)	Seed	28.08±0.09
69	*Lantana camera* L. (Verbenaceae)	Lantana (Aripoov)	Flower	27.90±0.25
70	*Oscimum sanctum* L. (Lamiaceae)	Holy basil (Tulsi)	Leaf	26.24±0.13
71	*Thespesia populnea* (L.) Sol. (Malvaceae)	Indian tulip tree (Puvarassu)	Seed	26.0 ±0.69
72	*Tricosanthus cucumeria* L. (Cucurbitaceae)	Snake gourd (Padavalam)	Seed	26.0 ±0.18

Sl. No	Plant species (family)	Common name (Vernacular name)	Plant part used	%Inhibition (Mean±S.E)
73	*Heliconia caribaea* Lam. (Heliconiaceae)	Lobster claw	Leaf	25.47±0.26
74	*Ricinus communis* L. (Euphorbiaceae)	Castor bean (Aavanakku)	Seed	24.96 ±0.13
75	*Couroupita guianensis* Aubl (Lecythidaceae)	Cannon ball tree (naagalingam)	Seed	24.62±0.46
76	*Fittonia verschaffeltii* (Coem.) Regel (Acanthaceae)	Mosaic plant	Leaf	23.25±1.13
77	*Santalum album* L. (Santalaceae)	Sandal wood(Chandanam)	Seed	23.16±0.45
78	*Immpatients flaccida* Arn. (Balsaminaceae)	Pink Orchid balsam	Seed	22.87±0.53
79	*Anethum graveolens* L. (Apiaceae)	Dill (Chatakuppa)	Seed	20.85±0.79
80	*Bauhinia blakeana* Dunn. (Fabaceae)	Hongkong Orchid tree	Seed	22.25±0.91
81	*Bauhinia tomentosa* L. (Fabaceae)	Yellow bell (Manja mandaram)	Seed	22.40±0.22
82	*Bauhinia acuminate* L. (Fabaceae)	Dwarf white Orchid (Mandaaram)	Seed	22.13±0.13
83	*Biophytum sensitivum* (L.)DC (Oxalidaceae)	Sensitive plant (Mukkutti)	Leaf	22.17±0.22

Sl. No	Plant species (family)	Common name (Vernacular name)	Plant part used	%Inhibition (Mean±S.E)
84	*Lagenaria siceraria* (Molina) Standl (Cucurbitaceae)	Bottle gourd (Pechura)	Seed	21.92±0.31
85	*Scoparia dulcis* L. (Planaginaceae)	Sweet broom weed	Seed	21.54±0.35
86	*Passiflora foetidae* L. (Passifloraceae)	Bush passion fruit(Poochapalam)	Seed	21.0±0.31
87	*Andrographis paniculata* (Burm.f.) Nees (Acanthaceae)	Kariyat (Kiriyat)	Seed	21.0±0.42
88	*Swietenia mahagoni* (L.) Jacq. (Meliaceae)	Mahagony	Seed	19.56±0.12
89	*Callistemon lanceolatus* R.Br (Myrtaceae)	Bottle brush	Leaf	19.27±0.27
90	*Withania somnifera* (L.) Dunal (Solanaceae)	Aswagandha(Amukkuram)	Seed	19.25±1.05
91	*Ensete superbum* (Roxb.) Cheesman (Musaceae)	Rock banana (Kalluvazha)	Seed	19.22±0.18
92	*Curcuma longa* L. (Zingiberaceae)	Turmeric (Manjal)	Seed	18.91±0.56
93	*Ixora coccinea* L. (Rubiaceae)	Ixora (Chethi)	Seed	18.60±0.98

Sl. No	Plant species (family)	Common name (Vernacular name)	Plant part used	%Inhibition (Mean±S.E)
94	*Passiflora pomifera* L. (Passifloraceae)	Passion fruit	Seed	18.40±0.16
95	*Casuarina equisetifolia* L. (Araceae)	Whistling pine(Sampirani)	Seed	17.87±0.33
96	*Spathiphyllum montanum* (R.A.Baker) Grayum (Araceae)	Fragrant peace lily	Leaf	18.03±0.05
97	*Artemisia vulgaris* L. (Asteraceae)	Mugwort	Leaf	17.09±0.38
98	*Bougainvillea glabra* Choisy (Nyctaginaceae)	Bougainvilleab(Kadal asupoov)	Leaf	17.01±0.14
99	*Aegle marmelos* L. (Rutaceae)	Bael (Koolakam)	Seed	16.53±0.19
100	*Clitoria ternatea* L. (Fabaceae)	Butterfly pea (Sangu pushpam)	Seed	15.62±0.33
101	*Mussaenda erytrophylla* Schumach and Thonn (Rubiaceae)	Ashanti blood (Vellilathali)	Leaf	15.14±0.31
102	*Mussaenda glabrata* (Hook.f.) Hutch. Ex (Rubiaceae)	Dhoni tree (Vellila)	Flower	13.42±0.53
103	*Bacopa monnieri* (L.) Pennell. (Plantaginaceae)	Water hyssop(Brahmi)	Leaf	13.70±0.46

Sl. No	Plant species (family)	Common name (Vernacular name)	Plant part used	%Inhibition (Mean±S.E)
104	*Calophyllum inophyllum* L. (Calophyllaceae)	Sulthan champa (Pinna	Seed	13.61±0.22
105	*Epipremnum aurem* (Linden and Andre) (Araceae)	Pothos(Money plant)	Seed	13.59±0.41
106	*Monstera deliciosa* Liebm. (Araceae)	Split leaf Philodendron	Leaf	13.46±0.36
107	*Alstonia scholaris* L. (Apocynaceae)	Scholar tree(Daivappala)	Seed	12.54±0.57
108	*Trichosanthus dioica* Roxb. (Cucurbitaceae)	Parval (Patolam)	Seed	12.50±0.26
109	*Cynodon dactylon* (L.) (Poaceae)	Bermuda grass (Belikaruka)	Leaf	12.26±0.27
110	*Elettaria cardamomum* L (Zingiberaceae)	Cardamom(Elakkai)	Seed	11.63±0.62
111	*Ravoulfia serpentine* L. (Apocynaceae)	Sarpagandha (Suvapavalforiyan)	Seed	11.05±0.28
112	*Canthium angustifolium* Roxb. (Rubiaceae)	Narroe leaved canthium (Katu- karas walli)	Seed	10.94±0.46
113	*Punica granatum* L. (Lyhtraceae)	Pomegranate(Madhala naranga)	Seed	10.68±0.58

Sl. No	Plant species (family)	Common name (Vernacular name)	Plant part used	%Inhibition (Mean±S.E)
114	*Aristolochia indica* L. (Aristolochiaceae)	Indian Birthwort (Eeswaramooli)	Seed	10.61±0.54
115	*Clerodendrum infortunatum* L. (Lamiaceae)	Hill glory bower (Peruku)	Leaf	10.14±0.23
116	*Rubus ellipticus* Smith (Rosaceae)	Yellow Himalayan raspberry (Cheemullu)	Fruit	8.65±0.57
117	*Phyllanthus niruri* L. (Phyllanthaceae)	Stone breaker (Keezhanelli)	Fruit	8.57±0.73
118	*Ensete superbum* Roxb. (Musaceae)	Rock babana (Kalluvazha)	Leaf	7.63±0.64
119	*Delonix regia* (Boj.ex Hook.) Raf. (Fabaceae)	Flame tree (Gulmohar)	Seed	6.98±0.30
120	*Nerium oleander* L. (Apocynaceae)	Oleander (Arali)	Leaf	6.16±0.13
121	*Zanthoxylum rhesta* (Roxb.) DC. (Rutaceae)	Indian prickly ash (Kothumurikku)	Seed	4.83±0.50
122	*Rosa indica* L. (Oleaceae)	Rose (Panineer)	Leaf	2.89±0.27
123	*Jasminum officinale* L. (Oleaceae)	Jasmine (Mullapoo)	Leaf	2.31±0.21

Values are Mean ± Standard Error (n=6)

100% gut enzyme activity corresponds to 408 U (408 nMole ml^{-1} min^{-1}).

One unit of enzyme is the amount of enzyme which converts 1nMole of substrate per millilitre per minute

During screening plants for protease inhibitor, to compare the plant extracts, inhibition per unit weight of tissue was considered that is one grm of tissue per ml buffer.

The Highest inhibition was exhibited by the seeds of *Ardisia solanaceae* with 88.6±1.19% inhibition and *Hibiscus acetosella* which inhibited the larval gut protease activity of *S.litura* to the extent of 88.61±0.40%. Among the 52 plants with inhibition above 40%, 11 plant extracts were reported for the first time to contain protease inhibitor.

4.2 PROTEINASE K TREATMENT OF PLANT EXTRACTS

Out of 52 plant extracts with inhibition above 40%, 14 plant extracts were subjected to proteinase K treatment based on their large scale availability. The results of proteinase K treatment of selected plant extracts were shown in Table 4.2

Table 4.2: Proteinase K treatment of selected plant extracts

Sl. No	Plant species	%Inhibition (Mean±S.E)		p value	Protein inhibitor Present (+)/ absent (-)
		Control (Untreated)	Test (After proteinase K treatment)		
1	*Ardisia solanaceae*	87.03 ±1.20	17.63±1.22	0.001	+
2	*Hibiscus acetosella*	86.97±0.40	6.06±0.24	0.006	+
3	*Eleusine coracana*	76.37±0.23	11.75±0.52	0.002	+
4	*Calopogonium mucunoides*	85.13±0.13	18.94±0.25	<0.0001	+
5	*Accacia concina*	73.42±0.18	13.41±0.20	0.001	+
6	*Abelmoschus manihot*	68.90±0.78	12.00±0.67	0.001	+
7	*Coccinia grandis*	68.82±0.49	19.10±2.24	0.003	+
8	*Syzygium samarangenese*	67.29±1.02	61.72± 1.80	0.001	-
9	*Mimusops elengi*	58.83±0.31	14.56±0.88	0.001	+
10	*Piper nigrum*	56.91±0.66	18.45±0.60	0.001	+
11	*Amaranthus dubius*	47.96±0.31	17.45±1.17	0.002	+
12	*Prunus cerasus*	46.78±0.33	41.45±0.59	0.001	-
13	*Abutilon indicum*	43.16±0.28	13.66±0.07	<0.0001	+
14	*Helicterus isora*	40.28±0.12	30.57±0.46	<0.0001	-

Values are Mean ± Standard Error (n=6). Plus sign (+) = proteinaceous inhibitor present, minus (-) sign =Major inhibitor is non proteinaceous .

Plant extracts treated with proteinase K were checked for their protease inhibition activity after inactivating the proteinase K activity by incubating the reaction mixture at 96 ºC for 5 minutes. The percentage inhibition obtained was compared with that of control.

Proteinase K treatment revealed that out of 14 plant extracts screened, 11 plant extracts were proteinaceous in nature as there is a drastic and significant reduction in their protease inhibition after proteinase K treatement. Plant extracts with proteinaceous protease inhibitors include *Ardisia solanaceae*, *Hibiscus acetosella*, *Eleusine coracana*, *Calopogonium mucunoides*, *Accacia concina*, *Abelmoschus manihot*, *Coccinia grandis*, *Mimusops elengi*, *Piper nigrum*, *Amaranthus dubius* and *Abutilon indicum* and that from the remaining 3 plant extracts were of mainly non-proteinaceous in nature (Table 4.2). The inhibitors present in *Syzygium samarangenese*, *Prunus cerasus* and *Helicterus isora* are non- proteinaceous in nature as they retained most of their inhibition even after treating with proteinase K, but there is a small, but significant, reduction in the protease inhibition. Out of the 11 plants containing proteinaceous protease inhibitors, *Hibiscus acetosella* seed extract (86.97±0.40%) was selected for further purification and characterization based on their high inhibition against larval gut protease of *S.litura*, availability and as there are no reports of the presence of protease inhibitor from this plant.

4.3 PURIFICATION OF PLANT PROTEASE INHIBITOR FROM THE SEEDS OF *HIBISCUS ACETOSELLA*

Standard protein purification methods were used for the purification of protease inhibitor from the seed extract of *Hibiscus acetosella*. The purification steps employed were ammonium sulfate $(NH_4)_2SO_4$ precipitation, ion exchange chromatography and trypsin affinity chromatography.

4.3.1 Ammonium sulphate precipitation

Soluble proteins from the seed extract of *Hibiscus acetosella* were precipitated out with 0-30%, 30-50%, and 50-70% ammonium sulphate concentrations and their inhibition against the larval gut protease of *Spodoptera litura* assessed (Table 4.3 and Figure 4.1)

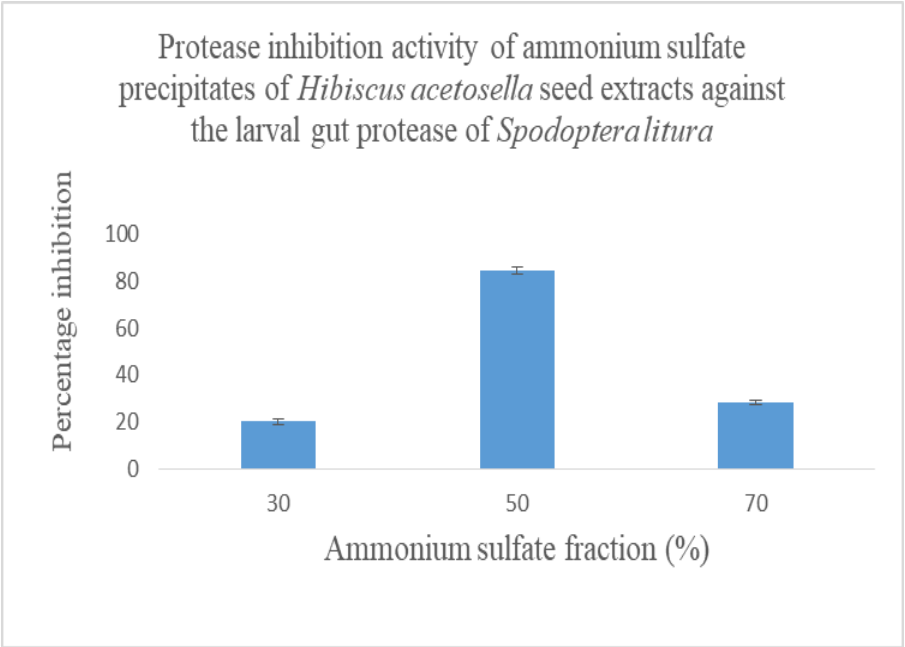
Table 4.3 Percentage inhibition exhibited by crude extract and different ammonium sulphate fractions of *Hibiscus acetosella* against the larval gut proteases of *Spodoptera litura.*

Sl.No	Sample	Protein concentration (µg/µl)	Mean % inhibition ± S.E
1	Crude extract	9.67	88.61±0.40
2	0-30% ammonium sulphate fraction	5.94	20.14±0.50
3	30-50% ammonium sulphate fraction	6.94	84.43±0.62
4	50-70% ammonium sulphate fraction	2.89	28.11±0.45

Values are Mean ± Standard Error (n=6)

Out of the three ammonium sulphate fractions, 30-50% ammonium sulphate fraction gave highest inhibition against the larval gut protease of *Spodoptera litura* (84.43±0.62%), followed by 50 -70% fraction (28.11±0.45%) and 0-30% fraction (20.14±0.50%) (Fig 4.1)

Fig 4.1: Percentage inhibition of different ammonium sulfate fractions from *H.acetosella*



Values are Mean ± Standard Error (n=6)

4.3.2 Ion exchange chromatography

Ammonium sulphate precipitate with the highest inhibition (30-50% fraction) was dialysed against bicarbonate buffer pH 9.0 (3 changes) followed by Tris buffer pH 8.1(1 change) and subjected to ion exchange chromatography in Source Q column of Biorad in NGC Quest Plus FPLC system. In a typical experiment 5 ml (48.35 mg) of

the 30-50% ammonium sulphate fraction dialysed as mentioned above was loaded on to Source Q cation exchange column with a flow rate of 0.2 ml min^{-1}. The column was equilibrated with Tris buffer pH 8.1 prior to sample application. After sample application, the column was washed with the equilibration buffer. The bound proteins were eluted using a continuous gradient from 0 to 500 mM NaCl in Tris buffer pH 8.1 and 0.5 ml fractions were collected. During elution two protein peaks were eluted out. First peak was eluted at 250 mM- 290 mM NaCl concentration and the second peak eluted at 470- 500 mM NaCl concentration (Figure 4.2). Protease inhibition assay of fractions from the two peaks were done and it was found that the inhibitor was seluted in the first peak (250-290 mM NaCl concentration) (Table 4.4).

Fig 4.2 Run report of ion exchange chromatography

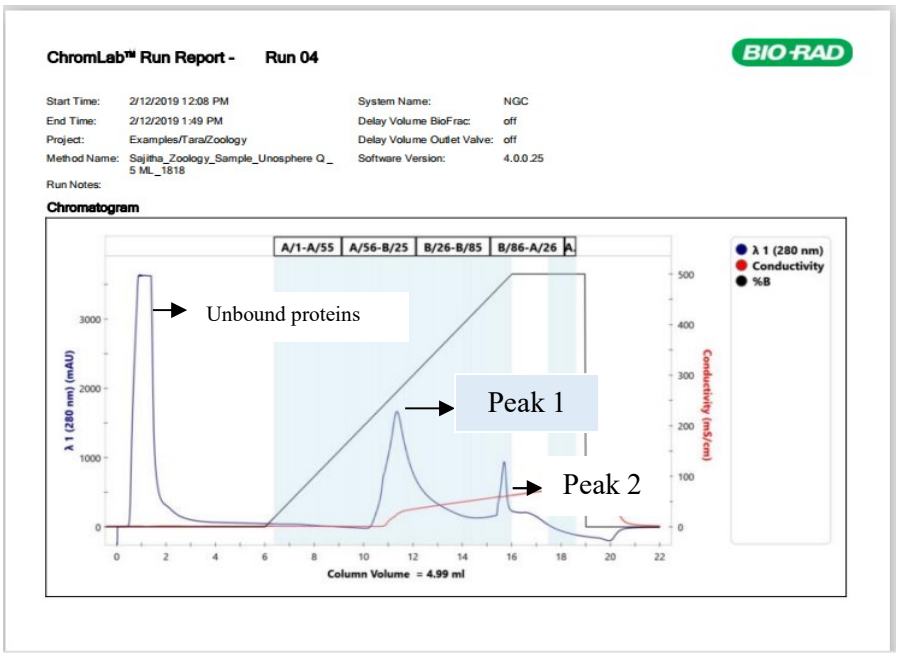


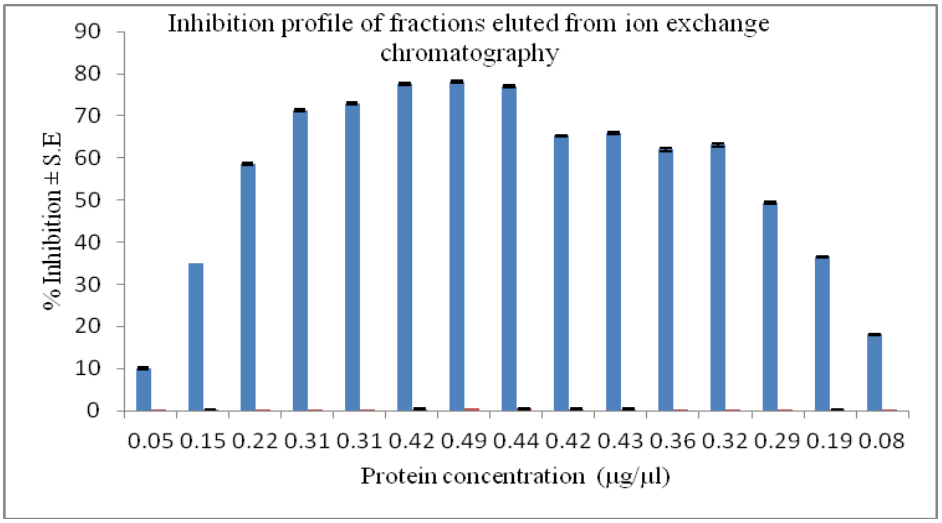
Table 4.4 Percentage inhibition against *S.litura* larval gut protease activity from fractions from two peaks eluted from Source Q ion exchange column

Peak /NaCl concentration	Fractions eluted	Mean % inhibition ± S.E
Peak 1 250-290 mM NaCl	F 1	10.79 ± 0.25
	F 4	35.05 ±0.05
	F 8	59.27 ±0.34
	F12	73.43 ± 0.23
	F 14	78.13 ± 0.22
	F 20	78.53 ± 0.20
	F 25	77.05 ± 0.21
	F 30	66.19 ± 0.23
	F 34	62.93 ± 0.43
	F 38	50.11 ± 0.25
	F 40	36.85 ± 0.13
	F 46	18.39 ± 0.12
Peak 2 470- 500 mM NaCl	F 1	4.30 ± 0.55
	F 6	3.62 ±0.51
	F 12	3.96 ± 0.25
	F 20	2.64 ± 0.54

Values are Mean ± Standard Error (n=6)

From the table it is clear that the inhibitor was eluted out in the first peak.

Fig 4.3 Protease inhibition and the corresponding protein concentration of different elutes from the peak 1



Values are Mean ± Standard Error (n=6)

Fig 4.3 depicts the protease inhibition and the corresponding protein concentration of different elutes from the peak 1 of the ion exchange chromatography. The percentage inhibition increased with increase in protein concentration of elutes. The FPLC elutes gave a clear peak with the highest inhibition in the fraction with the highest protein concentration (0.49 µg/ µl).

4.3.3 Trypsin affinity chromatography

The fractions eluted from Source Q ion exchange column with high inhibition were pooled and concentrated using amicon UF-3kDa membrane and loaded (4.455 µg protein) on to the trypsin affinity column (5 ml) for further purification. After loading the column was washed with 100mMTris buffer pH 8.0 and elution was done with 100 mM HCl. The eluted fractions were neutralized immediately with 0.5

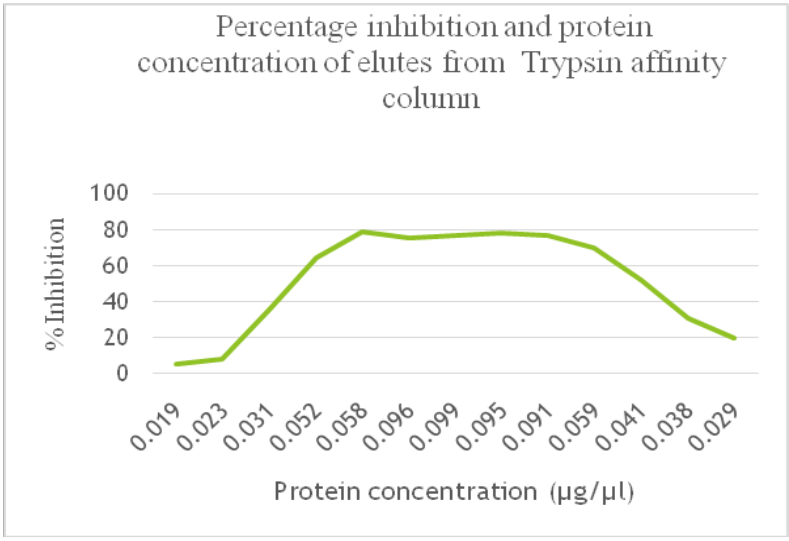
M NaOH. Percentage inhibition of eluted fractions were assessed using larval gut proteases of *S.litura* (Table 4.5 and Fig 4.4)

Table 4.5 Percentage inhibition of the fractions eluted from trypsin affinity column towards larval gut proteases of *Spodoptera litura*

Sl.No	Fraction eluted	Mean % inhibition ± S.E
1	F 1	5.67 ± 0.08
2	F 3	36.03 ± 0.09
3	F 5	79.09 ± 0.23
4	F 8	78.20 ± 0.19
5	F 12	31.24 ±0.09
6	F 15	14.20 ± 0.11

Values are Mean ± Standard Error (n=6)

Fig 4.4 Protease inhibition and the corresponding protein concentration of different elutes from the trypsin affinity column



Values are Mean ± Standard Error (n=6)

From the inhibition profile of the fractions eluted from the trypsin affinity column (Figure 4.4), it is clear that the percentage inhibition increased with increase in protein concentration of elutes. The trypsin affinity elutes gave a clear peak with the highest inhibition in the fraction with the highest protein concentration (0.058 µg/ µl).

The purity of the inhibitor was checked by SDS-PAGE and the inhibitor appeared as a single protein band confirming its purity and homogeneity (Figure 4.5). The inhibitor purified from the seed extract of *Hibiscus acetosella* was named as *Hibiscus acetosella* Protease Inhibitor (HAPI).

Table 4.6 Percentage inhibition in each purification step

Sl. No.	Sample	Protein concentration (µg/µl) (Amount of protein in assay(µg))	Total inhibitory activity (U)	Specific inhibition activity (U/ mg)	Fold purification	Mean percentage inhibition± S.E
1	Crude extract	9.67 (97)	350	36.10	1	82.88±0.57
2	Ammonium sulfate fraction (50%)	4.20 (42)	316	75.20	2.08	84.5±0.86
3	Fplc fraction pooled and concentrated	0.297 (2.9)	440	1517.24	42.14	78.51±1.08
4	Elute after trypsin affinity (Purified inhibitor)	0.058 (0.58)	330	5690.00	158.10	84.61±0.97

Values are Mean ± Standard Error (n=6)

Table 4.7 Protein concentration of the inhibitor at each purification stage required for 50% inhibition of larval gut protease activity of *S.litura*

Sl. No	Sample	Protein concentration(µg/µl) (Amount of protein in assay(µg))	Mean percentage inhibition	Specific inhibition activity (U/µg)
1	Crude extract	7.438 (74.4)	51.32	36.10
2	Ammonium sulfate fraction (50%)	2.1075 (21.1)	48.96	75.20
3	Fplc fraction pooled and concentrated	0.0594 (0.6)	53.26	1517.24
4	Elute after trypsin affinity (Purified inhibitor)	0.012 (0.12)	51.69	5690.00

After each purification step, there was an increase in percentage inhibition (Table 4.6).

One unit of gut enzyme activity was defined as the amount of enzyme (gut protein) that digested 1nMole of azocasein per milliliter of the reaction mixture per minute under the assay conditions.

There was an increase in specific inhibition activity of the inhibitor (Total activity /total amount of protein) after each purification step (Table 4.6 and 4.7)

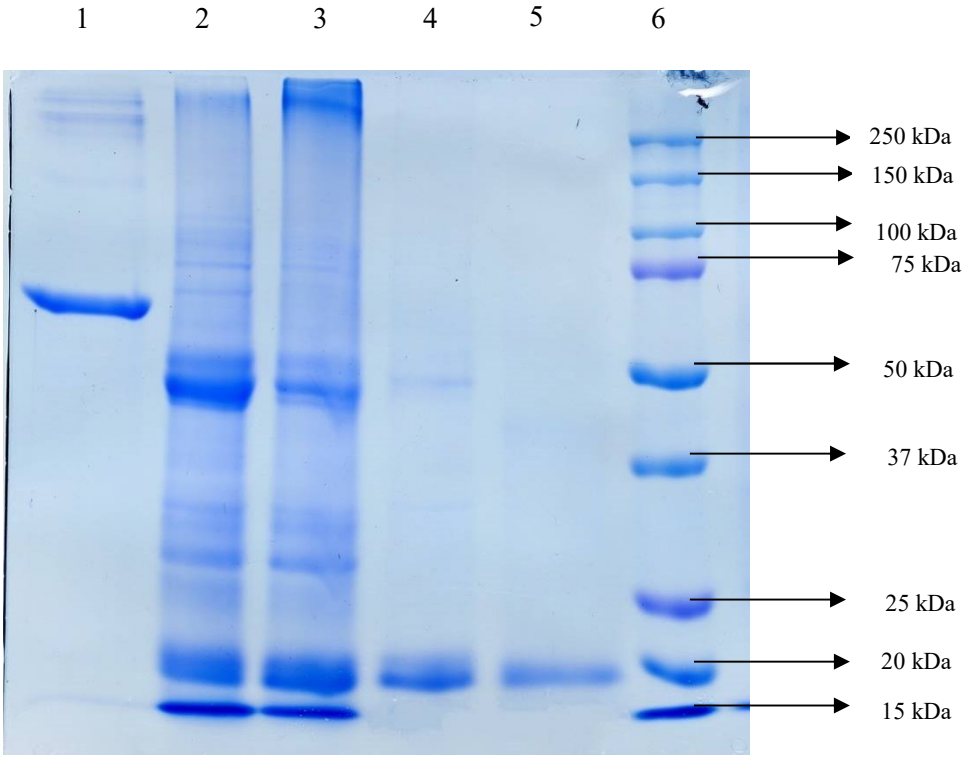
Of the different ammonium sulfate fractions, 50 % ammonium sulfate fraction gave the highest inhibition (84.5±0.86%) at the concentration of 4.2µg/µl, which represents 3.5 fold purification compared to the crude extract. Fractions eluted from ion exchange chromatography exhibited 35.7 fold (Table 4.7) purification compared to ammonium sulfate fraction and that from trypsin affinity showed 4.91 fold purification compared to ion exchange purification. The purified inhibitor from trypsin affinity achieved 600 fold more purification than that of the crude extract. The yield of the purified inhibitor was 2.2 µg of inhibitor /g of seed.

4.4 Characterization of *Hibiscus acetosella* protease inhibitor (HAPI)

4.4.1 Determination of subunit molecular weight

The sub unit molecular weight of the inhibitor isolated from *H.acetosella* was determined from SDS-PAGE from a plot of log molecular weight of molecular weight marker and their relative mobility and using the relative mobility of the inhibitor. The sub unit molecular weight of HAPI was found to be 20 kDa (Figure 4.5).

Figure 4.5 SDS-PAGE showing the purification profile of the inhibitor from the seed extract of *Hibiscus acetosella*

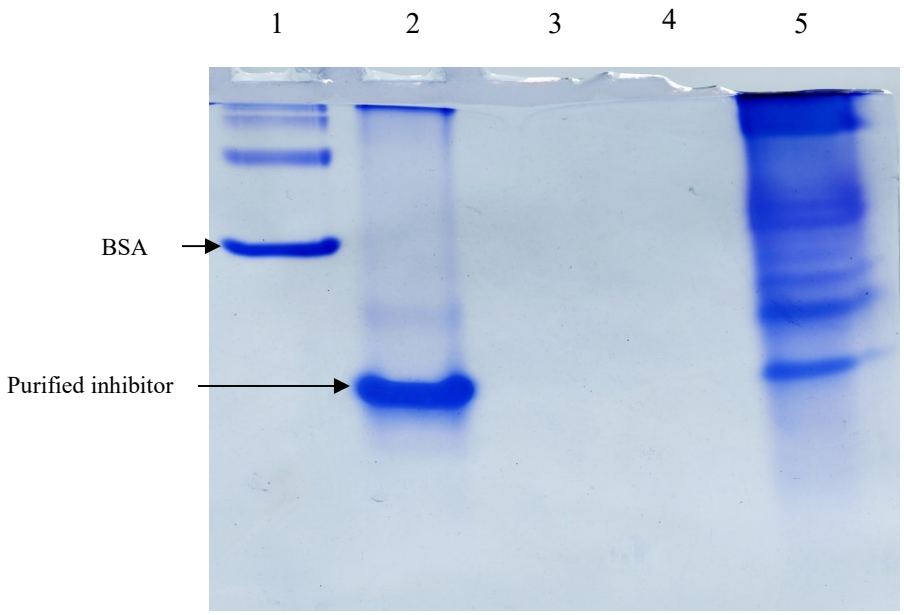


10% SDS-PAGE (under reducing conditions and stained with Coommassie Brilliant blue) of proteins eluted at different stages of purification of *Hibiscus acetosella* Protease Inhibitor (HAPI). Lane 1. BSA, Lane 2. Crude seed extract of *Hibiscus acetosella*(50µg), Lane 3. 50% ammonium sulphate fraction (30 µg), Lane 4. Elute from Source Q column (10 µg), Lane 5. Purified inhibitor from trypsin affinity column (10µg) and Lane 6. Protein marker.

4.4.2 Native PAGE (Alkaline PAGE)

Native PAGE was done to know the mobility of the purified inhibitor under non-denaturing conditions, in its native state. The inhibitor did not move in Acid PAGE (Data not shown) and moved only in Alkaline PAGE. In Alkaline PAGE the inhibitor moved below the band of BSA

Fig 4.6 Alkaline PAGE of the inhibitor purified from the seed extract of *Hibiscus acetosella*

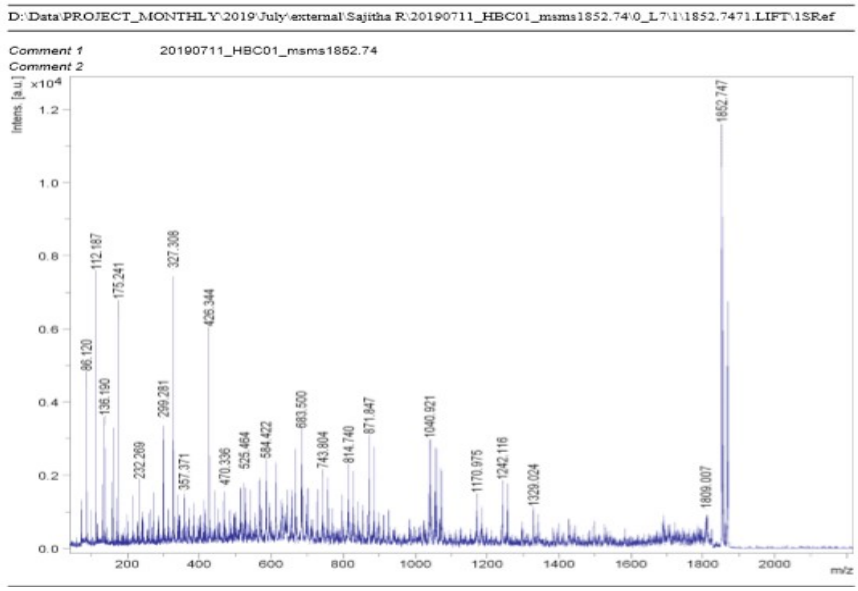


Alkaline PAGE was done with 7.5% separating gel and fixed and stained. Lane 1.BSA, Lane 2. Purified inhibitor, Lane 3 and 4. Dye alone and Lane 5. 50% ammonium sulphate fraction of *Hibiscus acetosella* seed extract.

4.4.3 Identification of the inhibitor protein by mass spectrometry

MALDI TOF/TOF analysis of SDS-PAGE separated protein revealed that it is a new protein as the data from mass spectrometry does not match with data from any other reported proteins in the data base. Figure 4.8 (a), Figure 4.8 (b) and Figure 4.8 (c) show the MALDI TOF/TOF analysis spectrum of the purified inhibitor and Table 4.9 shows the mass list of the peptides from the purified inhibitor.

Fig 4.7 a. Spectrum obtained from MALDI TOF analysis of the purified inhibitor



MALDI TOF spectrum obtained from the peptides of the purified inhibitor

Acquisition Parameter

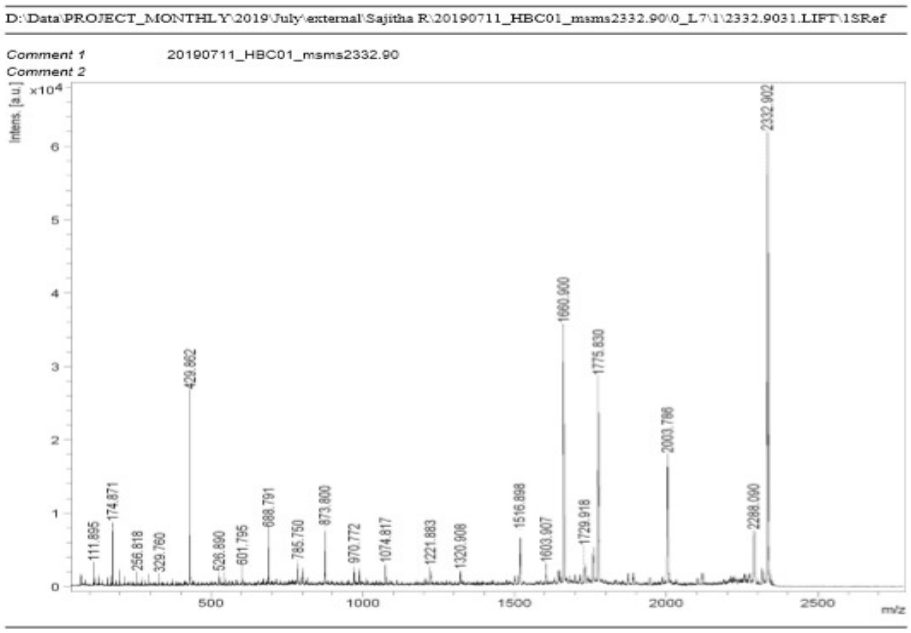
Date of acquisition	2019-07-11T16:09:41.531+05:30
Acquisition method name	D:\Methods\flexControlMethods\Specification\LIFT_service_3.fti
Aquisition operation mode	Reflector
Voltage polarity	POS
Number of shots	4500
Name of spectrum used for calibration	
Calibration reference list used	

Instrument Info

User	BDALDE
Instrument	FLEX-PC
Instrument type	ultraflexTOF/TOF

Bruker Daltonics flexAnalysis printed: 7/11/2019 5:49:02 PM

Fig 4.7 b. Spectrum obtained from MALDI TOF analysis of the purified inhibitor



MALDI TOF spectrum obtained from the peptides of the purified inhibitor

Acquisition Parameter

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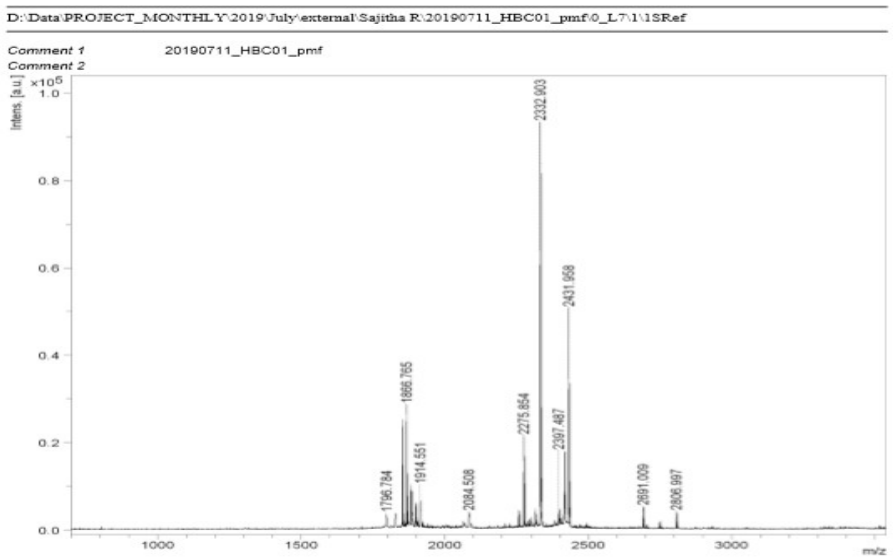
Number of shots	4500
Name of spectrum used for calibration	
Calibration reference list used	

Instrument Info

User	BDALDE
Instrument	FLEX-PC
Instrument type	ultraflexTOF/TOF

Bruker Daltonics flexAnalysis printed: 7/11/2019 5:56:12 PM

Fig 4.7 c. Spectrum obtained from MALDI TOF analysis of the purified inhibitor



MALDI TOF spectrum obtained from the peptides of the purified inhibitor

Acquisition Parameter

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Acquisition method name	D:\Methods\flexControlMethods\Specification\RP_700-3500_Da.par
Acquisition operation mode	Reflector
Voltage polarity	POS
Number of shots	2000
Name of spectrum used for calibration	
Calibration reference list used	

Instrument Info

User	BDALDE
Instrument	FLEX-PC
Instrument type	ultraflexTOF/TOF

Bruker Daltonics flexAnalysis printed: 7/11/2019 5:49:28 PM

Table 4.8 Mass list of peptides obtained from MALDI TOF/TOF **analysis of the purified inhibitor**

m/z	time	Intens.	SN	Quality Fac.	Res.	Area	Rel. Intens.	FWHM	Chi^2	Bk. Peak
1796.784	66892.79	1808.974	9.675	3608.282	900.384	9690.907	0.028	1.996	44220.023	0
1827.092	67450.45	2536.266	12.368	2444.556	1468.019	9837.011	0.040	1.245	127023.417	0
1852.747	67918.91	23229.942	88.943	30653.375	15936.775	8540.419	0.363	0.116	1002637.924	0
1866.765	68173.49	26460.286	100.581	17110.081	15462.139	10222.435	0.414	0.121	2804928.351	0
1869.739	68227.39	5272.518	18.388	1722.579	13450.620	2088.884	0.082	0.139	421234.804	0
1882.749	68462.62	5964.846	21.063	1443.799	8910.001	3645.973	0.093	0.211	1352003.079	0
1884.734	68498.44	4096.987	13.816	803.562	6993.634	3042.367	0.064	0.269	807839.894	0
1885.254	68507.82	3380.781	11.138	452.870	3072.412	5542.188	0.053	0.614	532150.423	0
1898.750	68750.82	3889.332	13.037	1302.429	10274.448	1980.602	0.061	0.185	399957.373	0
1900.720	68786.21	2534.463	7.762	179.781	5685.265	2133.023	0.040	0.334	368675.895	0
1914.551	69034.21	3339.468	16.555	2833.504	1249.255	15261.036	0.052	1.533	214658.921	0
2084.508	72011.36	2225.538	10.347	2845.085	1494.022	9410.777	0.035	1.395	88809.498	0
2257.836	74924.69	3907.302	14.561	2089.643	15487.058	1681.519	0.061	0.146	284113.212	0
2275.854	75221.01	15795.360	65.792	11747.582	14982.969	7806.627	0.247	0.152	1794752.397	0
2278.845	75270.09	2528.073	8.631	733.758	21841.249	684.394	0.040	0.104	469661.422	0
2315.876	75875.03	3003.436	10.985	1389.419	9588.935	2002.846	0.047	0.242	189482.329	0
2332.903	76151.56	63909.756	286.144	22868.646	12237.848	41125.063	1.000	0.191	21487722.086	0
2334.889	76183.75	23246.611	102.562	5431.262	20974.867	8333.375	0.364	0.111	7746699.574	0
2397.487	77191.37	2311.115	8.406	186.400	3945.943	3622.303	0.036	0.608	643749.587	0
2403.242	77283.34	2410.338	9.586	301.008	2731.322	5660.354	0.038	0.880	613664.977	0
2415.790	77483.50	3133.655	12.472	244.567	10604.460	2356.987	0.049	0.228	3662530.158	0
2417.589	77512.15	11860.188	54.606	14400.416	4850.855	19367.735	0.186	0.498	1857646.117	0
2431.958	77740.62	34632.280	165.667	26019.918	12454.449	23086.703	0.542	0.195	5941452.305	0
2691.009	81749.51	3885.580	21.857	8139.755	18040.945	2019.509	0.061	0.149	129868.418	0
2748.012	82605.39	1369.876	7.416	1408.476	13232.097	892.723	0.021	0.208	29985.873	0
2806.997	83481.73	2885.816	17.989	9584.946	17241.863	1639.315	0.045	0.163	62567.678	0

Table 4.9 shows the mass list of peptides from the purified inhibitor. The peptide sequence analysis showed that none of the protein in the database matches with the peptide sequence

4.4.4 Checking the glycosylation status of the inhibitor

To check whether the purified protein is glycosylated or not, after running SDS-PAGE the gel was stained with periodic acid Schiff's reagent (PAS). Ovalbumin, a positive control (glycosylated protein) and BSA, a negative control (non glycosylated protein) were also run along with the purified inhibitor. The PAS staining of the SDS-PAGE separated inhibitor of HAPI revealed that it is a non-glycosylated protein (Figure 4.8).

Figure 4. 8 Periodic acid Schiff's staining (PAS staining) of the SDS- PAGE separated purified inhibitor)

10% SDS-PAGE (under reducing conditions and stained with Periodic Acid Schiff's reagent) Lane 1. Ovalbumin, Lane 2. 50% ammonium sulphate fraction, Lane 3. BSA and Lane 4. Purified inhibitor

4.4.5 Study of thermal stability of the purified inhibitor

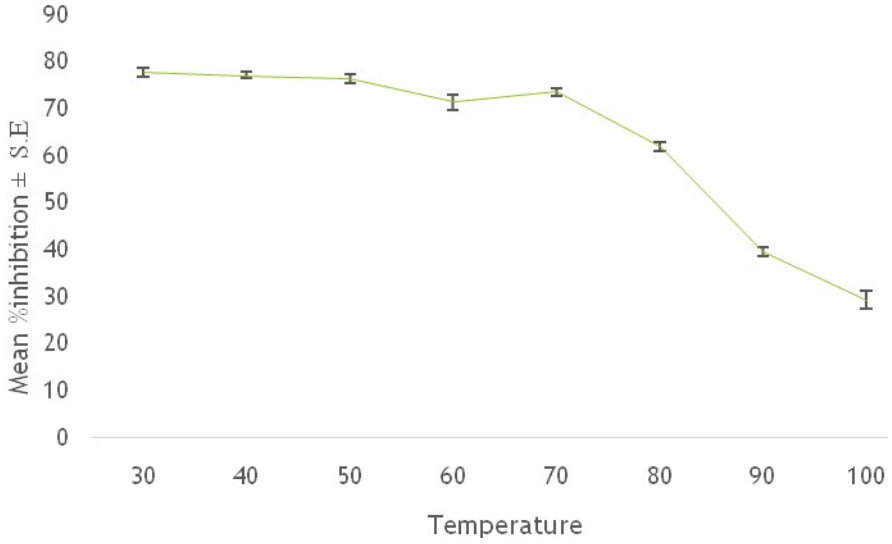
Effect of temperature on the protease inhibition of the purified inhibitor was evaluated by incubating the inhibitor at different temperatures ranging from 30 ºC to 100 ºC for 30 minutes. The residual protease inhibition was assessed using protease inhibition assay with gut enzyme of larval *S.litura* as the enzyme source (Table 4.10 and Figure 4.9)

Table 4.9 Effect of Temperature on the protease inhibitor against larval gut proteases of *Spodoptera litura*

Temperature (ºC)	Mean % inhibition ± S.E
30	77.70 ± 1.02
40	76.99 ± 0.68
50	76.38 ± 0.93
60	71.31 ± 1.63
70	73.55 ± 0.83
80	61.82 ± 0.94
90	39.52 ± 0.90
100	29.12 ± 1.93

Values are Mean ± Standard Error (n=6)

Figure 4.9: Effect of Temperature on the protease inhibitor activity against larval gut proteases of *Spodoptera litura*



Values are Mean ± Standard Error (n=6)

The inhibitor was found to be stable up to a temperature of 50 ºC and then declined in activity retaining an inhibition of 73.54±0.83 % even at 70 ºC. Thus the inhibitor is stable up to the temperature of 70 ºC.

4.4.6 Detrmination of pH optimum for of the purified inhibitor

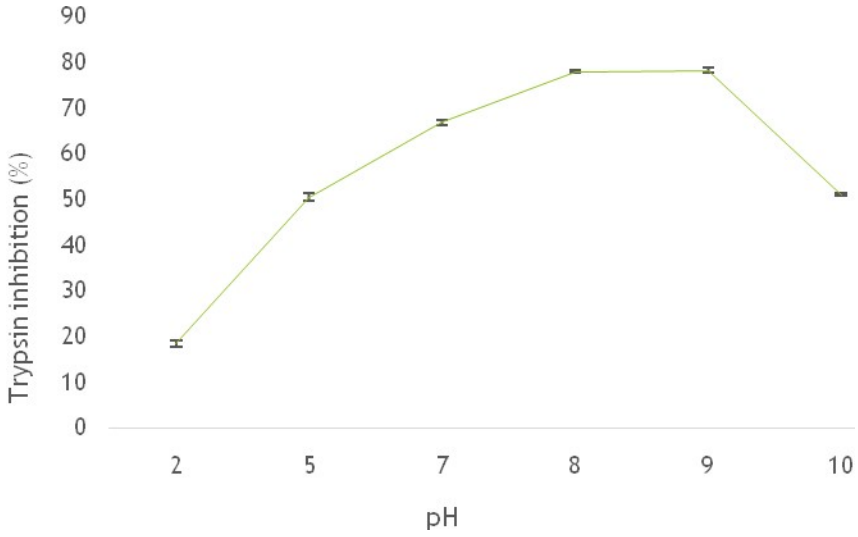
Optimum pH for the maximal protease inhibition of the purified inhibitor was determined by evaluating its inhibiton at different pH (2.0, 5.0, 7.0, 8.0, 9.0 and 10.0), with appropriate controls. The inhibition at different pH was represented in Table 4.11 and Figure 4.10

Table 4.10 Effect of pH on the inhibition of the purified inhibitor against the larval gut proteases of *Spodoptera litura*

pH	Mean % inhibition ± S.E
2.0	18.32 ± 0.63
5.0	50.46 ± 0.89
7.0	66.84 ± 0.55
8.0	77.97 ± 0.20
9.0	78.20 ± 0.62
10.0	51.06 ± 0.34

Values are Mean ± Standard Error (n=6)

Figure 4.10 Effect of pH on the inhibition of the purified inhibitor against the larval gut proteases of *Spodoptera litura*



Values are Mean ± Standard Error (n=6)

The inhibitor maximally inhibits the gut proteases of *S.litura* in the pH range 8.0-9.0. As the pH of gut of *S.litura* is around pH 9.0, the inhibitor will work in the gut of *S.litura*. From Figure 4.10 it is clear that the inhibition by the purified inhibitor was less above and below pH range 8.0- 9.0.

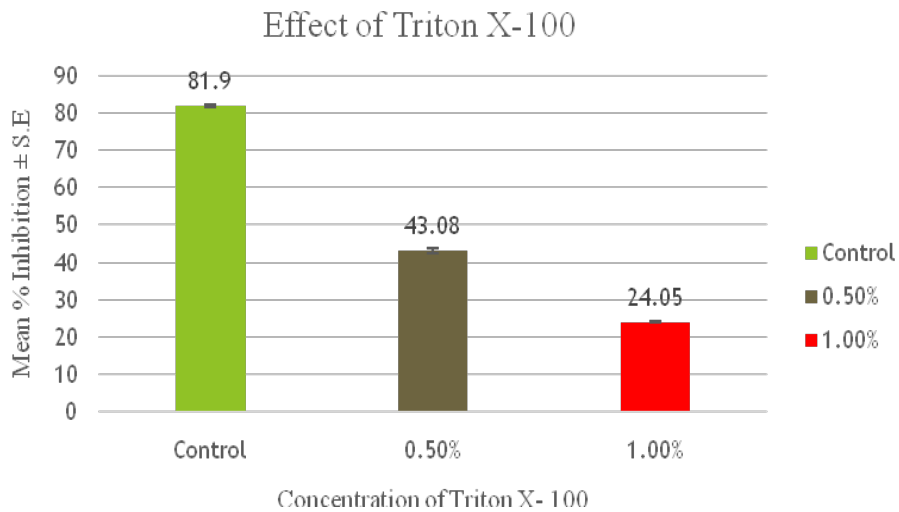
4.4.7 Effect of detergents, reducing and oxidizing agents on the protease inhibition by the purified inhibitor

To study the effect of detergents on protease inhibition of the purified inhibitor, SDS and Triton X-100 were used. For studying the effect of reducing and oxidizing agents on the purified inhibitor, β-mercaptoethanol and DMSO was used.

4.4.7.1 Effect of Triton X 100

The inhibitor was incubated with 0.5 and 1% w/v Triton X-100 for 30 minutes, later it was dialyzed against bicarbonate buffer pH 9.0 and the residual protease inhibition against trypsin was assessed. Appropriate controls were also maintained

Figure 4.11 Effect of Triton X-100 on protease inhibition activity of the purified inhibitor



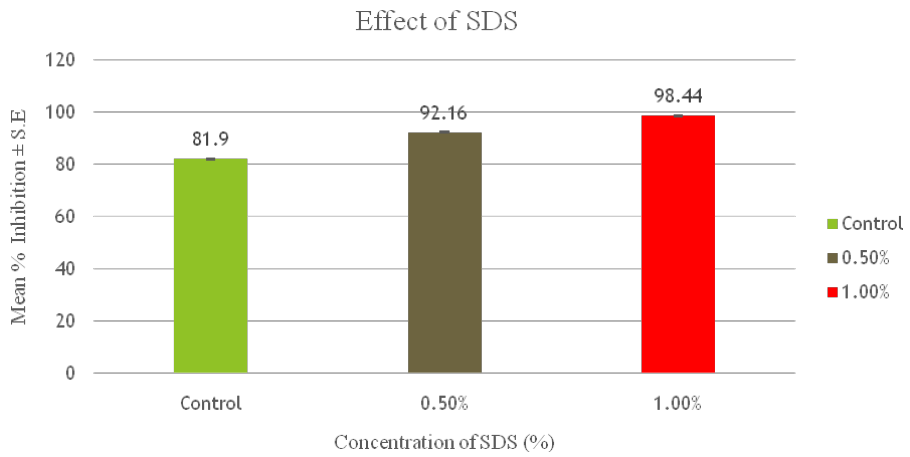
Values are Mean ± Standard Error (n=6)

Triton X-100 negatively affected the protease inhibition (PI) activity, where the residual inhibitory activities were decreased to 53% and 30% up on treating with 0.5 and 1% Triton X-100 respectively, compared to control (Figure 4.11)

4.4.7.2 Effect of SDS

The inhibitor was incubated for 30 minutes with 0.5 and 1% w/v SDS, later it was dialyzed against bicarbonate buffer pH 9.0. The residual protease inhibition was assessed using protease inhibition assay against trypsin. Appropriate controls were also maintained.

Figure 4.12 Effect of SDS on the protease inhibition activity of the purified inhibitor



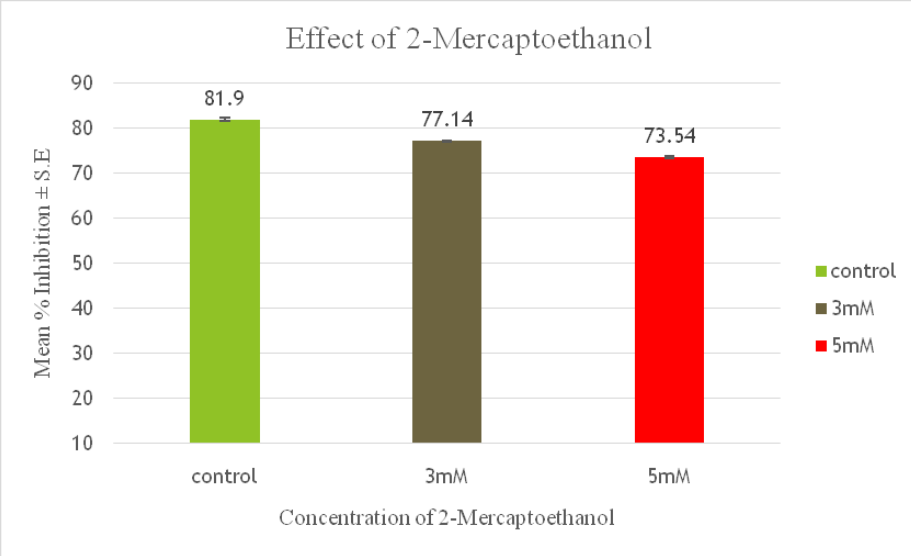
Values are Mean ± Standard Error (n=6)

Figure 4.11 shows that the protease inhibition activity of purified inhibitor increased 15% and 23% on treating with 0.5 and 1% SDS respectively compared to the control.

4.4.7.3 Effect of β mercaptoethanol

The effect of reducing agent β-mercaptoethanol was analyzed by incubation of the inhibitor with 3 and 5 mM of β-mercaptoethanol for 30 minutes and assessing the residual protease inhibition.

Figure 4.13 Effect of β- mercaptoethanol on the protease inhibition activity of the purified inhibitor



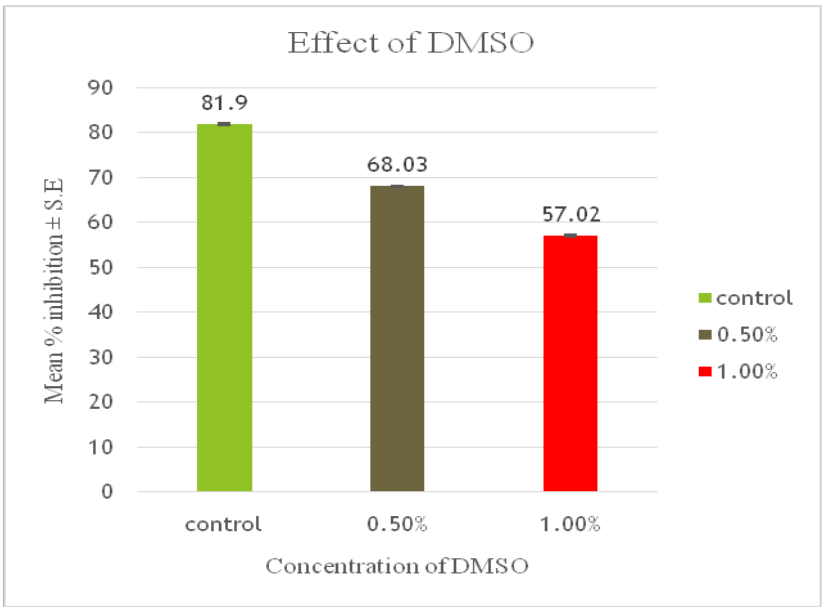
Values are Mean ± Standard Error (n=6)

There was only a slight decrease in inhibition on treatment with the reducing agent β-mercaptoetnanol (β- ME). The PI activity was reduced only 3 % and 5% on treating with 3mM and 5mM β-ME respectively compared to control (Figure 4.13).

4.4.7.4 Effect of Dimethyl sulphoxide (DMSO)

The inhibitor was incubated with 0.5 and 1% (v/v) Dimethyl sulphoxide (DMSO) for 30 minutes and the residual protease inhibition against trypsin was assessed.

Figure 4.14: Effect of DMSO on the protease inhibition activity of the purified inhibitor



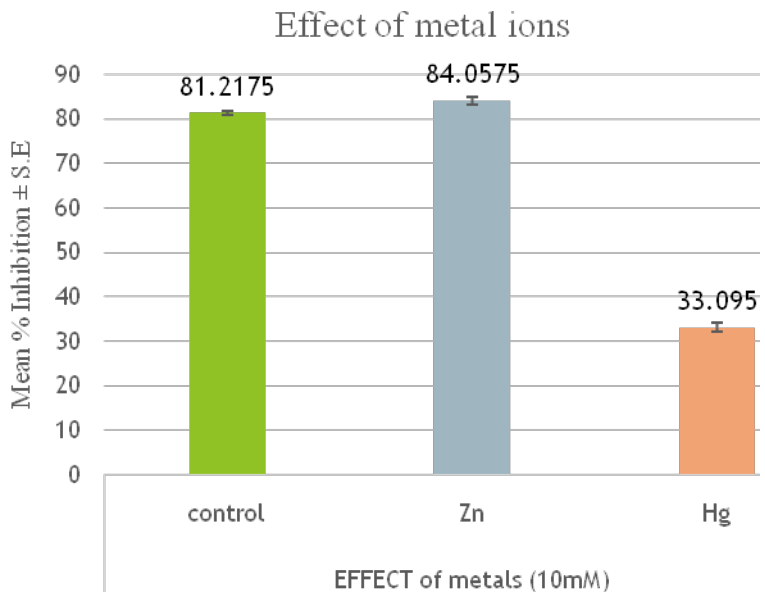
Values are Mean ± Standard Error (n=6)

The effect of oxidising agent, DMSO, on the PI activity was revealed from Figure 4.14 .It was found that 16 % reduction in activity of the purified inhibitor was found up on treating with 1mM DMSO and 29% reduction on treatment with 5mM of DMSO

4.4.8 Effect of metal ions on protease inhibition activity of the purified inhibitor

The metal ions selected for this are Zn^{+} and Hg^{2+}. The inhibitor was incubated with 10 mM of each metal ion for 30 minutes. The residual protease inhibition was assessed after the incubation (Figure 4.15)

Fig 4.15: Effect of Metal ions on the protease inhibition activity of the purified inhibitor



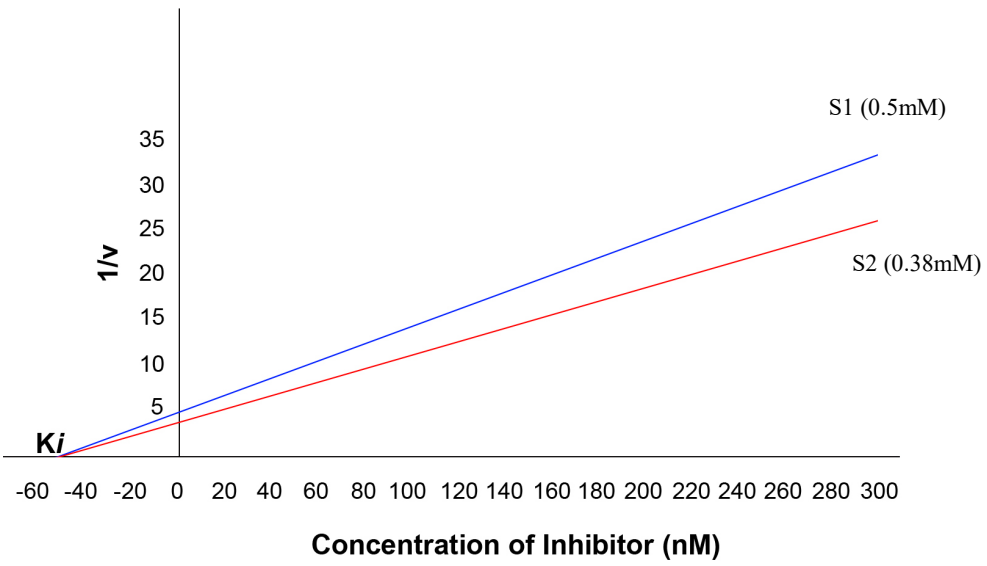
Values are Mean ± Standard Error (n=6)

The activity of protease inhibitor increased from 81.21 ± 0.52 % to 84.05 ± 0.88 % on treatment with Zn^{2+} ions and activity of the inhibitor was reduced to 33.09 ± 0.91 % up on treatment with Hg^{2+} ions.

4.4.9 Determination of Ki value and nature of inhibition

For the determination of Ki value different inhibitor concentrations were tested with two different substrate (BAPNA) concentrations using trypsin as the enzyme. The reciprocal of velocity (1/v) *versus* concentration of protease inhibitor for each substrate concentration was plotted and Ki was calculated from the interception of the two lines and the mechanism of inhibition was also inferred from the plot.

Fig 4.16: Dixon's plot



The Ki value calculated from Dixon's plot is found to be 52 nM for the inhibitor for trypsin. From the graph the nature of inhibition was found to be non-competitive.

4.4.10 *In vivo* effect of the purified inhibitor on fourth instar larvae of *Spodoptera litura*

The fourth instar larvae were fed with 1.16 µg of the purified inhibitor twice in a day by applying on to the leaves of *Ricinus communis* and dried before feeding. The experiment was carried out till the larvae entered into prepupal stage/ until all the test larvae were dead/ adult emerged, whichever is the earlier. Effect of the purified inhibitor on the larval weight gain (Table 4.12 and Figure 4.17) and survival rate (Table 4.13) was noted.

4.4.10.1 Effect of purified inhibitor on larval weight gain

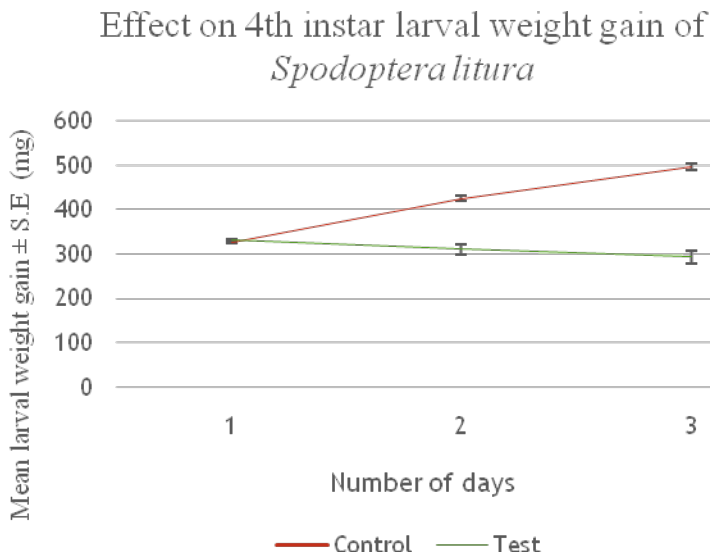
Table 4.11 Effect of purified inhibitor on larval weight gain

4th instar larvae (larval days)	**Control Mean weight±SE(mg)**	**Test Mean weight±SE(mg)**
1st **day**	326.55±2.10	331.45±2.53
2nd **day**	425.5±6.21	310.10±12.50*
3rd **day**	496.5±6.40	292.95±14.91*

Values are Mean ± Standard Error n=20(done in triplicates)
*p<0.0001

A significant reduction in larval weight gain was noted in test compared to control

Fig 4.17: Effect of the purified inhibitor on larval weight gain



Values are Mean ± Standard Error (n=6)

In the test, when the larvae fed with purified inhibitor (2.32 µg) exhibited drastic reduction in the larval weight gain compared to control. In the control after 3 days of feeding experiment the larvae attained 496.5±6.40 mg weight, whereas in the test the larvae attained only 292.95±14.91 mg weight. This reduction in weight may be the consequence of anti- metabolic effect of the inhibitor on the digestive physiology of *S.litura* larvae exerted by inhibiting the gut enzymes.

4.4.10.2 Effect of purified inhibitor on survivial rate of *Spodoptra litura*

The feeding experiment was continued till the prepupal stage/ larval death whichever is the earliest. Survival rate of the larvae, pupae

and adult forms of *S.litura* was recorded and compared with the control (Table 4.13).

Table 4.12 Survival rate of *Spodoptera litura* at different stages upon feeding with the purified inhibitor

Mean percentage death between 4th - 6th instar larvae ±S.E		**Mean percentage death at Pre pupa and pupa stages ±S.E**		**Mean percentage adult emergence ±S.E**	
Control	Test	Control	Test	Control	Test
Nil	11.06±5.26	1.08±3.21	83.21±5.38	97.85±3.48	5.5±8.97

Values are Mean ± Standard Error
n=20(done in triplicates)

A total of 11% of the larvae were dead in different larval instars and 83% of mortality was recorded in prepupal and pupal stages of *S.litura*. Only 5.5% of the larvae emerged as adults from the test where as in the control 97% the larvae emerged as adults.

DISCUSSION

5. DISCUSSION

5.1 SCREENING OF PLANT EXTRACTS FOR IDENTIFYING PROTEASE INHIBITORS AGAINST LARVAL GUT PROTEASE OF *SPODOPTERA LITURA*

In this study we screened 120 plants for checking the presence of protease inhibitors against the larval gut protease of *Spodoptera litura*. Out of the 120 plants screened 52 plants showed inhibition above 40% against the 5th instar larval gut protease of *S.litura.* The highest inhibition (88.6±1.19%) was shown by the seed extract of *Ardisia solanaceae* Roxb. and *Hibiscus acetosella* which inhibited the larval gut protease activity of *S.litura* to the extent of 88.61±0.40%. Among the 52 plants with inhibition above 40%, 11 plant extracts were reported for the first time to contain protease inhibitor. To the best of our knowledge there is no report of protease inhibitors from *Ardisia solanaceae* Roxb*, Calopogonium mucunoides* Desv, *Acacia concinna* D C*, Syzygium samarangenese , Myristica fragrans* Houtt*, Mimosa diplotrica, Mimusops elengi* L. *, Piper nigrum* L.*, Amaranthus dubius* Mart ex Thell, *Prunus cerasus* L. and *Abutilon indicum* L.

The highest inhibition is given by the seed extract of *Ardisia solanaceae* (88.60±0.50 %). Pratap Chandran reported that methanolic and aqueous extracts of *A.solanaceae* leaves showed potent ability to chelate iron (II) ions in a dose-dependent manner (Pratap Chandran et al., 2013). The iron (II) chelating activity of the leaf extract is highly significant as the transition metal ions contribute to the oxidative

damage in neurodegenerative disorders, like Alzheimer's disease and Parkinson's disease (Bush, 2003). The alcoholic leaf extract of *Ardisia solanaceae* has been reported to possess hepatoprotective activity which was confirmed by the prevention of prolongation in pentobarbital sleeping time by this extract due to CCl_4 induction (Kumar, 2014). It has been reported to have antioxidant, thrombolytic and cytotoxic activities. But the active compounds responsible for these activities are yet to be discovered (Amin et al., 2015).

Calapogonium mucunoides seed extract inhibited the gut protease activity of *S.litura* to the extent of 85.55±0.13%. Ndemangou *et al*., reported that ten isoflavonoids isolated from different parts of this plant exhibited urease inhibitory activity which directs its application in the treatment of stomach ulcers after *invivo* studies (Ndemangou et al., 2012). This result was further confirmed by the study of Osmund, where a substantial gastric cytoprotection was noted when the ethanolic leaf extract of *C.mucunoides* was administered orally to wistar rats with ethanol induced ulcer (Osmund et al., 2014). Leaves of *C.mucunoides* were used for treating diarrhea and leaf decoction is used as an anti-scorbutic drink. It was also reported to be used in eradicating measles and chicken pox when mixed with palm oil (Borokini and Omotayo, 2012).

The seed extract of *Acacia concinna* inhibited the larval gut protease activity to an extent of 73.84±0.57%. The pods of this plant are used traditionally for many skin diseases, cough, as a laxative and for dandruff treatment. The bark contains high levels of Saponins, which are foaming agents that are found in several other plant species

(Segelman and Famsworth 1969). The saponin of the bark has spermicidal activity against human semen (Gupta et al., 2013).

The larval gut protease activity of *S. litura* was inhibited to an extent of 67.03±1.02 by the seed extract of *Syzygium samarangenese* Gurib- Fakim reported that the leaves, fruits, bark and roots of *Syzigium samarangenese* were used to treat many diseases like diabetes mellitus, bronchitis, asthma and inflammatory disorders (Gurib- Fakim, 1991). The ethanolic leaf extract of *S. samarangenese* showed higher amount of flavonoids and proanthocyanidin (Majumdar et al., 2017), which impart the antifungal, antiviral, antitumour, antibacterial, antiallergic, anti- inflammatory properties along with scavenging free radicals and reactive oxygen species (Ozgen et al., 2010).

Myristica fragrans seed extract inhibited the larval gut proteases of *S. litura* to 65.12±1.01 Jaiswal *et al.,* reported that the dried seed of this plant is of most importance both in terms of commercial as well as pharmacological uses (Jaiswal et al*.,* 2009).The seed extract exhibits antimicrobial (Takikawa et al., 2002*;*Rani and Khullar, 2004; Mahadey et al., 2005), anti-depressant (Dhingra and Sharma, 2006), memory enhancing (Parle et al., 2004), Hepatoprotective (Morita et al., 2003) and pesticidal activity (Jung et al., 2007; Jaiswal and Singh, 2009). But the compound responsible for the insecticidal activity is unknown.

The larval gut protease activity of *S.litura* was inhibited by the seed extracts from *Mimosa diplotrica* up to 62.83±1.06%. Uyi et al.,

reported that the aqueous root extract (10% w/v) of *Mimosa diplotrica* repelled 100% of *Macrotermes* species by using filter paper impregnation technique and exhibited 100% mortality after 36 hours of exposure period (Uyi et al., 2018). A multifunctional water- soluble polysaccharide isolated from the seeds of *Mimosa diplotrica* showed multifunctional antioxidant activity and was found to retain moisture effectively in comparison with glycerol and hyaluronic acid (Rana et al., 2013).

Seed extract of *Mimusops elengi* was found to inhibit the larvla gut protease activity of *S. litura* to an extent of 57.95±1.23%. Bark of this plant is used to treat gum and teeth diseases (Basavaraj et al., 2010), the flowers cures liver disorders, headache and smoked flower is good for treating asthma(Manjeshwar et al., 2011) and the seeds cure nasal congestion and headache (Bharat et al., 2010).

Piper nigrum seed extracts inhibited the gut protease activity of *S.litura* larvae to an extent of 57.95±1.23%. It is traditionally used as a medicine for cough and cold. Piperine the 8/582563..bioactive compound in pepper is reported to have immune-modulatory, an/ti-carcinogenic, anti-inflammatory and hepatoprotective effect (Darshan et al., 2004).

The seed extracts of *Amaranthus dubius* was found to inhibit the larval gut protease of *S. litura* up to 48.88±0.311%. The leaves and stem of this plant is widely used as food. It is also used to treat constipation, anemia, kidney problems, stomach ache (Patel, 2013), fever and haemorrhage (Schippers, 2002)

The larval gut extract of *S. litura* was inhibited by the seed extract of *Prunus cerasus* to an extent of 47.46 ± 1.14%. The seeds of this plant are known its immune modulatory, antioxidant, anti-diabetic and anti-inflammatory activities and also it is reported to enhance sleep (Imtyaz et al., 2016).

Abutilon indicum which inhibited the*S.litura* larval gut protease activity to the extent of 43.16±0.28 has many medicinal uses. This plant is mainly used in Siddha medicines.It is used as a laxative, aphrodisiac, diuretic, demulcent. The roots are used for leprosy treatment, leaves as sedative and for piles and flowers are used to increase semen (Reyad-ul- ferdous, 2015).

Presence of protease inhibitors were reported from the plant extracts of *Eleusine coracana* (Shivaraj and Pattabhiraman, 1981), *Coccinia grandis* (Pramanik et al., 2019), *Mucuna pruriens* (Borde et al*., 2012), Celosia cristata and Linum usilatissimum (Rosu ana* et al*.,* 2010; Lorenc et al., 2001). Though the protease inhibitors were reported from these plants, there are no reports of protease inhibition against insect gut enzymes from these plants.

Hibiscus acetosella Welw. ex Hiern is an annual to a perennial shrubby plant which appear to be annual in extreme dry and cold seasons. This plant belongs to the Malvaceae family. It is commonly known as “African rosemallow”, “Cranberry hibiscus”, “Pulivenda”. It is native to South Africa and is consumed as green vegetables by peoples in sub Saharan Africa, Latin America, Asia and Western Europe (Tsumbu et al., 2012). In South India it is found in

Muzhappilangadi beach Kannur district and Tiruchirapalli district of Tamil Nadu. The leaves are added to salads or used in soups and stews.

H. acetosella leaves contain major compounds like polyphenols, caffeoyl-hydroxycitric acid and neochlorogenic acid which prevented Reactive Oxygen Species production in cellular models and on myeloperoxidase (MPO) which is involved in inflammation(Kapepula et al., 2017). The leaf extract of *H. acetosella* were confirmed for its anti-radical and anti-inflammatory,response of neutrophils (Tsumbu et al., 2012; Brain *et* al., 2014;Thungmungmee et al.,2015). The flower extract of this plant is reported to contain phenolic and flavonoid content with antioxidant capacity. Thus the flower extract was concluded to serve as a potential source of natural bioactive compounds, and also can have applications in skin-care products, natural coloring agent and dietary supplements (Thungmungmee et al., 2019)

Among the plant extracts with greater than 40% inhibition, without any reports of presence of protease inhibitor and proteinaceous nature of inhibitor was found in *Hibiscus acetosella* seed extract. The seed extract of *Hibiscus acetosella* inhibited the larval gut protease activity of *S.litura* to the extent of 88.61±0.40%. Thus *Hibiscus acetosella* Welw. ex Hiern seeds were selected for the isolation of protease inhibitors against larval gut proteases of *Spodoptera litura*.

5.2 PURIFICATION OF PROTEASE INHIBITOR FROM *HIBISCUS ACETOSELLA*

Hibiscus acetosella which inhibited the larval gut protease of *Spodoptera litura* to the extent of 88.61±0.40 was selected for the purification of the protease inhibitor for the following reasons. In some plants like *Syzygium samarangenese* (61.72± 1.80%) and *Prunus cerasus* (41.45±0.59%) the protease inhibitor is non proteinaceous in nature even though there is no report on the protease inhibitors from these plants. Purification of plant protease inhibitors which are proteinaceous in nature has an advantage over non proteinaceous protease inhibitors where the PI gene can be cloned and expressed in the host plants which will be a feasible strategy of insect pest management.

Ammonium sulfate fractionation was the first step done towards the purification of the protease inhibitor from the seeds of *Hibiscus acetosella*. This was done to remove the non-protein components in the seed extract and to enhance the proteinaceous protease inhibitor in the fractions precipitated. From the seed extract of *H.acetosella*, proteins were precipitated using three different concentrations of ammonium sulfate (0–30%, 30-50% and 50-70%). Among them, highest inhibition (84.43±0.62) was observed for 30-50% fraction followed by 50-70% fraction, (28.11±0.45) and last by 0-30 % fraction (20.14±0.50). Similarly, in the case of purification of protease inhibitors from *Tamarindus indica* (Tamarind trypsin inhibitor, TTI) (Araujo et al., 2005), *Butea monosperma* (*Butea monosperma* Protease Inhibitor, BmPI) (Jamal et al., 2014), *Allium*

sativum (*Allium sativum* Protease Inhibitor, ASPI) (Shamsi *et al*., 2016) and *Ciser arietinum* (*Ciser arietinum* Trypsin inhibitor, CATIN) (Karthik *et al*., 2019), 30-60% ammonium sulfate fractions showed highest inhibition against trypsin compared to other fractions. There was 3.5 fold purification of the inhibitor protein on protein basis in 30-50% ammonium sulfate fraction compared to the crude extract. Similar fold purification was obtained (4.18 fold) for the ammonium sulfate fraction with highest inhibition (30-50% fraction) while purifying the protease inhibitor from *Acacia nilotica* (Babu et al., 2012).

Ammonium sulfate fraction (30-50%) containing 48.35 mg of protein was loaded on to Source Q cation exchange column equilibrated with 25mM Tris buffer pH 8.1. Two peaks were eluted during the elution, of which the first peak contained the protease inhibitor. First peak was eluted at 250 mM- 290 mM NaCl. In the purification of trypsin inhibitor from Sapindus Trifoliatus also, the inhibitor was eluted with the 0.3 M NaCl during the ion exchange chromatography (Gandreddi et al., 2015).

For further purification, ion exchange fractions with high inhibition were pooled and concentrated in amicon UF-3kDa membrane and loaded (4.455 µg protein) on to the trypsin affinity column (5 ml**)**.The protease inhibitor was eluted out and a single band corresponding to 20 kDa was obtained was obtained with this step. It is reported that in many cases Trypsin affinity purification method is enough for obtaining pure protein (Mello et al., 2001; Rai et al., 2008).

Fifty percentage enzyme activity of the larval gut protease of *S.litura* was inhibited by the purified inhibitor at a protein concentration of 0.012µg/µl, whereas the crude extract required for 50% inhibition of the gut enzyme activity was 7.438µg/µl. After ammonium sulfate precipitation there was a 3.5 fold purification of the inhibitor compared to the crude extract.The fold of purification of protease inhibitor obtained for ion exchange chromatography was 35.7 fold purification compared to ammonium sulfate fraction. The purified inhibitor from trypsin affinity achieved 600 fold more purification than that of the crude extract. The yield of the purified inhibitor was 2.2 µg of inhibitor /g of seed.

The specific inhibitory activity of the purified inhibitor is 5690 U/mg.There was an increase in specific inhibitory activity of the inhibitor from 3608 U/ mg (Crude) to 56897 U/ mg (Purified Inhibitor).

5.3 CHARACTERIZATION OF *HIBISCUS ACETOSELLA* PROTEASE INHIBITOR

5.3.1 Determination subunit molecular weight

The sub unit molecular weight of the purified inhibitor determined from SDS-PAGE was found to be 20 kDa. The native protein on Alkaline PAGE moved below BSA which indicates that the inhibitor might be a monomer in the native state. The protein did not move in Acid PAGE (Data not shown). Most of the PIs reported from plants are small molecules with relative molecular masses ranging between 5-25 kDa (Singh and Rao, 2002). Serine protease inhibitors

fall under two families Kunitz type and Bowman- Birk type. These two families of serine protease inhibitors differ in their molecular weight, number of reactive sites and cysteine content (Richardson, 1997). Kunitz type inhibitors have low cysteine content , single reactive site and molecular weight ~20 kDa, whereas Bowman-Birk type inhibitors have high cysteine content, two reactive sites and have molecular weight ranging from 8kDa- 10kDa (Richardson, 1991).

A 20 kDa serine protease inhibitor which inhibited the trypsin activity with a Ki $5.3x10^{-10}$ M was reported from the seeds of *Diomorphandra mollis* (Macedo et al., 2000). A Kunitz proteinase inhibitor with molecular weight 20 kDa was reported from *Archidendron ellipticum* seeds.This inhibitor was found to inhibit the 5^{th} instar larval gut protease activity of *Spodoptera litura* (Bhattacharya et al., 2006). Shah *et al.,* reported a 20 kDa serine protease inhibitor from *Solanum tuberosum*, having hemagglutination activity (Shah *et al.,* 2016).Another Kunitz type protease inhibitor Okra protease inhibitor (OPI), purified from the seeds of *Abelmoschus esculentus* is also having a molecular weight of 21 kDa (Datta et al., 2019). Thus molecular weight of most of the Kunitz type protease inhibitors is ~20 kDa. From this the protease inhibitor purified from *H.acetosella* may be grouped into Kunitz- type serine protease inhibitor.

5.3.2 Identification of the inhibitor protein by mass spectrometry

Protein sequence analysis was done by mass spectrometry (MALDI TOF/TOF). Data of the peptides generated from the protein

did not match with peptides from other proteins in the database indicating that this is a new protein. The purified protease inhibitor from *Hibiscus acetosella* was named as *Hibiscus acetosella* Protease Inhibitor (HAPI)

5.3.3 Checking the glycosylation status of the inhibitor

The glycosylation status of the inhibitor was checked by running SDS-PAGE and followed by staining the gel with periodic acid Schiff's reagent (PAS). Ovalbumin and BSA, were used as positive (glycosylated protein) and negative control (non-glycosylated protein) respectively while running SDS-PAGE. The PAS staining revealed that the inhibitor (HAPI) is a non- glycosylated protein. Type-2 cystatins which inhibit C 1 proteases are generally non-glycosylated with the exception of human cystatins F and E/M which are glycosylated proteins. Human cystatins S, SA and SN present in saliva, tears,urine, muscle, liver and seminal plasma are all non-glycosylated proteins (Dickinson, 2002).

5.3.4 Study of thermal stability of the inhibitor

Thermal stability of the inhibitor was determined by incubating the inhibitor at different temperatures for 30 minutes followed by checking the residual protease inhibition. The inhibitor was found to be stable up to a temperature of 50 ºC and then declined in activity retaining an inhibition of 73.54±0.83 % even at 70 ºC. Thus the inhibitor is stable up to the temperature of 70 ºC. After 70 ºC there was a gradual decline in the protease inhibition activity and retains 29.12 ± 1.93% inhibition at 100 ºC. Most of the PIs isolated from plants are

heat stable (Saini, 1989, Godbole et al., 1994). The stability at high temperature may be attributed by its rigid structure stabilized by disulphide linkages as it was suggested in the case of PI isolated from pea seeds (Li de la Sierra et al., 1999).

A protease inhibitor, BTCI (Black eyed pea Trypsin/ Chymotrypsin inhibitor) with a molecular weight of 9 kDa, purified from *Vinga unguiculata* was found to be stable even at 75 ºC with the loss of only 20% of its antitryptic activity. This protease inhibitor was concluded to be a Bowman-Birk type serine protease inhibitor as it can withstand harsh temperature and pH conditions (Silva et al*.,* 2001). A 67 kDa protease inhibitor purified from *Cucumis trigonus* Roxburghi was reported to retain 90% of its trypsin inhibition activity even after incubating at 65 ºC (Ullah et al., 2006). Rufino et al., reported that a 21 kDa trypsin inhibitor purified from *Pithecellobium dulce* (Manila tamarind) seeds retained about 90% of its PI activity between the temperature range 37 ºC -100 ºC (Rufino et al., 2013).

5.3.5 Determination of pH optimum for the purified inhibitor

The pH optimum required for the maximum inhibition of the gut protease activity by the purified inhibitor was determined by checking the inhibition at different pH (2.0, 5.0, 7.0, 8.0, 9.0, and 10.0). The inhibitor maximally inhibits gut proteases of *S.litura* in the pH range 8.0-9.0. The gut pH of most lepidopteran pests falls in the alkaline pH range (Johnston et al., 1991). As the pH of gut of *S.litura* is around pH 9.0, the inhibitor will work well in the gut of *S.litura*.

Similar results were accounted by Godbole et al., where the trypsin inhibitor from the seeds of pigeon pea maintained its complete activity between pH 7.0- pH 10.0, but when subjected to acidic pH (pH 3.0- 5.0) most of its PI activity was lost (Godbole et al., 1994). A protease inhibitor isolated from seeds of *Madhuca indica*, MiTI exhibited a stable PI activity predominantly in alkaline conditions pH 6.0-pH 11.0 and also inhibited the midgut proteases of *Helicoverpa armigera* (Jamal et al., 2014). Jamal et al., accounted another protease inhibitor from the seeds of *Butea monosperma*, BmPI which shows trypsin inhibitory activity over a broad pH range (pH 4.0-pH 10.0). This inhibitor was recorded to cause 90% reduction in midgut protease activity of *Helicoverpa armigera* larvae (Jamal et al., 2015).

5.3.6 Effect of detergents, reducing and oxidizing agents on the protease inhibition by the purified inhibitor

To check the effect of detergents Triton X-100 and SDS were used. The effect of reducing agent β- mercaptoethanol and oxidizing agent DMSO on the protease inhibition activity of the purified inhibitor was checked.

Detergents are commonly used to solubilize proteins from any biological molecules or lipid membranes or to maintain the protein solubility in the solution (Meenu Krishnan and Murugan, 2015).

5.3.6.1 Effect of Triton X-100

The inhibitor was incubated with 0.5 and 1% w/v Triton X-100 for 30 minutes, later it was dialyzed against bicarbonate buffer pH 9.0.

The residual protease inhibition activity was assessed using protease inhibition assay. Appropriate controls were also maintained. Triton X-100 negatively affected the protease inhibition (PI) activity, where the residual inhibitory activities were decreased to 53% and 30% on treatment with 0.5 and 1% Triton X-100 respectively, compared to control. This reduction in PI activity may be due to many factors like conformational changes with a moderately high α helix content, with most of the hydrophobic residues associated with the detergents or linking of detergents with specific binding points of native proteins or cooperative association of protein with the detergents without major conformational changes by affecting the major amino acids required for inhibiting the enzyme activity (Choi et al., 2005).

Similar results were recorded by Meenu Krishnan and Murugan, where the trypsin inhibition activity of the purified protease inhibitor from the fruits of *Solanum acculeatissimum* Jacq were reduced to 55% upon treatment with 0.5% w/v of Triton X 100 (Meenu Krishnan and Murugan, 2015). Shamsi et al., reported that the residual protease inhibition activity of the protease inhibitor purified from *Allium sativum* (garlic), *Allium sativum* Protease inhibitor (ASPI), was decreased to ~ 58% on treating with 0.5% w/v of Triton X 100 (Shamsi et al., 2016). Another protease inhibitor *Rhamnus frangula* Protease Inhibitor (RfIPI) from *Rhamnus frangula* was found to lose 45% of its PI activity on treating with Triton X 100 compared to control. This reduction in the PI activity was concluded as a result of reduction in hydrophobic interactions (Bacha et al., 2017).

5.3.6.2 Effect of SDS

The inhibitor was incubated for 30 minutes with 0.5 and 1% w/v SDS, later it was dialyzed against bicarbonate buffer pH 9.0. The residual protease inhibition activity was assessed using protease inhibition assay. Appropriate controls were maintained. It was noted that the protease inhibition activity of purified inhibitor increased 15% and 23% on treating with 0.5 and 1% SDS respectively compared to the control.

Shamsi et al., reported that the PI activity of *Allium sativum* Protease inhibitor (ASPI) was enhanced to ~143% in the presence of 1% SDS compared to control (Shamsi et al., 2016). In the case of a protease inhibitor from *Rhamnus frangula*, RflPI the effect of SDS increased the PI activity to 70% compared to control, which was concluded to be due to the stabilizing acitivity of SDS (Bacha et al., 2017).

5.3.6.3 Effect of β-mercaptoethanol

The effect of reducing agent β-mercaptoethanol was analyzed by incubating inhibitor with 3 and 5mmol of β-mercaptoethanol for 30 minutes. The residual protease inhibition activity was assessed using protease inhibition assay. Appropiate control experiments were also carried out. The PI activity of the purified inhibitor reduced only 3 % and 5% on treating with 3mM and 5mM β-ME respectively compared to control.

Similar results were observed by Shamsi et al., where the residual protease activity of the inhibitor, *Allium sativum* Protease inhibitor (ASPI), was barely affected by incubating with β-mercaptoethanol (1.0-3.0 mM) even after three hours (Shamsi et al., 2016). In contrast to this Bijina *et al*., reported that the residual protease activity of protease inhibitor from *Moringa oleifera* was enhanced to 50% upon treating with β-mercaptoethanol (Bijina et al., 2011). Similarly Meenu Krishnan and Murugan noted an increase in the PI activity of *Solanum aculeatissimum* Protease Inhibitor (SAPI) with 1mM and 2mM β-mercaptoethanol to 110% and 119% respectively. Beyond this concentration β-mercaptoethanol reduced the PI activity which was concluded as it may be due to the distortion of the intramolecular disulphide bridges in the reactive site loops which normally ensure functional stability of the inhibitor (Meenu Krishnan and Murugan, 2015).

5.3.6.4 Effect of Dimethyl sulphoxide

To check the effect of oxidizing agent on the activity of inhibitor, it was incubated with 0.5 and 1% (v/v) Dimethyl sulphoxide (DMSO) for 30 minutes. Controls were also maintained. Protease inhibition assay was carried out to check the residual protease inhibition. It was found that 16 % reduction in activity of the purified inhibitor was found up on treating with 0.5% DMSO and 29% reduction on treatment with 1% of DMSO.

Similar results were observed in the case of *Allium sativum* Protease Inhibitor (ASPI), where the PI activity was reduced to ~ 21%

with 5% DMSO (Shamsi et al., 2016). A concentration dependent decrease in the antitryptic activity (33% and 7%) was noticed in *Solanum aculeatissimum* Protease Inhibitor (SAPI) upon treatment 0.5% and 4% DMSO (Meenu Krishnan and Murugan, 2015). But in the case of protease inhibitor from *Phaseolus vulgaris*, when it was incubated with 1% and 4% DMSO, a significant stability was observed in PI activity of the inhibitor, retaining 90% and 53% of its inhibitory activity (Puntambekar and Dake, 2017).

5.3.7 Effect of metal ions on protease inhibition activity of the purified inhibitor

The metal ions selected for this are Zn^{2+} and Hg^{2+}. The inhibitor was incubated with 10 mM of each metal ion for 30 minutes. The residual protease inhibition was assessed after incubation. The activity of protease inhibitor increased from 81.21 ± 0.52 % to 84.05 ± 0.88 % on treatment with Zn^{2+} ions. But activity of the inhibitor was reduced to 33.09 ± 0.91 % up on treatment with Hg^{2+} ions. The metal ions like Mg^{2+} and Zn^{2+} were known to maintain the secondary and tertiary structure of cysteine PIs. However, a decrease in the PI activity may be caused by the involvement of carboxylates of glutamate and aspartate amino acids in the binding of bivalent cations to metalloproteins (Greenwood et al., 2002; Jack et al., 2004).

Similar results were observed by Puntambekar and Dake, where the protease inhibition activity of the inhibitor from *Phaseolus vulgaris* was marginally increased upon treatment with 10 mmol Zn^{2+} (Puntambekar and Dake, 2017). Bacha et al., accounted that there was

a significant increase in the PI activity of *Rhamnus frangula* protease inhibitor (RfIPI) when incubated with Zn^{2+} and Hg^{2+} also. This increase in the PI activity was concluded to be due to the binding of metal ions to the PIs to stabilize its spatial conformation (Bacha et al., 2017). The PI activity of the protease inhibitor from *Moringa oleifera* leaves were markedly enhanced on treating with Zn^{2+} and Hg^{2+} (Bijina et al., 2011).

5.3.8 Determination of Ki value and nature of inhibition

For the determination of Ki value different inhibitor concentrations were tested with two different substrate (BAPNA) concentrations using trypsin as the enzyme. The reciprocal of velocity (1/v) *versus* concentration of protease inhibitor for each substrate concentration was plotted and Ki was calculated from the intersection of the two lines and the mechanism of inhibition was also inferred from the plot. The Ki value calculated from Dixon's plot was found to be 52 nM for the inhibitor for trypsin. Low Ki value of HAPI indicates that it exhibits high affinity towards trypsin and this was in compliance with other Kunitz-type PIs having antitryptic activity. Thus it further confirms that the inhibitor may be a Kunitz type serine protease inhibitor (Bhat and Pattabiraman, 1989). From the graph the nature of inhibition was found to be non-competitive. Most of the protease inhibitors exhibit non competitive type inhibition kinetics (Vogel et al., 1968).

The kinetic study of the Kunitz type inhibitor from *Allium sativum*, ASPI, revealed that it is a non competitive inhibitor with low Ki value of 0.12µM (Shamsi et al., 2016). Similar results of protease

inhibitor with non competitive mode of inhibition and low Ki value (0.02 µM) was recorded by Karthik et al., in the protease inhibitor purified from *Ciser arietinum* Trypsin inhibitor (CATIN) (Karthik *et al*., 2019). The trypsin inhibitors purified from *Jatropha curcas* L. (Costa *et al*., 2014), *Coccinia grandis* L.(Satheesh and Murugan, 2011) and *Erythrina velutina* (Machado et al., 2013) also have non competitive mode of inhibition kinetics.

5.4 *IN VIVO* EFFECT OF THE PURIFIED INHIBITOR ON FOURTH INSTAR LARVAE OF *SPODOPTERA LITURA*

The fourth instar larvae were fed with 1.16 µg of the purified inhibitor twice in a day by applying on to the leaves of *Ricinus communis* and dried before feeding. The experiment was carried out till the larvae entered into prepupal stage/ until all the test larvae were dead/ adult emerged, whichever is the earlier. Effect of the purified inhibitor on the larval weight gain and survival rate was recorded. A significant reduction in larval weight gain was noted in test compared to control. In the test, when the larvae fed with purified inhibitor (2.32 µg) exhibited drastic reduction in the larval weight gain compared to control. In the control after 3 days of feeding experiment the larvae attained 496.5±6.40 mg weight, whereas in the test the larvae attained only 292.95±14.91 mg weight. This reduction in weight may be the consequence of anti- metabolic effect of the inhibitor on the digestive physiology of the larvae exerted by inhibiting the gut enzymes, which directly affects the utilization of amino acids essential for its growth and development (Telang et al., 2003). In the feeding experiment a total of 11% of the larvae were dead in different larval instars and 83%

of mortality was recorded in prepupal and pupal stages of *S.litura*. Only 5.5% of the larvae emerged as adults from the test where as in the control 97% the larvae emerged as adults.

Similar results were reported in the case of SBTI (soybean trypsin inhibitor), where the larvae maintained on diet with SBTI gained significantly lower weight compared to the control group (McManus and Burgess, 1995). Same results were noted by the effect of the purified PIs from *Eugenia jambolana trypsin inhibitor* (EjTI) (Singh et al., 2014), *Madhuca indica* Trypsin inhibitor (MiTI) (Jamal et al., 2014) and by another Kunitz type inhibitor Tamarind trypsin inhibitor (TTI) (Pandey and Jamal, 2014) on the growth and development of *H.armigera.*

SUMMARY

6. SUMMARY

Spodoptera litura Fabricius (Lepidoptera: Noctuidae) commonly called Tobacco cutworm, Cotton leaf worm, or Tropical army worm, is a polyphagus pest which has a wide host range of more than 150 host species. Protease inhibitors (PIs) in plants are known to be part of defense that plants utilize to counteract the adverse effects from herbivore and pathogen attack

In the present study 120 plants were screened for identifying plants containing protease inhibitors. Out of this 52 plants showed inhibition above 40% against 5th instar larval gut proteases of *Spodoptera litura*. Among the 52 plants 11 plant extracts were reported for the first time to contain protease inhibitor. Though rest of the plants was reported to contain protease inhibitor, but there is no report on their effect on insect pests including *S.litura*. Fifteen plant extracts were subjected to Proteinase K treatment which revealed that the inhibitor from 12 plant extracts were proteinaceous in nature and that from the remaining 3 plant extracts were of non-proteinaceous in nature. *Hibiscus acetosella* was selected for further purification of protease inhibitor as no protease inhibitors were reported from it. The inhibitor (HAPI) was purified from *H.acetosella* by ammonium sulfate fractionation, ion exchange chromatography and trypsin affinity chromatography.The inhibitor is a non-glycosylated protein with a subunit molecular weight of 20 kDa and in native state it moves in Alkaline PAGE but not in Acid PAGE.MALDI TOF TOF analysis of HAPI revealed that the inhibitor is a novel plant protease inhibitor.

Stability studies of HAPI revealed that. Maximum inhibition (78.20±0.20%) was between pH 8.0-pH 9.0. The inhibitor was stable up to a temperature of 50ºC. Presence of detergent, Triton X 100, reduced the inhibition, whereas SDS increased the inhibition.There was a slight decrease in inhibition on treatment with the reducing agent β-mercaptoetnanol.The oxidizing agent DMSO decreased the inhibition.The activity of HAPI was increased from 81.21% to 84.05% on treatment with Zn^{2+} ions and activity of the inhibitor was reduced to 33.09% up on treatment with Hg^{2+} ions.The Ki value calculated from the Dixon's plot is found to be 52nM for trypsin. The nature of inhibition was found to be non-competitive for trypsin.The inhibitor inhibited the gut protease activity of *S.litura* to the extent of 84.61±0.97%. The purification of HAPI was 600 fold more than that of crude extract with a yield of 2.2 µg/g seed. *In vivo* effect of HAPI on *S.litura* was found to result in 11% of the larval death in different larval instars and 83% of mortality in prepupal and pupal stages of *S.litura*. Only 5.5% of the larvae emerged as adults from the test whereas in the control 97% the larvae emerged as adults. Thus the inhibitor purified in this study from *Hibiscus acettocella* (HAPI) has the potential to use in pest control of *S.litura* and other pests where the predominant gut protease is trypsin-like serine peroteases.

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