IDENTIFICATION, PURIFICATION AND CHARACTERIZATION OF PLANT PROTEASE INHIBITORS AGAINST LARVAL GUT PROTEASES OF *SPODOPTERA MAURITIA* (BOISDUVAL) (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

REMYA P.P.

DEPARTMENT OF ZOOLOGY
UNIVERSITY OF CALICUT
2019

CERTIFICATE

This is to certify that the thesis entitled "**IDENTIFICATION, PURIFICATION AND CHARACTERIZATION OF PLANT PROTEASE INHIBITORS AGAINST LARVAL GUT PROTEASES OF *SPODOPTERA MAURITIA* (BOISDUAL) (LEPIDOPTERA: NOCTUIDAE)**", is an authentic record of research work carried out by **Mrs. Remya, P.P.**, 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.

C. U. Campus
Date: 02-12-2019

Dr. Kannan V.M.
(Supervising teacher)

Dr. KANNAN V.M., Ph.D.
PROFESSOR
DEPARTMENT OF ZOOLOGY
UNIVERSITY OF CALICUT
KERALA 673635, INDIA

CERTIFICATE

This is to certify that all corrections and suggestions from the adjudicators have been incorporated in the thesis entitled **"IDENTIFICATION, PURIFICATION AND CHARACTERIZATION OF PLANT PROTEASE INHIBITORS AGAINST LARVAL GUT PROTEASES OF *SPODOPTERA MAURITIA* (BOISDUVAL) (LEPIDOPTERA: NOCTUIDAE)"** submitted by Remya, P.P, Department of Zoology, University of Calicut.

Dr.Kannan.V.M, (Ph.D Supervisor)

Professor, Department of Zoology,

University of Calicut.

Dr. KANNAN V.M., Ph.D.
PROFESSOR
DEPARTMENT OF ZOOLOGY
UNIVERSITY OF CALICUT
KERALA 673635, INDIA

DECLARATION

I, REMYA,P.P., do hereby declare that the present work in this thesis entitled "**IDENTIFICATION, PURIFICATION AND CHARACTERIZATION OF PLANT PROTEASE INHIBITORS AGAINST LARVAL GUT PROTEASES OF *SPODOPTERA MAURITIA* (BOISDUAL) (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.

Calicut University,
Date: 02-12-2019

Remya, P.P.

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Remya, P.P.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
2-ME	2-mercarptoethanol
Acid PAGE	Acid Polyacrylamide Gel Electrophoresis
BAPNA	Nα-benzoyl-DL-arginine-4-nitroanilide hydrochloride
BSA	Bovine Serum Albumin
CNBr	Cyanogen bromide
Da	Dalton
DEAE	Diethylaminoethyl
g	Gram
Ha	Hectares
hrs	Hours
kDa	Kilo Dalton
M	Molar
mg	Milligram
ml	Millilitre
mM	Milli Molar
Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium bicarbonate
Ni –IMAC	Nickel Immobilized Metal Affinity Chromatography

PBS 7.4	Phosphate-Buffered Saline, pH 7.4
PIs	Protease Inhibitors
PPIs	Plant Protease Inhibitors
SDS-PAGE	Sodium Dodecyl Sulphate-Poly Acrylmaide Gel Electrophoresis
SE	Standard Error
SpPI	*Spatholobus parviflorus* Protease Inhibitor
TBS 8.0	20 mM Tris buffer pH 8.0
TEMED	N,N,N',N'- tetramethyl ethylene diamine
Tris	Tris(hydroxymethyl)aminomethane
µg	Microgram
µL	Microlitre

INTRODUCTION

The world population will be reaching approximately 8 billion by the year 2025 (U.S. Census Bureau, 2018). For feeding the world population by the year 2050, we need to increase our overall food production at least by 70%. In developing countries like India, the annual increase in population is more than that of the annual increase in food production. To meet the increasing demand we should increase the current food production.

Insects are the largest class of living creatures in the phylum Arthropoda and are among the most diverse group of animals on earth. They represent more than half of all known living organisms and existed over a million years prior to the emergence of human population. Insects are adapted to live in almost all types of environment. They play a significant role in the ecosystem, and some of them are beneficial with many different roles including that in pollination while some others are classified as pests.

Agriculture is the lynchpin of the Indian economy, and it contributes to a significant share in Gross Domestic Product (GDP). But a number of pests and pathogens, which include insects, fungi, bacteria, viruses, weeds, birds, rodents etc., cause considerable yield loss. Among the insects a large number of insect species are destructive and adapted to feed on a wide variety of plants and infest animals, thereby causing considerable loss in agricultural production leading to severe loss to our economy, and adversely affect public

health. According to Atwal and Singh, any animal species would attain the status of a pest when its population exceeds a certain level of economic injury to its host, and when its existence conflicts with the welfare, convenience and profit of man (Atwal and Singh, 2014).

Rice is the foremost source of nourishment for the world population and world paddy production in 2017 is 759.6 million tonnes (FAO, April 2018). In Asia the estimate of rice production in 2017 is 686.7 million tonnes and in India it is estimated as 166.5 million tonnes during the same period. (FAO, April 2018). Final estimates of paddy cultivation area and production in Kerala during 2017-2018 is 58278 Ha and 148913 tonnes respectively (Directorate of Economics and Statistics, Kerala, 2017-18). In the last few decades rice cultivation is falling at an alarming rate (Mukesh, 2015). Among the different causes of reduction in rice cultivation in Kerala include changes in the climatic condition, lower profitability of paddy cultivation, shifting of paddy cultivation with other crops etc. (Mukesh, 2015), and crop loss due to pest infestation.

Among the different pest of paddy, *Spodoptera mauritia* (Boisd.) (Lepidoptera: Noctuidae) remains one of the most serious pest of paddy sometimes causing considerable damage to the crops. It is a sporadic pest and is distributed in many parts of the world. In India it is found in all the rice growing areas especially along the west coast and delta in Kerala and Tamil Nadu (David and Ananthakrishnan, 2004). It has attained the status of a major pest of rice in Eastern India, especially in Orissa, Chattisgarh, Jharkhand and Bihar (David and Ananthakrishanan, 2004; Tanwar *et al*., 2010).

There were recent reports of armyworm attack in Kerala, one at Thrissur, around 125.45 Ha of paddy field were destroyed (Muringatheri, 2016) and the other from Kuttanad paddy field affecting about 4500 Ha of paddy field (Pillai, 2017). Control measures against *S. mauritia* include chemical methods such spraying pesticides, biological control methods using parasitoids such as tachinids and by cultural methods like flooding the nursery. Majority of the pesticides are not specific and they also affect non-target organisms. Most of them are not easily degradable and the residues persist in soil leading to accumulation through food chain and through drinking water to humans posing substantial health hazard. Intensive application of pesticide contaminates the whole ecosystem and leads to the loss of biodiversity. Thus there is a need for developing more ecofriendly pest management systems.

Protease inhibitors (PIs) inhibit proteases and are effective in preventing the growth of larvae of many pests by inhibiting their gut protease activity. They have been isolated and characterized from many organisms, including plants, animals and microbes (Valueva and Mosolov, 2004). Plants are good sources of protease inhibitors, which are defensive proteins and protect plants from diseases, pests and herbivores. Plant protease inhibitors are generally small proteins, mostly occurring in storage tissues, such as tubers and seeds, and also found in the aerial parts of plants (Leo *et al*., 2002). They are also induced in response to injury or attack by insects or pathogens (Ryan, 1990). Plant protease inhibitors act as anti-metabolic proteins, which interferes with the digestive process of phytophagous insects by

inhibiting the proteases present in the gut, causing a significant reduction in the availability of amino acids necessary for their growth and development (Lawrence and Koundal, 2002). Thus there is growing interest to exploit PPIs for management of insect pests of agricultural importance. But development of resistance by insects towards PPIs is an impediment in achieving the goal. Thus it is important to identify better plant protease inhibitors to overcome the resistance. In this study we screened plant extracts to identify extracts containing protease inhibitors and purified and characterized plant protease inhibitor against larval gut proteases of *Spodoptera mauritia*.

The objectives of the study are:

1. To screen different plants to identify plant extracts containing protease inhibitors against gut proteases of *Spodoptera mauritia* larvae.

2. To isolate and purify plant protease inhibitor against larval gut proteases of *Spodoptera mauritia.*

3. To identify and characterize the isolated plant protease inhibitor.

REVIEW OF LITERATURE

2.1 AGRICULTURE

Agriculture plays an important role in the global economy as it is the heart of the economic system of many countries. In the countries like India, agriculture is of great importance as 54.6% of Indian population depends on agriculture and agro-industries for livelihood. Moreover, it contributes 17% to India's Gross Value added during 2015-2016 (Annual Report, Govt. of India, 2016-2017). Among different crops, rice is one of the most important and the first cultivated crop in Asia and consumed by majority of the Indian population and it provides instant energy.

Rice is the staple food grain crop in many states in India including Kerala. In Kerala, Thrissur, Palakkad and Kuttanad are the main centres where large-scale cultivation of rice is done. Once paddy cultivation was a part of the proudness of Kerala, but now the area under rice cultivation has been continuously declining at a threatening rate over the years (Mukesh, 2015). One of the reasons for reduction in rice production is pest attack. Though there was a lot of improvement in the agriculture sector due to the green revolution, unforeseen problems are introduced. As a result of the green revolution, intensive rice monoculture systems were practiced and it promoted a favourable environment for the pest (Pingali and Garpacio, 1997). Rathee and Dalal reported that 15-25% yield losses in principal major food and economic crops were caused by insect pests (Rathee and Dalal, 2018).

2.2 INSECT PLANT INTERACTIONS

Arthropods are the most widespread and diverse groups under kingdom Animalia with a worldwide estimate of approximately 4-6 million species (Novotny *et al.,* 2002). Among them, insects are the earliest terrestrial herbivores and acted as major selection agents on plants. Insects were living together with plants for more than 350 million years and they co-evolved to avoid each other's defense mechanisms (War *et al.,* 2012). Insects play a major role in both aquatic and terrestrial ecosystems as herbivores, predators, saprophages, parasites and pollinators. There have been a number of studies to investigate insect-plant interactions. The coupled evolution of insects and plants is described in the book 'Insect-Plant interactions' (Bernays, 2018). Some interactions like pollination are beneficial to both plants and insects and in such cases, the co-evolution leads to the development of very specific mutualism.

2.3 INSECTS AS PESTS

Putman defined Pests as those species that cause significant economic damage that can be a major problem for agriculture (Putman, 1989). In farming, they are considered as pests if the damage they cause to crop or livestock is sufficient to reduce both the yield and quality of the harvested product by an extent that is undesirable to the farmer. According to Krattiger around 15% loss of the total production in the agricultural sector is due to the pre-harvest infestation by insect pests (Krattiger, 1997). Therefore, the management of insect pest is

crucial in sustained crop production and economic development of the country.

2.4 PESTS OF PADDY

It was reported in literature that around 800 species of insects are there in the paddy ecosystem and among them 100 species were considered as pests, however, only one by fourth of them are considered as deliberate pests (Pathak, 1968; Khush, 1997; Sogawa *et al.,* 2003; Pathak and Khan, 1994). In India almost 21-51% crop losses have been estimated to be due to the incidence of rice pests especially stem borers, gall midges, plant hoppers and sporadic pests. The abundance of the pest and pest attack vary from place to place and from year to year (Pasalu *et al.*, 2004).

2.4.1 *Spodoptera mauritia* as a pest

Among different pests of *Oriza sativa*, *Spodoptera mauritia* (Boisduval) (Lepidoptera: Noctuidae) is considered to be a sporadic pest which is commonly known as armyworm or rice swarming caterpillar. *Spodoptera mauritia* occasionally causes serious losses to rice crops. It is a seasonal pest, usually occurs on paddy field from July to September, and is widely recorded in Indian subcontinent, eastern and southern Asia and Australia. Larvae appear in large swarms of thousands and destroy the whole field of paddy and then march on to the next field.

2.4.2 Pest status of *Spodoptera mauritia*

Spodoptera mauritia is widely distributed in the East and southern Asia, Indian subcontinent and in the Australian Region. An outbreak of *Spodoptera mauritia* in Malaysian Borneo (Sarawak) in 1967 affected 25,000 acres of lowland and upland rice (Rothschild, 1969). It has been reported in Bangladesh as a serious rice pest throughout the country (Tanwar *et al.,* 2010). In South India, it is very common in all the rice flourishing tracts, specifically along the West Coast and Delta tracts. Earlier it was considered as a minor pest of rice, but for the last decade, it has emerged as a serious major pest of rice seedlings especially in Eastern India particularly in Jharkhand, Orissa, Bihar and Chattisgarh. The larvae of *S. mauritia* are nocturnal. It actively feeds on the leaf margins at night and leaves only the midribs, thus completely defoliates the rice plant as if grazed by cattle. The loss in paddy yield due to the larval outbreak ranges from 10 to 20%. During off season of rice cultivation, the larvae were able to migrate to alternate host plants, for example, *Ischaemum aristatum* and at the end of the off season, they make a full-scale comeback in the nursery stages of paddy.

2.4.3 Pest control strategies for *Spodoptera mauritia*

The infestation of *S. mauritia* starts at the onset of monsoon and larvae generally preferred on crops which are less than 20-25 days old (Tanwar *et al.,*2010). Larvae feed at night on the leaves and defoliate the plant completely.

During off-season, larvae survive on alternate host plant, *Ischaemum aristatum* and migrate in swarms of thousands to paddy field destroying the entire crop within a short period of time. Regular monitoring, mechanical and cultural means of pest control and use of pesticides and biological control are important components of the pest management.

2.4.3.1 Regular monitoring

Monitoring of the pest is essential for identifying its presence at the early stages of the crop cycle. It can be done by the middle of April as the pest appears in May. Moth population can be further confirmed by using insect light traps as the moths are attracted towards light. Presence of *S. mauritia* can also be monitored by digging the soil up to 6-9 inches where the larvae burrow for pupation (Tanwar, *et al.,* 2010).

2.4.3.2 Cultural and mechanical control:

This includes alternate crop rotation, deep ploughing of the field in summer to expose the larvae and pupae for predatory birds, removal of excess nurseries and weeds from the field, flooding the nurseries and small fields to bring out the larvae to the surface and can be collected by means of special woollen combs. Let a team of ducks graze into the paddy field. It will feed on the larvae and use of bamboo perches enhances predation by birds. In case of a severe outbreak, the spreading of larvae from one field to next can be prevented by digging a trench around the infested field wherever possible (Tanwar, *et al.,* 2010). Collection of larvae with hand net or sweeping basket and collection of adults by using the light trap also can be done. Larvae are

poor swimmers and in flooded fields, they are forced to stay on the crop which they defoliate. Application of kerosene in the stagnant water will also kill the larvae.

Spraying of following pesticide in the paddy during the evening at the early stage is effective. The chemical pesticides used per hectors are 2.5 litres of chlorpyriphos 20 EC, 2.0 litres quinalphos 25 EC, 1.0 litres of triazophos 40 EC and 600 ml of dichlorvos 76 SL.

2.4.3.3 Biological control

Biological control involves deploying the diverse natural enemies of the pest like predators, parasites and viruses.

a) Parasites

There are reports of many parasites parasitizing on different stages of *S. mauritia.* Two species of egg parasites were found parasitizing *S. mauritia* eggs. One species is the scelionid, *Telenomus nawai* Ashmead, which attacks *S. mauritia* is reported from the Aina Haina and Waialae-Kahala areas of Honolulu (Beardsley, 1955). A survey over a period of about one year showed that 60 to 95 per cent of the egg masses were parasitized by *T. nawai*. First reported braconid egg-larval parasite attacking *S. mauritia* in Hawaii was *Chelonus texanus* (Cresson) (Van, 1955). *Apanteles marginiventris* (Cresson) which appears to be the most important natural enemy of *S. mauritia* in Hawaii was also reported (Van, 1955). Three species of tachnid flies, *Chaetogaedia monticola* (Bigot), *Achaetoneura archippivora* (Williston) and *Eucelatoria armigera* (Coquillett) were found to be

parasitizing *S. mauritia* larvae in Hawaii (Beardsley, 1955; Tanada, 1957). A number of parasites have been recorded from *S. mauritia* around the world which include *Macrocentrus* species from Fiji (Lever, 1946), *Echthromorpha conopleura* Krieger from the pupae in Guam, (Swezey, 1946), *Chelonus carbonator* Marshal from India (Ballard, 1921).

b) Predators

Two species of minute ants were found to attack healthy eggs, they are *Monomorium floricola* Jordan and big-headed ant, *Pheidole megacephala* (Fabricius) (Tanada, 1957). The major vertebrate predators of *S. mauritia* found to predate on the larvae include *Corvus splendens* (common crow), *Corvus macrorhynchos* (jungle crow), *Bubulcus coromondus* (Eastern cattle egret), *Ardeola grayii* (paddy bird), *Amaurornis phoenicurus* (white- breasted water hen) and *Acrido therestristis* (common mynah) (Tanwar *et al.,* 2010).

c) Nuclear Polyhedrosis Virus (NPV)

A polyhedrosis virus, Nuclear Polyhedrosis Virus (NPV) attacking *S. mauritia* was first reported in Hawaii (Bianchi, 1944). If they infect in the early stage, the larvae die before the fourth instar and usually begin to turn pale 2 to 4 days prior to death and, at death, have a whitish or creamy appearance. After death larvae immediately darken in colour. Older instars gradually turn slightly pale with a pinkish tinge several days before death, but otherwise remain nearly as dark brown as the healthy larvae. In South India, a disease of this armyworm was considered as probably of bacterial origin, but the symptoms recorded

by them resemble some what those of the nuclear polyhedrosis (Anantanarayanan and Ramakrishna, 1937).

2.5 PLANT DEFENCE MECHANISMS

Under natural conditions, wild plants produce both physical and chemical factors for partial protection against different insect pests (Boulter, 1993), whereas in some plants the expression of the defensive molecules were induced in response to attack or injury by pests (Jamal *et al*., 2013).

Physical factors of plants play an important role in the defence mechanism against pests. Mechanical protection on the surface of plants like toughened leaves, hairs (trichomes), spines and thorns are examples for direct defence against herbivores. Plants produce a wide range of secondary metabolites which are toxic to pathogens and herbivores as a defensive mechanism against the pests. Resistance in plants against pest attack can be mediated by a wide range of metabolic products, including primary and secondary metabolites. Primary metabolites include protease inhibitors, amylase inhibitors, chitinase inhibitors, lectins etc, and secondary metabolites include alkaloids, tannins, rotenoides etc (Macedo *et al.*, 2003). Chemical factors for the defence include alkaloids, terpaniodes, phenols, anthocyanin, quinones, saponins tannins, repellents and protease inhibitors produced by the plants to kill or retard the growth of the herbivores (Hanley, 2007).

Many toxins are produced in plants for defence against insect attack. Vicilins, a 7 S storage globulin protein present in the legumes

was shown to be detrimental to *Callosobruchus maculatus* and, later Macedo *et al.,* purified it from the seeds of *Vigna unguiculata* (Macedo *et al*., 1993, Macedo *et al.,* 1995). A toxic protein, called Canatoxin is isolated from jackbean, *Canavalia ensiformis* is toxic for *Callosobruchus maculates* as well as it is lethal for insects displaying cathepsin based digestion (Carlini *et al.,* 1997). A glycoprotein called as zeatoxin, isolated from zea mays affect the development and survival of the cowpea weevil (Macedo *et al.,* 2000).

Recently plant protease inhibitors, PPIs exhibiting insecticidal effects also emerged as an interesting and powerful tool against predators and pathogens (Jouanin et al., 1998). Digestibility reducers or anti- metabolic proteins, the protease inhibitors produced by the plants against digestive proteases of herbivore enemies played a significant role in plant defence mechanisms (Arimura, 2009).

2.6 PROTEASES

Proteases are the hydrolytic enzymes that break the specific peptide bonds, which link aminoacids together in a polypeptide chain in the target protein. The term proteases is used to describe both endopeptidases and exopeptidases, whereas the term proteinases are used more specifically for endopeptidases (Ryan,1990).They are present in almost all forms of living organisms having simple role in lower organisms and more advanced and complex role in higher organisms. Protease play a pivotal role in various complex physiological and pathological processes such as release of hormones and pharmacologically active peptides from precursor proteins,

activation of zymogens, cell growth and migration, tissue arrangement, morphogenesis in development, protein catabolism, transport of secretory proteins across membranes etc. In general extra cellular proteases play a critical role in the catabolism of large proteins into smaller molecules for succeeding absorption by the cells whereas intra cellular proteases are essential for the regulation of metabolism (Dubey, 2010). Proteases are very essential for the normal body function of the organisms and they are ubiquitous in nature (Barrett *et al.,* 2001).

2.6.1 Classification of proteases and their functions

Based on the site of action, proteases are categorised into two major groups, endopeptidases and exopeptidases. Exopeptidases acts on the peptide bond proximal to the carboxy or amino termini of the substrate, while endopeptidases cleave the peptide bonds away from the termini of the substrate. Based on the functional group present at the active site, proteases are classified into four major groups, ie, Serine protease, Cysteine proteases, Metallo proteases and Aspartic proteases (Bode and Huber, 1992; Barrett *et al*., 2012; Hartley, 1960). There are some miscellaneous proteases which do not come under the standard classification, e.g., ATP dependent proteases which require ATP for its activity (Menon and Goldberg, 1987).

2.6.1.1 Serine proteases

Serine proteases constitute the largest group of proteases and are characterized by the presence of a serine residue in their active site. They are abundant in eukaryotes, prokaryotes, archaea and viruses.

Serine proteases are characterized by the presence of common conserved catalytic triad of the three amino acids, serine, aspartate and histidine (Carter and Wells, 1988; Antao and Malcata, 2005). One of the features of serine protease is the occurance of zymogens, the inactive precursor from. Serine proteases are grouped into 20 families based on their structural similarities. Out of which, subtilisin family is the second largest, initially isolated from *Bacillus subtilis* and this family is universally distributed among eubacteria, archae bacteria, eukaryotes and viruses (Antao and Malcata, 2005).

Serine proteases involved in many physiological processes like digestion (eg. trypsin and chymotrypsin), immune responses (eg. complement factors B, C and D etc), blood coagulation (eg. Factors VIIa, IXa, X and XIIa), reproduction (Eg. acrosin) and fibrinolysis (plasmin, kallikrein, urokinase and tissue plasminogen activator) (Polgar, 1989). Serine proteases are also of commercial importance as some are used in biopharmaceuticals and in detergents (Anwar and Saleemuddin, 2000).

2.6.1.2 Cysteine proteases:

Cysteine proteases are otherwise known as thiol proteases that are characterized by the presence of nucleophilic cysteine thiol group in a catalytic triad or dyad consisting of cysteine and histidine in the order (Cys-His or His-Cys) which differs among the families (Vicik *et al.,* 2006, Turk, 2006, Barrett, 1994). They are commonly found in fruits like papaya, pine apple, fig and kiwi fruit. Papain was the first cysteine proteases purified, characterised and structure to be solved

from *Carica papaya*, the papaya fruit (Sajid, 2002). Trypanopain and Cruzipain (Cruzain) are cysteine proteases isolated from *Trypanosoma brucei* and *Trypanosoma cruzi* respectively (Lonsdale-Eccles *et al.,* 1995, Cazzulo *et al.,* 1990). Cytoplasmic calpains and the lysosomal cathepsins are the most important cysteine proteases in mammals (Otto and Schirmeister, 1997).

Cysteine proteases play important roles in plants such as plant growth and development, senescence and programmed cell death, accumulation of storage proteins such as in seeds, and also in storage protein mobilization.

2.6.1.3 Metalloproteases

Metalloproteases are the protease enzyme whose catalytic mechanism involves a metal ion at their active site. Most of the metalloproteases require zinc, but some use cobalt as the metal ion. They plays crucial role in many biological processes which include cell proliferation, differentiation and remodelling of the extracellular matrix, vascularisation, cell migration etc. (Chang and Werb, 2001). Matrix metalloproteases (MMPs) and metalloprotease-disintegrins (a disintegrin and metalloproteases- ADAMs) are the two closely related metalloprotease families. Matrix metalloproteases (MMP) are the specific class of extracellular matrix degrading proteases under the family of zinc atom dependent endopeptidases (Chang and Werb, 2001). Metalloprotease-disintegrins are transmembrane proteins that contain disintegrin and metalloprotease domains, indicative of cell adhesion and protease activities.

2.6.1.4 Aspartyl Proteases

Aspartyl proteases are proteases which have aspartate residues in the active site and usually function at acidic pH. They have isoelectric points in the range of pH 3.0 to 4.5. Hence its function limits to some specific sites in organisms. Aspartyl proteases have been categorized into three families, pepsin (A1), retropepsin (A2), and enzymes from pararetroviruses (Barrett, 1995). Pepsin, gastricsin and chymosin from stomach, cathepsins D and E from lysosomes, renin from kidney, and secretory proteases from fungi ie, rhizopus pepsin, penicillo pepsin, and endothia pepsin are some of the aspartyl proteases (Tang and Wong, 1987).

2.7 ROLE OF PROTEASES IN INSECTS

Proteases play a vital role in insect growth, development and reproduction, inflammation, pro-enzyme activation and detoxification/ toxin activation (Terra *et al.,* 1996). They play a crucial role in insect physiology. The first insect protease purified and characterized was a serine protease known as Cocoonase from silk moths and it enables the adult moth to emerge (Kafatos *et al*., 1967a, 1967b). Indrasith *et al.,* purified a serine protease from the egg of *Bombyx mori* that degrade egg yolk protein, vitellin to release amino acids essential for the embryonic development (Indrasith *et al.,* 1988). A 350-kDa protein called nudel protein secreted by the ovarian follicles contains a serine protease domain (Hong and Hashimoto, 1995; Turcotte and Hashimoto, 2002). Mutations in this domain in the Nudel protein produces a dorsalizing phenotype in *Drosophila* and can also result in

fragile egg shells (Hong and Hashimoto, 1996, LeMosy *et al*., 1998, LeMosy and Hashimoto, 2000). A Cysteine protease which digests yolk proteins was identified from *Drosophila melanogaster* and *Musca domestica* (Medina *et al*., 1988; Ribolla and De, 1995).

Serine proteases present in the insect haemolymph control several defence responses in insects, including antimicrobial peptide synthesis and coagulation of haemolymph and melanization. Gorman and Paskewitz identified five serine proteases from the hemolymph of the mosquito, *Anopheles gambiae* which regulate defense responses in the mosquito (Gorman and Paskewitz, 2001). Melanization, a defensive or immune response to wounding or infection in insects involves a cascade of serine proteases (Kanost and Gorman, 2008).

Proteases also play a critical role in insect physiology and food digestion and they are of great interest as a target for insect pest management (Christeller *et al.,* 1992). In the alimentary canal of insects, these enzymes hydrolyse the dietary proteins and release the amino acids which are then absorbed by epithelial cells of the insect mid gut. Role of proteases in insect digestive systems is more targeted to insect pest control strategies. Digestive proteases are responsible for the complete hydrolysis of proteins into amino acids. Serine proteases like trypsin or trypsin-like proteases are a major component of the lepidopteran digestive system (Srinivasan *et al.,* 2006). The predominant protease in the gut of *S. mauritia* is found to be serine proteases (Kannan *et al.,* unpublished observation).

2.8 PROTEASE INHIBITORS

In biological systems the action of proteases are restraint or inactivated by distinct mechanisms like proteolytic degradation or binding with an inhibitor molecule. Since the peptide bond hydrolysis catalysed by proteases is irreversible, a comprehensive regulatory network of protease inhibitors has evolved to ensure the correct spatial and temporal regulation of their activity (Fear *et al.,* 2007). In addition to the regulatory role in proteolytic acitvities, protease inhibitors also have a protective role in tissues and body fluids from degradation by unwanted or foreign proteolytic activities (Neurath, 1984). The recently researchers are attracted to the investigation of PIs as they can be worthwhile weapons in biomedical, biochemical, pharmacological and agricultural applications (Umezawa, 1982; Ahn *et al.,* 2004; Imada, 2005; Copeland, 2005).

As early as 1947, Mickel and Standish, explored the inability of certain insect larvae to grow normally on soybean products paved way for unveiling the role of PPIs in plant protection (Mickel and Standish, 1947). Later Lipke *et al.,* reported that the soybean trypsin inhibitors were toxic to the larval flour beetle, *Tribolium confusum* (Lipke *et al*., 1954). Plants exhibit both physical and chemical defensive mechanisms against pests and pathogens, the latter includes protease inhibitors which may be proteinaceous or non-proteinaceous in nature (Polya**,** 2003).

Proteinacious protease inhibitors are the protease inhibitors which are proteins and they are widely distributed in plants, animals

and microorganisms. Protease inhibitors form very stable complexes with proteolytic enzymes either reversibly or irreversibly to control the proteolytic activity. Non-proteinaceous protesase inhibitors are also present in plants. A number of synthetic protease inhibitors are also available in the market. Synthetic metallo peptidase inhibitors include the metal chelators, 1, 10-phenathroline, ethylene glycol tetraacetic acid (EGTA), and ethylene diamine tetraacetic acid (EDTA). Benzamidene hydrochloride (BHC), phenyl methane sulfonyl fluoride (PMSF), diisopropyl fluoro phosphates (DFP) and 3, 4- di chloro isocoumarin (DCI) are synthetic serine protease inhibitors. Synthetic cysteine protease inhibitors include N-ethylmaleimide (NEM), iodoacetamide, p-chloromercuribenzoate (PCMB) and iodoacetate. These synthetic protein inhibitors are useful in studying the role of proteases or for preventing proteolytic digestion of proteins.

2.8.1 Classification of plant protease inhibitors

Protease inhibitors are class specific and their reactions are based on the active amino acids present in the reaction center of the proteases. PPIs are divided into four classes such as cysteine protease inhibitors, aspartyl protease inhibitors, metallo-protease inhibitors and serine protease inhibitors (Koiwa *et al.,* 1997). Out of these, large numbers of PPIs are directed towards serine and cysteine proteases (Barrett, 1987, Turk and Bode, 1991) and very few are known for aspartic and metallo-proteases (Jouanin *et al.,* 1998; Oliveira *et al.,* (2003).

2.8.1.1 Cysteine protease inhibitors

Cysteine protease inhibitors or cystatins are ubiquitous in nature and are present in microorganism, animal and plant species. They specifically inhibit the activity of cysteine proteases and papain (Oliveira *et al.,* 2003). Colella *et al*. first described cystatins in the egg white of chicken and was later called egg white cystatin (Colella *et al.*, 1989). Cysteine protease inhibitors are classified into four families based on the primary sequence similarities, molecular masses, number of disulfide bonds and subcellular localization.

Family-1 cystatins are commonly known as stefins family. They are single chain proteins with molecular mass of around 11 kDa, which lack disulfide bonds and carbohydrates with around 100 amino acid residues (Barrett, 1986). Family-2 cystatin, contains about 115 amino acid residues with two intra-chain disulfide bonds near the carboxyl-terminus, and are glycosylated, secretory proteins with approximately molecular mass of 13-24 kDa (Abrahamson *et al*., 1987). Family-3 cystatin are also known as Kininogen family, which are larger than the members of the two other families and comprises the blood plasma kininogens. They are most complex cystatin molecules with high molecular mass of 60-120 kDa. Family-3 cystatin or Kininogen, play an important role in blood coagulation process (Salvesen *et al.,* 1986). Family-4 cystatin, called as the phytocystatins (Turk and Bode, 1991; Turk *et al.,* 1997), are known to have characteristics that are found commonly in most family-1 and –2 cystatins. Oryzacystain from rice seeds was the first cystatin of plant

origin and is similar in structure and function to chicken egg white cystatin (Barrett *et al.,* 1986). Phytocystatins can be classified in to two groups, one group of phytocystatins which comprise the majority of phytocyststins contains a single domain (Pernas *et al.,* 1998) and second group has multiple domains that are found in sunflower seeds, tomato leaves and potato tubers (Walsh and Strickland, 1993; Wu and Haard, 2000; Kouzuma *et al.,* 2000). Phytocystatins have been identified in monocot and dicot species, such as maize, rice, potato, soybean and apple (Kondo *et al.,* 1990; Abe *et al.,* 1991, Botella *et al.,* 1996; Gruden *et al*., 1997; Ryan *et al.,* 1998; Tian *et al.,* 2009). Phytocystatins present in tomato and potato confer resistance and protect the plants from cowpea weevils and Colorado potato beetles which employ cysteine proteases as important digestive enzymes (Gatehouse *et al.,* 1986; Amirhusin *et al.,* 2007; Wolfson and Murdock, 1987).

2.8.1.2 Aspartyl protease inhibitor

Aspartyl protease inhibitors inhibit the catalytic activity of aspartyl proteases that contains active site aspartate residue. They are relatively less studied due to their scarcity in nature. The best characterized aspartyl protease inhibitors are the potato cathepsin D inhibitor (PDI) which is bi-functional (it also inhibits serine proteases) (Keilova and Tomasek, 1976) and has considerable amino acid sequence similarity with the soybean trypsin inhibitor family (Mares, *et al.,* 1989). Recently from the phloem exudates of *Cucurbita maxima* (squash), a novel aspartyl protease inhibitor was purified and characterized (Christeller *et al*., 1998).

2.8.1.3 Metallo-protease inhibitors

The metallo-protease inhibitors from tomato and potato represent the metallo-carboxypeptidase inhibitor family (Rancour and Ryan, 1968, Hass *et al*., 1975, Graham and Ryan, 1981). Two matrix metallo protease inhibitors having a selective inhibitory effect on tumor cell invasion were purified by Shahverdi *et al*. from *Ferula persica* (Shahverdi *et al.,* 2006).

2.8.1.4 Serine protease inhibitors

Serine PIs are the most-studied and well characterized over the years, and are widely distributed in microorganisms, animals and plants. They are found throughout the plant kingdom. In dicots the families of Leguminosae and Solanaceae have the largest number of species with serine protease inhibitors, whereas in the monocots the family Graminae has the largest number of species with these inhibitors (Mello, 2001). Serine PIs are concentrated mainly in reproductive and storage tissues such as seeds and tubers, although they have also been found in roots, leaves and fruits (Xavier-Filho, 1992).

Kunitz-type and the Bowman– Birk type inhibitors are the two major serine protease inhibitors in plants. Kunitz type trypsin inhibitor was the first PPIs to be isolated and characterized (Birk, 2003). They have one or two polypeptide chains, a low cystine content (usually with four Cys residues in two disulfide bridges), approximately having a molecular mass of 18–22kDa, and one reactive site. Whereas Bowman–Birk type inhibitors have a lower molecular mass (8–10

kDa), high cystine content, two reactive sites and typically found in legume seeds (Birk, 1996; Birk, 2003). They can bind simultaneously and independently to two separate enzyme molecules, such as trypsin and chymotrypsin (Birk, 1985, 2003; Bode and Huber, 1992; McBride *et al.,* 2002; Qi *et al.,* 2005). Some of the plant serine PIs act as bifunctional molecules by inhibiting both trypsin and α- amylase (Strobl *et al.,* 1995; Haq *et al*., 2005).

2.8.2 Sources of protease inhibitors

Protease inhibitors are abundant and naturally occurring compounds that are found in various plants, animals and microorgamisms (Laskowski and Kato, 1980). PIs, in general, are small molecules which form complexes with proteases and inhibit their proteolytic activity thereby controlling the proteolytic events in living organisms (Laskowski and Kato, 1980; Neurath, 1984). Though they are present in animals and in microorganisms, most of the PIs found are well studied and characterized from plants (Richardson, 1991).

2.8.2.1 Protease inhibitors from prokaryotes

Irrespective of the complexity of organisms, proteases are necessary for the normal development of individual cell at every stage in life in respect of non-specific protein degradation as well as in biological process. Therefore precise control of the action of proteases is obviously crucial for the normal functioning of the individual cell which is achieved by the inhibitors for proteases, among other things. Irvin *et al.,* identified and characterized a serine protease inhibitor called thermopin from the *Thermobifida fusca,* a thermophyllic

prokaryote (Irving *et al.,* 2002). Thermopin in comparison with human antitrypsin have enhanced heat stability at 60 ^{0}C (Irving *et al.,* 2002*). Escherichia coli* trypsin inhibitor, Ecotin, was purified from the periplasmic space of *E. coli* by Chung *et al.,* using conventional methods (Chung *et al*., 1983). Ecotin inhibits trypsin, chymotrypsin, human serosal urokinase, rat mast cell kinase, pancreatic elastase etc, but not papain or pepsin (Chung *et al*., 1983).

2.8.2.2 Protease inhibitors from fungi

Protease inhibitors play a significant role in fungal innate defensive role against predators, pathogens, and parasites as well as in regulatory role in the specific inhibition of several proteases. Sabotic *et al.*, identified a serine protease inhibitor specifically a trypsin inhibitor called Cospin from the fruiting body of *Coprinopsis cinerea* (Sabotic 2012). A novel pepsin inhibitor of 22.3 k Da with a single subunit was isolated by Zhang *et al.* from the basidiomycetes fungi, *Coriolus versicolor* (Zhang *et al*., 2012). Dohmae et al. sequenced the complete amino acids of two isomeric endogenous serine protease inhibitors from the fruiting body of *Pleurotus ostreatus*, higher basidiomycetes (Dohmae *et al.,* 1995). A novel protease inhibitor of Hepatitis C Virus (HCV) was isolated from the fungus *Penicillium griseofulvum* (Chu *et al.,* 1999). Hanada *et al.,* reported a new thiol protease inhibitor, E-64 from the extract of *Aspergillus japonicas* solid culture and it has inhibitory activity against bromelains, papain etc. (Hanada *et al.,* 1978).

2.8.2.3 Protease inhibitors from Animals

Protease inhibitors, play a vital role in various physiological processes such as reproduction, protection from foreign bodies like viral infection, anticoagulant activity, regulation of both endogenous and exogenous proteases in tissues, etc. Most of the animal species ranging from lower animals to higher mammals and humans produced a variety of PIs from various tissues/ fluids including liver, pancreas, ova, seminal plasma, skin secretion, serum, venom, salivary glands and haemolymph (Ng *et al*., 2012).

A trypsin/ chymotrypsin inhibitor of 6.1 kDa, Kunitz type inhibitor, was isolated from the tropical sea anemone *Radianthus macrodactylus* (Sokotun *et al.,* 2006). Azzolini *et al.* reported a 6.53 kDa serine PI named 'HiTi' from the thoracic extract of the horn fly, *Haematobia irritans irritans* (Azzolini *et al.,* 2005). A multifunctional Kunitz type trypsin inhibitor, Boophilin, was isolated by Macedo *et al.,* from the cattle tick *Boophilus microplus* and it inhibits thrombin, trypsin and plasmin (Macedo, Ribeiro *et al.,* 2008). While screening medicinal leech *Hirudo medicinalis,* Yanes *et al.,* separated 13 protease inhibitors which show sequence similarity with known serine protease inhibitors (Yanes *et al.,* 2005). From the crustacean, the black tiger shrimp, *Penaeus monodon*, a 29 kDa Kazal type serine PI was reported (Somprasong *et al*., 2006). Xue *et al*., identified a 7.61 kDa serine PI with novel amino acid sequence from the mollusc, eastern oyster, *Crassostrea virginicia* (Xu *et al.,* 2006). Two serine PIs, antithrombin III and α 1- P1 were isolated from common carp fish, *Cyprinus carpio,* by Mickowska (Mickowska, 2009). From the ova of

odor frog, *Rana graham,* a single chain 14.4 kDa serine PI was isolated by Han *et al* (Han *et al.,* 2008). Slowinska *et al.,* isolated serine PI from the reproductive tract and seminal plasma of turkey, *Meleagris gallopavo.* A 7.65 kDa secretory trypsin inhibitor was isolated from pancreatic tissue of ostrich by Zhao *et al.* (Slowinska *et al.,* 2008; Zhao *et al.,* 1996). Three different protease inhibitors, α1- antichymotrypsin, α1- proteinase inhibitor and α2- macroglobulin from human plasma has been shown to inactivate a purified serine proteinase present in the fungus, *Aspergillus melleus* (Korzus *et al.,*1994). Human serum, amniotic fluid and urine contain a Kunitz-type protease inhibitor, Bikunin, having anti-metastatic activity through direct inhibition of plasmin activity as well as by inhibiting urokinase plasmin activator (Kobayashi *et al.,* 1995; Kobayashi *et al*., 2002; Kobayashi *et al*., 2001). Protease inhibitor from mosquito functioning as an anticoagulant during blood-sucking was also reported (Watanabe *et al.,* 2010).

2.8.2.4 Protease inhibitors from Plants

Plants are important source of large number of protease inhibitors. PIs are widely distributed in plant kingdom, and most of them that have been characterized are from Gramineae (Poaceae), Leguminosae (Fabaceae), and Solanaceae families (Brzin and Kidric, 1995; Richardson, 1991). PIs are one of the most significant elements of defence strategies in plants to combat various phytophagous pests like insects, mites, slugs *etc* and phytopathogenic micro-organisms like bacteria, viruses, parasites and fungi (Haq *et al.,* 2004, Ryan, 1990).

In storage tissues up to 10% of total protein content are contributed by the PPIs, but they are also detectable in leaves. They are also expressed in response to the attack by insects and pathogenic microorganism (Ryan, 1990). The defensive role of PPIs relies on inhibition of proteases present in insect gut or secreted by microorganisms (Lawrence and Koundal, 2002). Plant protease inhibitors were synthesized constitutively during normal development of plant or may be induced in response to herbivory and pathogen attacks (Ryan, 1990). Bowman-Brick type trypsin inhibitor was isolated and purified from the mechanically wounded leaves of Alfalfa by Brown and Ryan (Brown and Ryan, 1984).

Over the past decade, large number of protease inhibitors were isolated and purified from different tissues like seeds, leaves, fruits and tubers of plants of several families (Xavier-Filho and Campos, 1989, Richardson, 1991, Kendall; 1951, Wingate *et al.,* 1989). A trypsin inhibitor (PFTI) of 19 kDa was isolated and characterized from the seeds of *Plathymenia foliolosa*, by Ramos *et al.* and it exhibits significant inhibitory activity against larval midgut proteases of *Anagasta kuehniella* and *Diatraea saccharalis* (Ramos *et al*., 2008). Hilder *et al.* reported the protein and cDNA sequences of Bowman Brick protease inhibitor from the cowpea (Hilder *et al.*, 1989). The complete amino acid sequences of the trypsin and chymotrypsin inhibitor were done from cowpea seeds (Mohry and Ventura, 1987).

2.8.3 Properties of plant protease inhibitors

Plant protease inhibitors have high content of lysine and cysteine residues that form disulphide bridges which confer resistance

to wide pH ranges, heat, and proteolysis (Richardson, 1991, Greenblatt *et al*., 1989; Hung *et al.,* 2003). Many PPIs are heat stable due to the presence of disulphide bridges (Hung *et al*., 2003). From the seeds of *Brassica nigra,* a novel thermostable trypsin inhibitor was isolated by Genov *et al.,* (Genov *et al.,* 1997). A thermostable protease inhibitor, SmaPI which shows an inhibitory activity against extracellular 50 kDa metalloprotease, was isolated from *Serratia marcescens* and it was heat stable up to 30 min in boiling water bath (Kim *et al.,* 1995). In general molecular weight of PPIs varies from 4 to 85 kDa with majority in the range of 8 to 20 kDa (Hung *et al*., 2003). A novel serine protease inhibitor have been identified from the seeds of buckwheat *Fagopyrum esculentum* Moench and possessed high pH-stability in the pH range 2.0 to 12.0 and thermostability (Tsybina *et al*., 2004). Many new low molecular weight protein inhibitors of serine proteinases have been reported. Bowman-Birk type trypsin inhibitor purified from the seeds of *Brassica campestris* (BCTI) having molecular weight of 8 kDa was found to be heat stable (Hung *et al*., 2003).

2.8.4 Role of plant protease inhibitors in plants

Plant protease inhibitors may be synthesized during the normal course of development of the plant as well as in response to various stress conditions like insect attack, pathogens, wounding and environmental stresses such as high salinity (Solomon *et al.,* 1999, Koiwa *et al.,* 1997). Their physiological roles include the regulation of endogenous proteases during seed dormancy, reserve protein mobilization, and plant protection from the proteolytic enzymes of the

parasites and insects (Birk, 2003). They also serve as reserve food material.

2.8.5 Application of plant protease inhibitors

During the past few decades protease inhibitors have been the object of study in many disciplines such as in biology, medicine, health etc. In addition to the role of PPIs in agriculture, they may be of use in the management of diseases such as AIDS, cardiovascular diseases, osteoporosis, Alzheimer's diseases, Rheumatoid Arthritis, were proteases play a role in the development or progression of the diseases.

2.8.5.1 Protease inhibitors for controlling pests

Pests and pathogens are major threats to plant growth and development, resulting in heavy losses in crop quantity and quality. Plant resistance to pests and pathogens can be mediated by wide range of metabolic products, including both primary metabolites and secondary metabolites (Macedo *et al.,* 2003). Among them, plant protease inhibitors play an important role in defence of plants against pests and pathogens. Antinutritional effect of serine protease inhibitors against lepidopteran insect pests were reported in previous studies (Shulke and Murdock, 1983; Applebaum, 1985).

Plant protease inhibitor, CpTi, exhibits a broad range of activity including suppression of pathogenic nematodes like *Globodera pallida, G. tabaccum,* and *Meloido gyneincognita* (Williamson and Hussey, 1996). Growth of many pathogenic fungi including *Trichoderm sreesei* were adversely inhibited by cysteine PPIs from pearl millet (Joshi *et al.,* 1998). Trypsin/chymotrypsin inhibitors from

buckwheat inhibit the seed germination and mycelium growth of *Alternaria alternate* (Dunaevskii *et al*., 1997 Four serine PIs were reported from *Solanum nigrum* which differ significantly in substrate specificity, patterns of accumulation and their herbivory deterrent effects (Hartl *et al.*, 2011).

2.8.5.2 Genetically modified crops expressing plant protease inhibitors

Crop protection strategies in agricultural system primarily relies on the exclusive use of agrochemicals or pesticides The extensive chemical pesticide use resulted in rapid resistance development, and their non-selectivity leads to the imbalance of pests and natural predators which in turn make favours to pests (Metcalf. 1986). To reduce the harmful effects of these pesticides on environment and human health, genetically modified plants expressing PPI gene can be explored as an alternative to create pest resistant plants.

Development of GM crops have come a long way from the first transgenic tobacco plant produced (Hilder *et al.,* 1987). The Cowpea trypsin inhibitor, CpTi from *Vigna unguiculata* was the first PPI gene to be successfully transferred to the tobacco plant and showed significant resistance against tobacco hornworm (*Manduca sexta*) (Hilder *et al.,* 1987). In feeding trials under laboratory conditions, reduction in the biomass up to 50% in armyworm (*Spodoptera litura*) larvae fed on transgenic leaves expressing 3-5 µg of CpTi /g was observed by Sane *et al.* (Sane *et al.,* 1997).

Many of these protease inhibitors are rich in cysteine and lysine, which enhanced the nutritional quality (Ryan, 1998). The use of PPIs for raising GM crops with antifungal, antibacterial and antiviral properties will be an attractive way for plant protection. A novel protease inhibitor of 10 kDa, having antifungal and antibacterial activity was isolated and characterized from mung bean (*Phaseolus mungo*) (Wang *et al*., 2006).

Studies indicate that insect adapt to the protease inhibitors in different ways when they were fed with diet containing PIs or when they were reared on transgenic plants expressing PPIs. Even though these adaptations of insect to overcome the expression of protease inhibitors may limit the use of protease inhibitors, studies are carried out to overcome these problems. By genetic engineering technique, multi-domain inhibitors of cystein protease expressed in potato plant was found effective against western flower thrips, *Frankliniella occidentalis* larvae and it caused about 80% of death of larvae (Outchkourov *et al.,* 2004).

Co-expression of oryzacystatin I and II expressed in transgenic potato inhibits the larval development of Colorado potato beetle (*Leptinotarsa desimlineata*) (Cingel *et al*., 2017). Serine and cysteine protease inhibitors from barley expressed on tomato showed a notable weight reduction on the major tomato pest, *Tuta absoluta* (Hamza *et al.,* 2018). Thus expressing PPI in host plant is a new strategy for the pest control.

MATERIALS AND METHODS

3.1 CHEMICALS:

BAPNA (N-α-benzoyl DL- arginine-p-nitro anilide), Azocasein, Sepharose-4B, and Riboflavin were purchased from Sigma Aldrich, USA. Acrylamide, bis acrylamide, TEMED, methylene green, bromophenol blue and Amicon 3 kDa protein concentrator were obtained from Merck, Germany. Coomassie Brilliant Blue G-250, Coomassie Brilliant Blue R-250, BSA (Bovine Serum Albumin, fraction V) and Dialysis membrane from Himedia. 2- Mercapto ethanol (2-ME), cyanogen bromide (CNBr), Protein molecular weight Marker and β- alanine and, Ammonium sulphate from Sisco Research Laboratories Limited, Mumbai. Diethyl aminoethyl (DEAE) Sephadex A-50, Sephadex G-100 were products of Pharmacia fine Chemicals Uppala, Sweden. All other chemicals used were of analytical grade.

3.2 METHODS

3.2.1 Collection and rearing of *Spodoptera mauritia*

The adult moths of *Spodoptera mauritia* (Boisduval) (Lepidoptera: Noctuidae) attracted to light during night and were collected using an insect sweep net. They were collected locally from in and around campus of University of Calicut. They were brought to the laboratory and kept in glass beakers closed with muslin cloth. The moths were fed with diluted solution (10%) of honey soaked in cotton swabs. They were allowed to mate and the females laid eggs on the cloth covering of beakers within two days. The cloth with eggs was

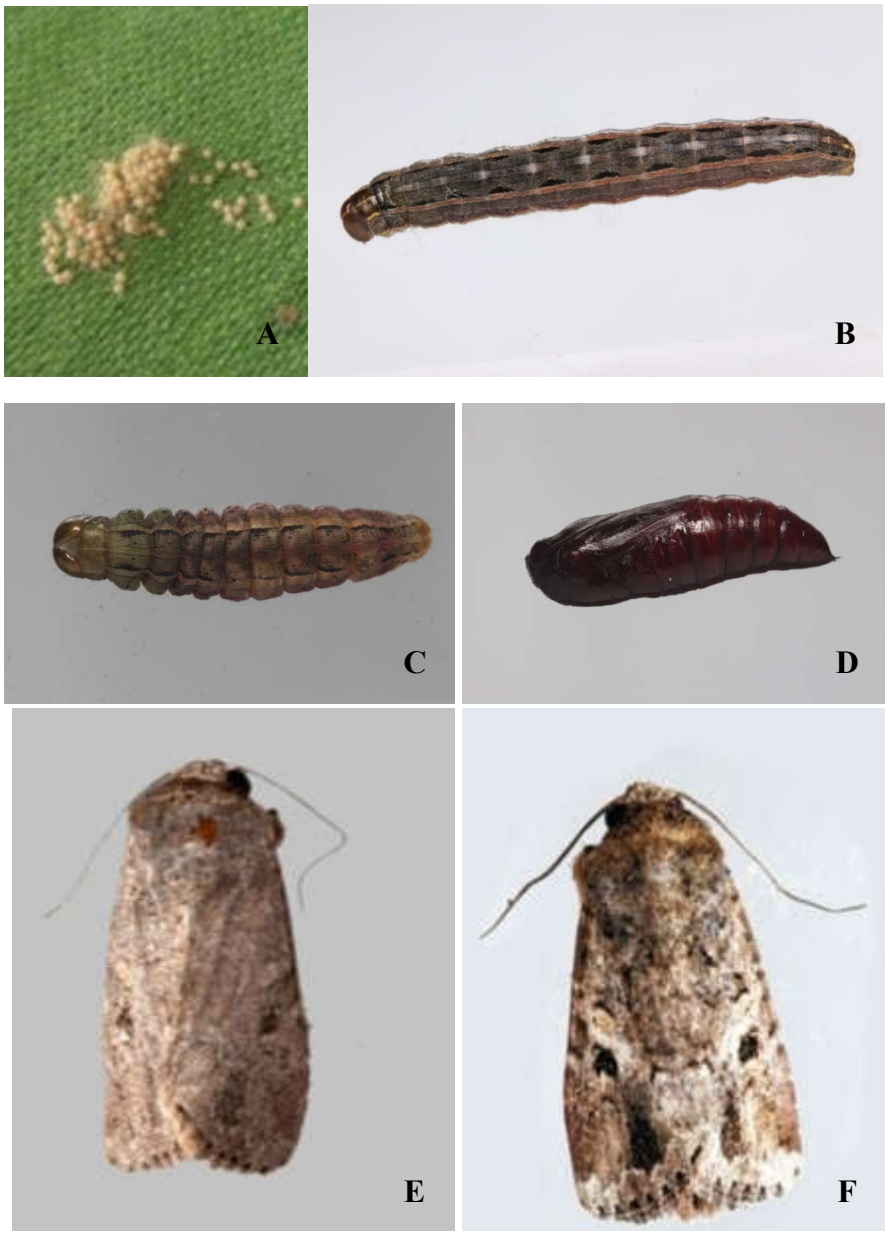
transferred to a new glass beaker. The fertilised eggs were hatched into larvae after three days of incubation. The newly hatched larvae in beakers were daily fed with fresh, tender leaves of grass, *Ischaemum aristatum,* collected from the paddy field, which is an alternate host plant of the *S. mauritia* larvae. The beakers were kept away from intense light. The colonies were kept clean, maintained at room temperature, relative humidity 90.0 ± 3.0% and 12:12 light:dark photoperiodic regime.

When the larvae were grown in size, they were transferred to plastic troughs which were covered with muslin cloth. During dry season, the muslin cloths covering the trough were moistened occasionally. Fresh leaves of *I. aristatum* were provided to the larvae every day after removing the faecal matter and maintained stock culture in the laboratory. Fifth instar larvae were used for the experimental purpose.

3.2.1.1 Lifecycle of *Spodoptera mauritia*

The *Spodoptera mauritia* larvae have six larval instars before pre pupation and pupation. Under natural condition on rice plant the life cycle of *S. mauritia* (Figure 3.1) completed in 25-40 days (Tanwar *et al.,*2010), where as in laboratory condition it is 19 to 23 days (Mathew, 2008).

Figure 3.1 Different stages of life cycle of *Spodoptera mauritia*



A. Egg; **B.** 5th Instar larva; **C.** Prepupa; **D.** Pupa; **E.** Adult female; **F.** Adult Male

First instar larvae

The newly hatched first instar larvae of *S. mauritia* congregated on the muslin cloth covering of the glass beaker or at the wall of the beaker in which it is hatched. The first instar larvae were light green in colour and were characterized by the presence of a large black head capsule. They were fed with fresh and tender leaves of grass *Ischaemum aristatum*, which is an alternate host plant. The newly hatched out larvae were descended by means of silken threads to the fresh leaves supplied for feeding the larvae. Larvae fed the green part of the leaves leaving behind the midribs and larval movements are in a leaping manner. On each segment of the body, small, wart-like, setigerous, pigmented tubercles were present in a cross-wise raw. The first instar larvae measured about 1 mm in length and 0.5 mm in width. The larval period was found to be 2 to 3 days and moults to the second instar.

Second instar larvae

The second instar larvae were characterized by the presence of three white longitudinal stripes on the dorsal surface of the body continuing from prothorax to the last abdominal segment. Larvae were pale green in colour. Two pairs of white longitudinal lateral lines were also present on the sides of the body, one being more prominent. Second instar larvae also descended using silken threads and the body retained the small wart like setigerous tubercles on each segment of the body. The second instar larval body measured about 2.5 mm in length

and 0.5 mm in width. Duration of the second instar larvae was about 2 to 3 days.

Third instar larvae

The third instar larvae were pale green in color, and they no longer used silken threads to descend to the leaves. They possessed three white longitudinal stripes, one on the dorsal side and the other two on the lateral side of the body extending from anterior to the posterior end. The third instar larvae also had dark reddish supraspiracular lines extending from anterior to posterior end. The wart- like tubercles disappeared and fully grown larvae measured about 6 mm in length and 1 mm width. After 2 to 3 days the larvae molted to fourth instar.

Fourth instar larvae

Fourth instar larvae were characterized by greyish-black colour and the three longitudinal stripes became dull white in colour. Two lateral reddish black lines were still visible, one on each side of the body. Black intermittent dots appeared dorso-laterally on each segment which broadened towards the later stages of the instar. The dorsum of the larvae was paler than the supraspiracular area and fully grown fourth instar larvae showed an average length of 15 mm and width of 2 mm. The larval period is about 2 to 3 days.

Fifth instar larvae

The fifth instar larvae also were greyish-black in colour like fourth instars. The larvae were characterized by double rows of

prominent black triangular markings present on the dorsolateral side bordered with narrow white stripes. The paired supraspiracular stripes became transparent pinkish in colour. The integument was slightly transparent so that the internal structures were visible. During this instars, testes/ ovary can be visible to sort out the male and female larvae. The total length of the fully grown fifth instar larvae measured about 20 mm in length and 4 mm in width. The larvae fed voraciously and grew quickly. The fifth instar larval period extended up to 3 days.

Sixth instar larvae

The newly moulted sixth instar larvae were characterized by greyish black in colour and the triangular markings became wider and darker than those of the fifth instar larvae. During the first three days after moulting the larvae fed voraciously and attained maximal body weight on day three. Fully grown larvae had an average length of 37 mm and width of 6 mm and they stopped feeding. Within a few hours of the cessation of feeding, the larvae emptied their gut by a behaviour known as "gut purge" i.e., massive excretion of fluid faeces. This marks the initiation of the post-feeding stage and soon after completion of the gut purge, larvae start the wandering behaviour which lasts for 24h. During this wandering period, larvae showed an average length of about 26 mm and a width of 4.5 mm. The wandering larvae transformed into the pre-pupal stage by day 5 and this stage was characterized by highly wrinkled larvae which underwent larval-pupal apolysis after 24 h. The body of the pre-pupae measured about 20 mm in length and 5 mm in width. The period of development of sixth instar larvae extended up to 6 days.

Pupal instar

Pupae were of the object type and measured 17 mm in length and 5 mm in width with dark brown colour. Adults were emerged within 7- 8 days after pupation. Female pupae took 7 days, whereas males took 8 days for the eclosion to adults.

Adult

The adult moths of *S. mauritia* were found to be medium-sized with a conspicuous spot on the forewings, which had a wavy pattern on the fringe and it exhibits a clear sexual dimorphism in their morphological characteristics. Males were dark grey in colour with white markings on forewings and were provided with large tufts of hairs on the forelegs whereas females were grey in colour but lacked both white markings and large tufts of hairs on the forelegs. The adults measured about 15-20 mm in length and had a wingspan of 30-40 mm (Tanwar *et al.,* 2010). The mating of adult moth takes place in the night within 24h after emergence. Egg laying was found to begin from 24h after mating and around 100 to 500 eggs were laid in mass. The eggs were covered with buff coloured silken hairs. The larvae were hatched out within 2 to 3 days. On the whole, in the life cycle of *S. mauritia*, the egg period lasted for 3 days, the larval period extended for 19 to 23 days and pupal period for 7 to 8 days.

3.2.2 Preparation of *Spodoptera mauritia* gut extract

Fifth instar larvae were anesthetized to dissect out the mid gut and it was stored at -20 °C until use. The gut was homogenized in 0.1

M bicarbonate buffer, pH 9.0 (1 ml/g of tissue) and were centrifuged at 10,000 x g at 4 °C for 10 minutes. The soluble protein recovered from the supernatant was stored as aliquots at -20 °C until use.

3.2.3 Collection of plants and preparation of the plant extract

Plant parts (seeds/ leaves) were collected from Kozhikode, Kannur and Malappuram district of Kerala, India. They were washed and soaked in bicarbonate buffer, pH 9.0 (2 ml/g tissue) and homogenized directly/ powdered with liquid nitrogen and then homogenized. The homogenates were centrifuged at 10,000 x g for 10 minutes at 4 ^{o}C. The supernatant containing soluble protein was used for protease inhibition assay.

3.2.4 Protease assay and protease inhibition assay

The protease assay and protease inhibition assay were done using Nα-Benzoyl-DL-Arginine-P-Nitro Anilide (BAPNA) / azocasein as substrate and trypsin / larval gut extract as enzyme.

Initially plant extracts were screened against trypsin using Nα-Benzoyl-DL-Arginine-P-Nitro Anilide (BAPNA) as substrate. The protease assay with trypsin as enzyme was carried out in a total volume of 1 ml with 8.4 µg/ml trypsin (Bovine), 603 µg/ml NaCl and 330 µg/ml BAPNA as substrate. Proteolytic activity was measured by continuous spectrophotometric rate determination method using UV Spectrophotometer at 405 nm for 5 minutes. For the protease inhibition assay, the plant extract was pre-incubated for 10 minutes with trypsin and assay done as described earlier.

In the second method we used Azocasein as substrate. This product consists of casein conjugated to an azo-dye. This serves as a general substrate for proteolytic enzymes. Degradation of the casein liberates free Azo-dye into the supernatant that can be measured quantitatively.

The protease assay was done by incubating, 5 µl of the gut extract with 0.015 µg/µl azocasein as substrate in a total volume of 20.2 µl, at 37˚C for 30 minutes. The reaction was stopped by adding 80 µl 5% TCA. After centrifugation, 50 µl supernatant was mixed with 150 µl of 0.5 M NaOH. The absorbance was measured calorimetrically at 440 nm using a Micro plate reader (Synergy HT, Bio Tek). In protease inhibition assay 10 µl of the plant extract was pre-incubated with the 5 µl of the gut extract and assay done as described in protease assay. Percentage inhibition is calculated by taking the absorbance of the gut extract as 100% activity. All assays were done in duplicate and the experiments were repeated three times.

Calculations

Absorbance of the control was subtracted from the absorbance of the inhibitor alone, and the value thus obtained represents the protease activity present in the plant extract. This value was subtracted from the absorbance in presence of the inhibitor and enzyme/ gut extract to get actual absorbance in the absence of any protease activity from the plant extract. The absorbance of the enzyme alone was taken as 100% enzyme activity. Based on this, the absorbance in presence of

the inhibitor was converted into percentage activity. Subtracted the value thus obtained from 100 to get percentage inhibition.

3.2.5 Proteinase K treatment of plant extracts

Plant extracts with higher inhibition was tested to assess whether the inhibitor is proteinacious in nature or not. Proteinacious nature of the inhibitor was assessed by overnight incubation of the plant extract (90 µl) with Proteinase K (0.231 mg) at 56 ^{0}C followed by the inactivation of the proteinase K by heating the mixture at 75 ^{0}C for 15 minutes. A buffer control and inhibitor alone control were also kept without Proteinase K for incubation. It was centrifuged 10000 x g for 1 minute and the supernatant was used for protease inhibition assay. Inhibitor control without any incubation was also done. All assays were done in duplicate and the experiments were repeated three times.

3.2.6 Statistical analysis

Statistical analysis was done by using SPSS software, version 16.

3.2.7 Estimation of protein

Protein concentration of samples was estimated using the Bradford's dye binding method (Bradford, 1976) or UV absorption at 280 nm. The concentration of protein in the sample was calculated from a standard curve prepared using BSA (fraction V) as standard.

Preparation of Bradford's reagent

Brilliant blue G-250 (0.06 %) was dissolved in 0.6 N HCl and filtered through Whatman No. 1 filter paper and stored in an amber coloured bottle.

3.2.8 Purification of protease inhibitor from the selected plant

The protease inhibitor was purified using Ammonium sulfate [$(NH_4)_2SO_4$] precipitation, DEAE-Sephadex ion exchange chromatography, gel filtration chromatography, trypsin affinity chromatography and Immobilized Metal Affinity Chromatography.

3.2.8.1 Selection of the plant

Spatholobus parviflorus (DC.) Kuntze seeds were selected for isolation and purification of PPI against larval gut proteases of *S. mauritia* as it gave more than 55% inhibition and the inhibitor is proteinaceous in nature. Samples were collected from Calicut district, Panthirikkara / Kakkad, Kerala, India. Seeds were removed from the seed pod and stored at -20 ^{0}C. The endosperm collected after the removal of the soft seed coat was used for the isolation and purification of protease inhibitor.

Spatholobus parviflorus belonging to the family Fabaceae, and sub family: Faboideae in the class Magnoliopsida. *Spatholobus parviflorus* (Figure: 3.2) is a strong woody climber and distributed in a wide geographic range from Nepal, Bhutan and India. In Kerala its leaf paste is used to treat conjuctivities (Vijayan *et al.*, 2007).

Figure 3.2. *Spatholobus parviflorus* (DC.) Kuntze

A. Leaf with fruit; **B.** Stem; **C.** Fruit and Seed.

3.2.8.2 Preparation of extract from the seeds of *Spatholobus parviflorus* (DC.) Kuntze

In a typical experiment 250 g of the endosperm was powdered with liquid nitrogen using mortar and pestle and it was homogenized in a mixer grinder with 500 ml of 0.1 M sodium bicarbonate buffer, pH 9.0. It was filtered through cotton gauze. The filtrate was then centrifuged at 10,000 x g for 10 minutes at 4 ^{0}C. The supernatant containing soluble protein was used for the next step of purification or stored at -20 ^{0} C until use.

3.2.8.3 Ammonium Sulfate precipitation

For ammonium sulfate precipitation, method of Englard and Seifter was used (Englard and Seifter, 1990). The plant extract containing soluble proteins obtained in the above step was subjected to ammonium sulfate precipitation. Differential precipitation of the protein was done at 4 ^{0}C with different ammonium sulfate concentrations. Zero to 30%, 30 to 50% and 50 to 70% ammonium sulphate fractions were prepared and assayed to determine the fraction containing the highest percentage of inhibition. Solid ammonium sulfate was added gradually to the soluble protein with constant stirring at 4 ºC to obtain 30% saturation. After the addition of the salt, it was kept stirring for 30 - 45 minutes. Then the samples were centrifuged at 12,000 x g at 4 ^{0}C for 10 minutes. The precipitate recovered, which contain proteins precipitated at 30% ammonium sulphate was kept on ice. To the supernatant ammonium sulphate was again added to get the higher salt percentage. The steps repeated up to 70% ammonium

sulphate fraction. Three separate precipitates were re-suspended in minimum volume of 0.1 M bicarbonate buffer pH 9.0 and taken in 3 separate dialysis bags. They were dialysed against 20 mM Tris buffer pH 8.0 containing, 50 mM NaCl for 24 h at 4 ^{0}C with four changes of buffer. After the completion of dialysis each fractions were centrifuged at 10,000 x g at 4^0 C for 10 minutes. Protease inhibition assay was carried out using these fractions. Protein concentration was estimated using Bradford's dye binding method.

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

3.2.8.4 Ion Exchange Chromatography

The ammonium sulphate fractionation with the highest inhibition was subjected to ion exchange chromatography at 4 ^{0}C. DEAE-Sephadex A50, an anion exchange resin, was used for the separation of inhibitor. For this DEAE Sephadex powder was soaked for 1 to 2 days at room temperature in 20 mM Tris buffer pH 8.0 containing 50 mM NaCl. The supernatant was decanted and replaced with fresh buffer several times during the swelling period. Pre-processed ion exchange resin was carefully packed in a glass column (25x1.5 cm) without any air bubbles. The column equilibrated with 20 mM Tris buffer pH 8.0 containing 50 mM NaCl. Then column was washed with the same buffer up to 10 column volumes. Then ammonium sulphate fraction with the highest percentage inhibition (30 to 50% Ammonium sulfate fraction) after dialysis was loaded carefully on to ion exchange column (150 mg protein). After loading all the

protein, the column was closed for 30 to 45 minutes for binding of the protein to the DEAE-Sephadex. Then the column washed with the 20 mM Tris buffer pH 8.0 containing 50 mM NaCl until protein free. Presence of protein in the wash and elute was detected by mixing with an equal volume of Bradford's reagent. The protein was eluted with a discontinuous gradient of NaCl (0.15 M, 0.3 M, 0.55 M and 1.05 M) in 20 mM Tris buffer pH 8.0. Two ml fractions were collected. The fractions were monitored for protein by measuring their absorbance at 280 nm. Inhibition of fractions was tested and peak fractions with high percentage inhibition and protein concentration were pooled. The pooled fractions were dialysed against 20 mM Tris buffer pH 8.0 containing 50 mM NaCl and concentrated using Amicon 3 kDa protein concentrator. Each pooled fraction samples were tested for the inhibitory activity against gut proteases of *S. mauritia.* It was used for further purification by gel filtration chromatography for the separation of inhibitor protein based on size.

3.2.8.5 Gel Filtration Chromatography

Gel Filtration Chromatography was performed following the method of Andrews, (Andrews, 1965). Sephadex G-100 was suspended in distilled water and allowed to hydrate for 1 to 2 days at room temperature. Fine particles were removed by decantation, then re-suspended in Phosphate-Buffered Saline, pH 7.4 (PBS 7.4). This was repeated until the gel is free of fine particles. Gel suspension was carefully poured in a column of 60 X 1.5 cm without air bubbles and allowed to settle under gravity. The column was opened with a slow flow rate until the packing is complete. Column was equilibrated with

PBS 7.4. Blue dextran 2000 was used to test the performance of the column and to measure the void volume of the packed gel filtration column.

The pooled fractions from ion exchange chromatography exhibiting the higher inhibitory activity was dialysed against PBS 7.4 and loaded on to Sephadex G-100 column. Two ml fractions were collected and the protein was monitored by measuring the absorbance at 280 nm. Peak fractions based on percentage inhibition and protein concentration were pooled and concentrated using Amicon 3kDa protein concentrator.

3.2.8.6 Trypsin Affinity Chromatography

Activation of Sepharose - 4B with Cyanogen Bromide (CNBr) and the coupling of trypsin to the CNBr activated Sepharose was done as per the protocol of Cuatrecass and Anfinsen (Cuatrecass and Anfinsen, 1971).

3.2.8.6.1 Preparation of CNBr Activated Sepharose

Sepharose 4B Gel was washed several times with distilled water under suction. 20 g of washed gel put into 40 ml 2 M sodium carbonate (Na_2CO_3) and 20 ml distilled water added. The mixture was kept stirring at 4 ^{0}C. Then 800 mg Cyanogen bromide (CNBr) dissolved in 2 ml Dimethyl formamide and was added to the gel while stirring and the activation continued for 5 minutes. The gel is immediately washed with cold 0.1 M sodium bicarbonate ($NaHCO_3$) at least 20 times gel volume. The gel is activated and ready for coupling.

3.2.8.6.2 Coupling of trypsin to CNBr-activated Sepharose gel

160 mg trypsin dissolved in 20 ml 0.1 M cold $NaHCO_3$ and added to the activated gel. Slurry of gel gently stirred for 18 hrs at 4 ^{0}C. Coupling was stopped by addition of 400 µl of 0.1 M ethanolamine HCl and stirring continued for 1 hour. The uncoupled trypsin was removed by washing with 0.1 M $NaHCO_3$, followed by washing with distilled water, then with 50 mM acetate buffer, pH 5.0, followed by distilled water and finally washed and equilibrated with PBS 7.4.

3.2.8.6.3 Loading of ion exchange fraction on to Trypsin-Sepharose column

The trypsin affinity chromatography was done at 4 ^{0}C. Fifty mg Protein eluted from ion exchange chromatography was loaded on to Trypsin – Sepharose affinity column (5 ml gel). During loading the column was closed for 30 to 45 minutes for binding of the protein to the trypsin. The unbound proteins were removed by washing with 20 mM Tris buffer pH 8.0 (TBS 8.0) containing, 150mM NaCl and the bound protein was eluted with 5 mM HCl and neutralized with 0.5 M NaOH. Five hundred micro litre fractions were collected and protease inhibition of the fractions tested. Fractions having higher protease inhibition against the gut proteases of *S. mauritia* were pooled, dialyzed in TBS 8.0 containing 150 mM NaCl and concentrated. Further purification was done on Immobilized Metal Affinity Chromatography (Ni-IMAC).

3.2.8.7 Immobilized Metal Affinity Chromatography (Ni -IMAC)

Metal affinity chromatography was done by batch method. The Ni - IMAC resin was equilibriated with TBS 8.0 containing 150 mM NaCl. For further purification 0.5 ml of the concentrated protein (1 mg/ml) from trypsin affinity chromatography was mixed with Ni-IMAC resin (0.25 ml). Incubated the mixture for 30 to 45 minutes at 4 ^{0}C and centrifuged at 2,000 x g for 5 minutes. The supernatant containing the unbound proteins were collected and saved (flow through). The gel was washed with TBS 8.0 containing 150 mM NaCl and eluted with the same buffer containing 0.25 M immidazole. The unbound protein (inhibitor) was concentrated using the 10 kDa Amicon protein concentrator. The concentrated protein was used for the protease inhibition assays and further characterization.

3.2.9 Characterization of purified protease inhibitor

3.2.9.1 Electrophoresis

Native or denaturing Poly Acrylamide Gel Electrophoresis (PAGE) was done to separate protein/sub units. Elute form different chromatography steps loaded on to the Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

3.2.9.1.1 SDS-PAGE

The samples were subjected to SDS-PAGE using 10% acrylamide gel in a Mini slab gel according to the method described by Laemmli (Laemmli, 1970). At each stage of purification, the inhibition

was assessed by enzyme inhibition assay and the purity was checked by 10% SDS-PAGE.

A. Reagents

1. Acrylamide solution

Acrylamide	:	30 g
Bis acrylamide	:	0.8 g

Make up to100 ml in distilled water, filtered and stored in amber coloured bottle.

2. Buffer 1.

Tris (0.614 M)	-	18.5887 g
SDS	-	410 mg
Distilled water	-	250 ml

pH was adjusted to 8.8 with HCl.

3. Buffer 2

Tris (0.147 M)	-	4.450 g
SDS	-	270 mg
Distilled water	-	250 ml

pH was adjusted to 6.8 with HCl.

4. Chamber buffer

Tris (0.25 M)	-	0.756 g
Glycine (0.192 M)	-	3.6 g
SDS (0.1%)	-	0.25 g

	Distilled water	-	250 ml
	pH was adjusted to 8.3.		
5.	Tracking dye	-	Bromphenol blue
6.	Fixative		
	50% methanol	-	100 ml
	Formaldehyde	-	75 µl
7.	Staining solution		
	Coomassie brilliant blue R-250	-	60 mg
	Methanol	-	44 ml
	Acetic acid glacial	-	12 ml
	Distilled water	-	44 ml
8.	Destaining Solution		
	Acetic acid glacial	-	7.5 ml
	Methanol	-	5 ml
	Distilled water	-	87.5 ml
9.	Ammonium per sulphate	-	15 mg/ml

B. Gel preparation (10% gel)

Reagents	**Separating gel**	**Spacer gel**
Acrylamide	3 ml	0.5 ml
Buffer 1	5.5 ml	Nil
Buffer 2	Nil	4.25 ml
Ammonium per sulphate	0.45 ml	0.25 ml
TEMED	0.01 ml	0.005 ml

After the run, gel was fixed with fixative for 45 minutes, stained for 45 minutes and destained till the bands appeared with clear background.

3.2.9.1.2 Non-denaturing electrophoresis (Acid PAGE)

Acid PAGE was done by the method described by Reisfeld *et al.* (Reisfeld *et al.*, 1962).

Reagents

Solution A: pH 4.3

1N KOH	-	48 ml
Glacial acetic acid	-	17.2 ml
TEMED	-	4 ml

Made up to 100 ml with distilled water.

Solution B: pH 6.6 to 6.8

1 N KOH	-	48 ml
Glacial acetic acid	-	2.87 ml
TEMED	-	0.46 ml

Made up to 100 ml with distilled water.

Solution C:

Acrylamide	-	30 g
Bis acrylamide	-	0.8 g

Made up to 100 ml with distilled water.

Solution D:

Acrylamide	-	20 g
Bis acrylamide	-	0.5 g

Solution E: Riboflavin- 4 mg dissolved in 100 ml distilled water.

Solution F: Methylene blue (Tracking dye), 0.005% solution

Solution G: Ammonium per sulphate- 2.8 mg/ml in distilled water.

Gel preparation

A. Separating gel (7.5% acrylamide)

1 part	-	Solution A
2 parts	-	Solution C
1 part	-	Distilled water
4 parts	-	Solution G

B. Spacer gel

1 part	-	Solution B
1 Part	-	Solution D
1 Part	-	Solution E
5 parts	-	Distilled water

Polymerization was achieved under fluorescent light.

Reservoir buffer

0.05% M β- alanine, pH adjusted to 4.5 with acetic acid. Fixing, staining and destaining were done as described for SDS-PAGE

3.2.9.2 Determination of subunit molecular weight

Sub unit molecular weight of the protein was determined by running the inhibitor on SDS-PAGE and calculated the molecular weight from a plot of log molecular weight verses mobility of standard protein markers and the mobility of the inhibitor.

3.2.9.3 Identification of the inhibitor protein

Plant protease inhibitor inhibiting the gut protease activity of *Spodoptera mauritia* larvae was identified by LC-MS/MS mass spectrometry. For this SDS-PAGE of purified protein was done and stained with colloidal Coomassie brilliant blue G-250 stain and protein band was excised from the gel and LC-MS/MS was done at Rajeev Gandhi Center for Biotechnology, Trivandrum in AB SCIEX 3200 QTRAP® LC-MS/MS System as per the protocol of Shevchenko *et al.,* (Shevchenko *et al.,* 2006). The excised gel was chopped and the gel pieces were soaked overnight with 13 ng of trypsin in ammonium bicarbonate solution for the extraction of peptides.

3.2.9.4 Effect of pH on inhibition

The effect of pH on the inhibitor over a range of pH was determined by incubating the inhibitor with 20 mM Sodium Phosphate (pH 6.0), Tris HCl (pH 8.0), Glycine NaOH (pH 10.0) and Disodium hydrogen phosphate-NaOH (pH 12.0). Appropriate controls were also kept.

3.2.9.5 Effect of temperature on the stability of the inhibitor

Temperature stability of the inhibitor in TBS 8.0 containing 50 mM NaCl at different temperatures was evaluated by incubating 80 µl of the protease inhibitor in a sealed tube at different temperatures (4 to 100 ºC) for 20 minutes. Then protease inhibition assay was carried out to assess the extent of loss of inhibition.

4.1 SCREENING PLANT EXTRACTS FOR TRYPSIN INHIBITOR

Plant parts from plants of different families were collected from Calicut, Kannur and Malappuram districts of Kerala. Some plant extracts were initially screened for the trypsin inhibition using BAPNA as substrate followed by screening of plants extracts with higher inhibition against gut proteases of *S. mauritia* using azocasein as substrate. Out of the 21 plants screened, 7 plants showed greater than 70% inhibition towards trypsin (Table 4.1).

Table 4.1. List of plant screened for protease inhibition against trypsin and their percentage inhibition

Sl. No.	Scientific Name/ Vernacular name	Plant parts used	Mean % inhibition ± SE
1	*Chrysophyllum cainito* L. (Golden leaf tree, Swarnapathri)	Leaf	89.35 ± 0.10
2	*Dalbergia latifolia Roxb.* (Black rose wood, Kariveetti)	Leaf	85.09 ± 0.40
3	*Nephelium lappaceum* L. (Rambuttan)	Seed	83.45 ± 0.05
4	*Cochlospermum religiosum* (L.) Alston (Silk cotton tree, Parappoola)	Seed	82.65 ± 0.09
5	*Anacardium occidentale* L. (Cashew-nut, Parangimavu)	Tender Seed	79.23 ± 0.04

6	*Samanea saman* (Jacq.) Merr. (Rain Tree, Mazhamaram)	Seed	78.27 ± 0.08
7	*Mucuna pruriens* (L.) DC. (Velvet bean, Naikkuruna)	Seed	71.34 ± 0.06
8	*Psidium guajava* L. (Guava, Pera)	Leaf	69.2 ± 0.09
9	*Alpinia calcarata* Roscoe (Snap ginger, Chittaratha)	Leaf	67.25 ± 0.12
10	*Syzygium cumini* (L.) Skeels (Black plum, Njaval)	Seed	61.10 ± 0.26
11	*Gliricidia sepium* (Jacq.) Kunth (Quick stick, Sheemakkonna)	Seed	56.90 ± 0.09
12	*Gnetum ula* Brongn. (Joint Fir, odal)	Seed	55.60 ± 0.09
13	*Pterocarpus santalinus* Blanco (Red sandalwood, Rakthachandanam)	Leaf	52.49 ± 0.01
14	*Aerva lanata* (L.) Juss. ex Schult. (Mountain knot-grass, Cherula)	Leaf	43.40 ± 0.40
15	*Theobroma cacao* L. (Cocoa)	Leaf	34.31 ± 0.32
16	*Amorphophallus hohenackeri* Engl. & Gehrm.	Seed	20.30 ± 0.08
17	*Bombax ceiba* Burm.f. (Indian Bombax, Elavu)	Seed	16.12 ± 0.51
18	*Colocasia esculenta* (L.) Schott (Taro, Chembu)	Leaf	09.38 ± 0.02
19	*Pouteria campechiana* (Kunth) Baehni (Egg fruit, Muttappazham)	Seed	05.41 ± 0.13
20	*Adhatoda vasica* Nees (Aadalodakam, Adhatoda)	Leaf	00.91 ± 0.01
21	*Piper longum* Blume (Pipli, Thippali)	Leaf	0.05 ± 1.28

Values are Mean ± Standard Error (n=6)

The highest inhibition of 89.35 ± 0.10% was observed for leaves of *Chrysophyllum cainit*o. The other plants having greater than 70% inhibition include extract of *Dalbergia latifolia* (85.09 ± 0.40%), *Nephelium lappaceum* (83.45 ± 0.05%), *Cochlospermum religiosum* (82.65 ± 0.09%), *Annacardium occidentale* (79.23 ± 0.04%), *Samanea saman* (78.27 ± 0.08%) and *Mucuna pruriens* (71.34 ± 0.06%), (Table 4.1).

4.2 SCREENING OF PLANT EXTRACTS FOR PROTEASE INHIBITOR AGAINST LARVAL GUT PROTEASES OF *SPODOPTERA MAURITIA*

Among the 21 plants screened for trypsin inhibitor, the plant showing greater than 70% inhibition and available in sufficient quantities (5 numbers) were again screened (*Chrysophyllum cainit*o, *Nephelium lappaceum, Cochlospermum religiosum, Samanea saman, Mucuna pruritia*) for inhibition of larval gut proteases of *S. mauritia* using azocasein as substrate. Further screening of plant extract was done with gut extract directly as the trypsin inhibition is not directly related to gut enzyme inhibition in many cases. In this way a total of 60 plants were screened for PIs against larval gut proteases of *S. mauritia* and they were listed with their percentage inhibition in Table 4.2. Thus the total number of plant extracts screened for PIs is 76 and those screened against larval gut proteases of *S. mauritia* is 60 including the five plant extracts initially screened using trypsin.

Table 4.2 List of plants screened for protease inhibition against larval gut proteases of *S. mauritia* and their percentage inhibition

Sl. No.	Scientific Name / Vernacular name	Plant parts used	Mean % inhibition ± SE
1	*Abelmoschus moschatus* Medik. (Musk mellow, Kasthurivenda)	Seed	75.61 ± 0.08
2	*Chrysophyllum cainito* L. (Golden leaf tree, Star apple)	Leaf	75.56 ± 1.12
3	*Areca triandra* Roxb. (Triandra palm)	seed	73.33 ± 0.04
4	*Abelmoschus manihot* (L.) Medik. (Sunset musk mallow)	Seed	72.57 ± 0.04
5	*Calophyllum inophyllum* L. (Indian laurel, Punna)	Seed	69.62 ± 1.03
6	*Baccaurea courtallensis* Müll.Arg. (Mootikaya, Mootippuli)	Seed	68.62 ± 0.46
7	*Artocarpus heterophyllus* Lam. (Jackfruit, Plavu)	Seed	66.02 ± 0.91
8	*Connarus monocarpus* L (Zebra wood, Puzhukkadilkaya)	Seed	61.96 ± 0.33
9	*Spatholobus parviflorus* (DC.) Kuntze (Bando lata, Athambuvalli)	Seed	60.36 ± 0.39
10	*Acacia intsia* (L.) Willd. (Mala inja)	Seed	60.02 ± 0.01
11	*Samanea saman* (Jacq.) Merr. (Rain Tree, Mazhamaram)	Seed	58.77 ± 0.88
12	*Hibiscus aculeatus F.Dietr.* (Pineland Hibiscus)	Seed	55.06 ± 0.92
13	*Zapoteca formosa subsp. rosei* (Wiggins) *H.M.Hern.* (Powder puff plant)	Seed	54.56 ± 0.08
14	*Mallotus tetracoccus* Kurz (Rusty kamala, Thavittuvatta)	Seed	53.00 ± 1.77

15	*Hopea parviflora* Bedd. (White Kongu, Thambakam)	Seed	52.24 ± 0.92
16	*Albizia lebbeck* (L.) Benth. (Womans tongue, Nenmani vaka)	Seed	49.72 ± 0.15
17	*Mucuna pruriens* (L.) DC. (Velvet bean, Naikkuruna)	Seed	47.30 ± 3.46
18	*Cochlospermum religiosum*(L) Alston (Silk-cotton tree, Parappoola)	Seed	46.62 ± 0.06
19	*Crotalaria pallida* Aiton (Ding ding, Kilukkichedi)	Seed	46.56 ± 0.94
20	*Clitoria ternatea* L. (Clitoria, Shankupushpam)	seed	46.12 ± 0.06
21	*Nephelium lappaceum* L. (Rambuttan)	seed	45.66 ± 1.28
22	*Solanum torvum* Sw. (Turkey Berry, Aanachunda)	Seed	45.01 ± 0.04
23	*Persea americana* Mill. (Avacado pear, Vennappazham)	Seed	44.38 ± 0.09
24	*Mukia maderaspatana* (L.) M.Roem. (Mukkalppeeram)	Seed	43.88 ± 0.07
25	*Ipomoea cairica* (L.) Sweet (Railway creeper, Kolambipoo)	Seed	42.53 ± 1.20
26	*Albizia chinensis* Merr. (Sau tree, Pottavaka)	Seed	42.07 ± 0.04
27	*Ricinus communis* L. (Castor oil plant, Aavanakku)	Seed	41.77 ± 3.28
28	*Thunbergia alata* Bojer ex Sims (Black eyed Susan, Karimkanni)	seed	40.82 ± 0.05
29	*Croton tiglium* L. (Croton oil plant, Kadalavanakku)	Seed	39.01 ± 0.48
30	*Passiflora foetida* L. (Pop vine, Poochapazham)	Seed	37.43 ± 1.36
31	*Jacquemontia pentantha* (Jacq.)G.Don (Sky-blue cluster vine)	Seed	37.10 ± 0.12

32	*Xanthium indicum* J.Koenig ex Roxb. (Burweed, Ottukai)	Seed	37.12 ± 0.02
33	*Cleistanthus patulus* Müll.Arg. (Lance leaved discosus feather foil, Navamaram)	Seed	35.30 ± 0.08
34	*Sapindus saponaria* L. (Wing leaf Soapberry)	Seed	34.98 ± 0.05
35	*Crotalaria retusa* L. (Rattle weed, Kilukilukki)	Seed	34.41 ± 2.14
36	*Sauropus androgynus* Merr. (Chikurmanis, Velicheera)	Seed	34.80 ± 0.09
37	*Mimusops elengi* L. (Bullet wood tree, Ilanji)	Seed	33.48 ± 4.50
38	*Careya arborea* Roxb. (Patana oak, Pezhu)	Seed	33.36 ± 0.40
39	*Lagerstroemia speciosa* Pers. (Banaba, Manimaruthu)	Seed	29.10 ± 0.02
40	*Aphanamixis polystachya* (Wall.) R.Parker (Rohituka tree, Chemmaram)	Seed	28.25 ± 0.12
41	*Asystasia chelonoides* Nees (Murikootipacha)	Seed	27.21 ± 0.25
42	*Debregeasia longifolia* (Burm.f.) Wedd. (Orange Wild Rhea, Kattunochi)	Seed	26.79 ± 2.16
43	*Annona squamosa* Delile (Custard apple, Aathachakka)	Seed	25.32 ± 0.09
44	*Colubrina travancorica* Bedd.	Seed	21.20 ± 0.02
45	*Merremia umbellata* (L.) Hallier f. (Yellow Wood Rose, Kolavaravalli)	Seed	19.45 ± 0.08
46	*Indigofera tinctoria L.* (Black henna, Neelayamari)	Seed	18.83 ± 0.02
47	*Canavalia maritima* Thouars (Beach bean, Manalamara)	Seed	16.02 ± 1.25
48	*Geophila repens* (L.) I.M.Johnst. (Karinkudungal)	Seed	15.97 ± 0.31

49	*Atylosia scarabaeoides* (L.) Benth. (Wild Red Gram, Wild cajanas)	Seed	15.83 ± 0.09
50	*Senna tora* (L.) Roxb. (Foetid cassia, Ponthakara)	Seed	15.47 ± 3.37
51	*Clerodendrum infortunatum* L. (Hill Glory Bower, Perivelam)	seed	15.32 ± 0.22
52	*Rubus ellipticus* Sm. (Yellow Himalayan Raspberry, Cheemullu)	Seed	13.34 ± 0.02
53	*Fioria vitifolia* (L.) Mattei (Grape leaved Mallow, Kattuvelluram)	Seed	12.62 ± 1.83
54	*Aristolochia indica* L. (Garudakkodi)	Seed	12.53 ± 0.32
55	*Flacourtia jangomas* Steud. (Puneala plum, Lavalolikka)	Seed	12.27 ± 2.81
56	*Canthium angustifolium* Roxb. (Canthium, Kattakkaramullu)	Seed	12.18 ± 0.02
57	*Passiflora quadrangularis* Triana & Planch (Gaint granadilla, Akasavellari)	seed	11.18 ±1.8
58	*Rauvolfia serpentina* Benth. ex Kurz (Serpantina, Sarppagandhi)	Seed	10.54 ± 3.4
59	*Anethum graveolens* Ucria (Dill, Chatakuppa)	Seed	10.25 ± 1.45
60	*Allamanda neriifolia* Hook (Bush allamanda)	Seed	08.12 ± 0.85

Values are Mean ± Standard Error (n=6)

From the 60 plants screened against larval gut proteases of *S. mauritia* 15 plants showed greater than 50% inhibition (Table 4.2.). The highest percentage of inhibition was shown by *Abelmoschus moschatus* (75.61 ± 0.08%). Other plant extracts with more than 50% inhibition against gut proteases of *S. mauriti*a include *Chrysophyllum cainito* (75.56 ± 1.12%), *Areca triandra* (Roxb.) (73.33 ± 0.04%), *Abelmoschus manihot* (L) (72.57 ± 0.04%), *Calophyllum inophyllum*

(69.62 ± 1.03%), *Beccaurea courtallensis* (68.62 ± 0.46%), *Artocarpus heterophyllus* L (68.02 ± 0.91%), *Connarus monocarpus* (61.96 ± 0.33%), *Spatholobus parviflorus* (60.36 ± 0.39%), *Accasia intysia* (60.02 ± 0.01%), *Samanea saman* (Jacq.) Merr. (58.77 ± 7.88%), *Hibiscus auleatus* (55.06 ± 0.92%), *Zapoteca formosa* subsp. *rosei* (Wiggins) H.M. (54.56 ± 0.08 %), *Mallotus tetracoccus* (53.40 ± 1.77%) and *Hopea parviflorus* (52.24 ± 0.92%).

4.3 PROTEINASE K DIGESTION

Out of the 15 plants showing greater than 50% inhibition in the screening, 10 plants which were available in sufficient quantities were selected for proteinase K treatment to check whether the inhibitor is proteinaceous or non proteinaceous in nature. Table 4.3 shows the results of the proteinase K digestion of the selected plant extracts.

Table 4.3 Effect of Proteinase K treatment of plant extracts on *S. mauritia* gut protease inhibition

Sl No	Plant used	Control Mean% inhibition ± SE	Test Mean % inhibition ±SE	*p* value	Proteina-ceous inhibitor
1	*Chrysophyllum cainito* L.	75.1 ± 0.72	74.51 ± 0.75	0.12	-
2	*Baccaurea courtallensis Müll.Arg.*	72.16 ± 0.38	71.01 ± 0.38	0.11	-
3	*Areca triandra Roxb.*	65.61 ± 0.35	64.67 ± 0.31	0.07	-
4	*Abelmoschus manihot* (L.) Medik.	61.09 ± 0.45	20.78 ± 0.56	0.001	+
5	*Samanea saman* (Jacq.) Merr.	54.68 ± 0.45	18.50 ± 0.39	0.001	+
6	*Calophyllum inophyllum* L.	54.73 ± 0.47	52.79 ± 0.68	0.06	-
7	*Spatholobus parviflorus* (DC.) Kuntze	53.98 ± 1.03	15.16 ± 0.36	0.001	+
8	*Hopea parviflora* Bedd.	53.65 ± 0.77	49.72 ± 1.18	0.032	-
9	*Connarus monocarpus* L	52.29 ± 0.43	51.71 ± 0.27	0.08	-
10	*Acacia intsia* (L.) Willd.	43.34 ± 0.78	42.75 ± 0.29	0.07	-

Values are Mean ± Standard Error (n=6). Plus sign (+) indicates that the inhibitor is proteinaceous in nature, whereas minus (-) sign represents the inhibitor is non-proteinaceous one.

Plant extracts treated with proteinase K and after stopping the reaction, the protease inhibition assessed and compared with untreated.

Proteinase K treatment showed that the inhibitor from three plants are mainly proteinaceous in nature as major share of the inhibition is lost on proteinase K treatment. They include *Abelmoschus manihot* (L), *Samanea saman* (Jacq.) Merr and *Spatholobus*

parviflorus (DC.) Kuntze, the remaining seven may be non-proteinaceous in nature. Seven plants retained most of their inhibition even after proteinase K treatment indicating that the major inhibitor may not be a protein or is inhibiting proteinase K. They include *Chrysophyllum cainito, Beccaurea courtallensis, Areca triandra, Calophyllum inophyllum, Hopea parviflorus, Connarus monocarpus* and *Accasia intysia.* The protease inhibitor present in *Spatholobus parviflorus* (DC.) Kuntze seed extract is proteinaceous in nature. No protease inhibitor is reported from this plant and is showing considerable inhibition (60.36 ± 0.39%) towards the larval gut proteases of *S. mauritia.* Thus the seed extract from *Spatholobus parviflorus* was selected for further purification and characterization of the inhibitor.

4.4 PURIFICATION OF PROTEASE INHIBITOR FROM *SPATHOLOBUS PARVIFLORUS* SEEDS

Standard protein purification methods were employed for the purification of protease inhibitor. The inhibitory protein was purified up to homogeneity employing ammonium sulfate $(NH_4)_2SO_4$ precipitation, ion exchange chromatography, gel filtration chromatography, trypsin affinity chromatography and Immobilized Metal Affinity Chromatography.

4.4.1 Ammonium Sulfate precipitation

Soluble proteins in the seed extract of *Spatholobus parviflorus* were precipitated with three different ammonium sulfate concentrations (0-30%, 30-50% and 50-70%). Of the three different

ammonium sulfate fractions 30 to 50% ammonium sulphate fraction gave the highest inhibition of 59.92 ± 0.99% against the larval gut proteases of *S. mauritia* (Table 4.4.).

Table 4.4 Inhibition of larval gut protease activity of *S. mauritia* by different ammonium sulfate fractions of extract from seeds of *Spatholobus parviflorus*

Sl No.	Sample used	Protein concentration (mg/ml)	Mean % inhibition ± SE
1	Crude extract	10.60	57.36 ± 1.39
2	0-30 % $(NH_4)_2$ SO_4 fraction	6.25	49.28 ± 1.12
3	30-50 % $(NH_4)_2$ SO_4 fraction	7.43	55.92 ± 1.99
4	50-70 $(NH_4)_2$ SO_4 fraction	1.25	10.98 ± 0.08

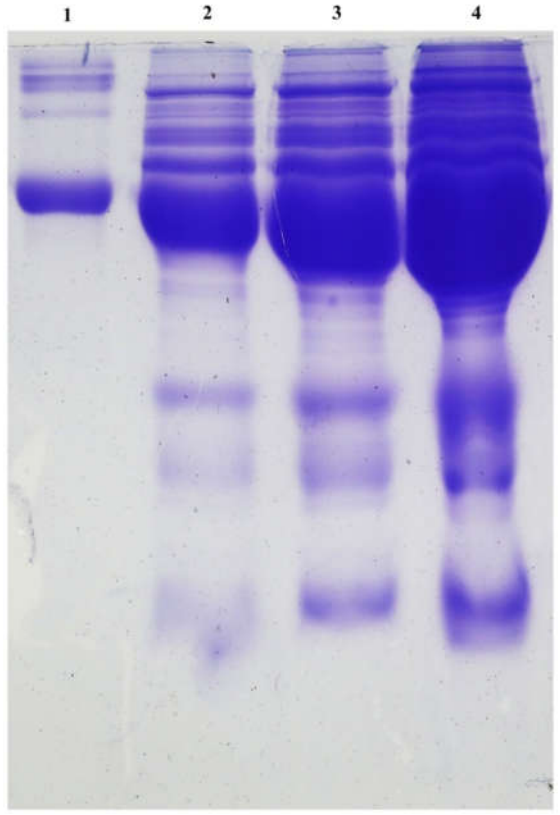
Values are Mean ± Standard Error (n=6)

Soluble protein from the seed extract of *Spatholobus parviflorus* was precipitated with different concentrations of ammonium sulfate and the inhibition of the fractions towards the larval gut protease of *S. mauritia* assessed.

Of these different fractions, 30-50% ammonium sulfate fraction got the highest percentage inhibition of 55.92 ± 1.99% followed by 0-30% fraction, (49.28 ± 1.12) and 50-70% fraction (10.98 ± 0.08). On ammonium sulfate fractionation (30–50%), there is 1.5 fold enrichment of the inhibitor protein compared to the crude extract on a protein basis. A representative figure of protein profile of the ammonium sulfate fractions on SDS-PAGE is shown in figure 4.1. The protein

profile of the ammonium sulfate fractions indicate that there are a number of protein bands with some of them having very high intensity.

Figure 4.1 SDS-PAGE of Ammonium sulfate fractions of *Spatholobus parviflorus* seed extract



10% SDS-PAGE of ammonium sulphate fractions from the seed extract of *S. parviflorus*. Gel was run under reducing conditions and stained with Coommassie Brilliant blue. Lane (1). BSA (1.5 µg), Lane (2). Crude Extract, Lane (3). 0-30% fraction, Lane (4). 30-50% fraction.

4.4.2 Ion Exchange Chromatography

Ammonium sulfate precipitate (30-50% fraction) after dialysis was subjected to ion exchange chromatography using DEAE Sephadex. In a typical experiment 10 ml (150 mg protein) of the

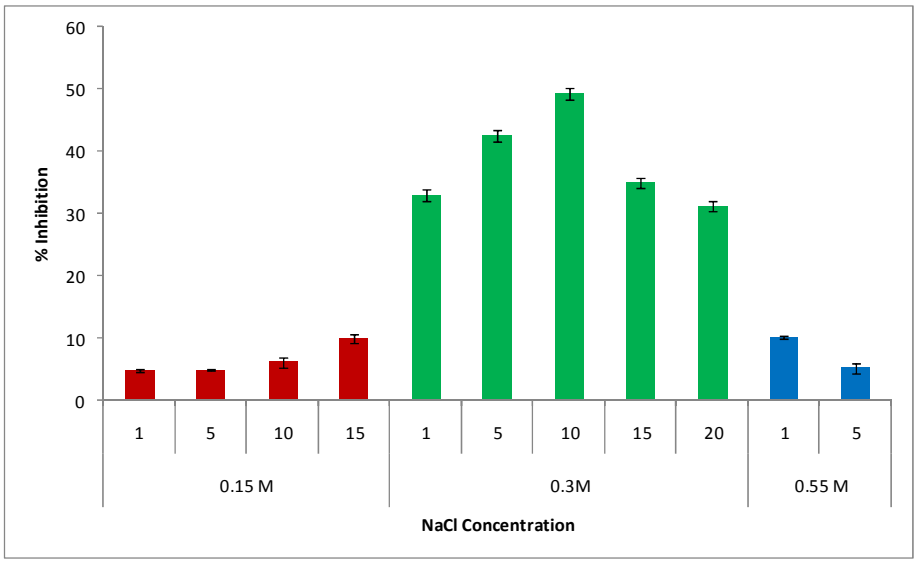
ammonium sulfate fraction in 20 mM Tris pH 8.0 containing 50 mM NaCl was loaded on to DEAE Sephadex column (25 X 1.5 cm). After loading the column, the flow was stopped for binding for 30-45 minutes. The column was washed with Tris buffer pH 8.0 containing 50 mM NaCl. The bound protein was eluted with 0.15M, 0.3 M, 0.55 M and 1.05 M and NaCl successively. Protease inhibition assay was carried out to identify fractions containing inhibitor and the maximum inhibition was observed in 0.3 M NaCl fractions. Table 4.5 and Figure 4.2 show the DEAE fractions with their percentage inhibition.

Table 4.5 Inhibition of larval gut protease activity by fractions eluted from DEAE- Sephadex column

NaCl Concentration	Eluted Fractions	Mean % inhibition ± SE
0.15 M	E1	4.89 ± 0.24
	E5	4.92 ± 0.15
	E10	6.18 ± 0.85
	E15	10.00 ± 0.64
0.3 M	E1	32.94 ± 0.98
	E5	42.40 ± 0.93
	E10	49.20 ± 0.95
	E15	35.00 ± 0.75
	E20	31.20 ± 0.87
0.55 M	E1	10.10 ± 0.25
	E5	5.20 ± 0.87

Values are Mean ± Standard Error (n=6)

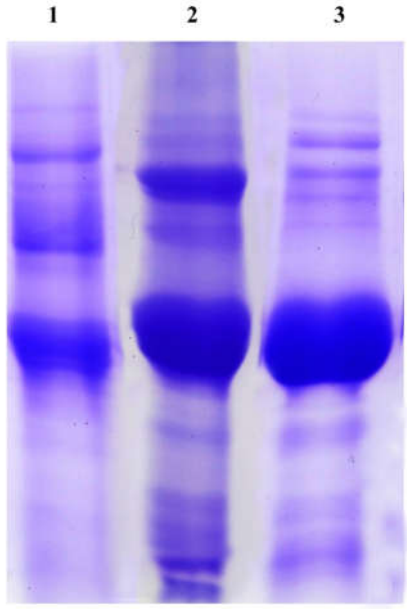
Figure 4.2 Histogram showing inhibition of larval gut protease activity by fractions eluted from DEAE- Sephadex column



Values are Mean ± Standard Error (n=6)

Peak fractions with inhibitor was pooled, concentrated using Amicon 3.0 kDa membrane and dialyzed against 20 mM Tris buffer containing 50 mM NaCl, pH 8.0 to remove excess NaCl. Figure 4.3 shows the protein profile on SDS-PAGE of protein after ion exchange chromatography.

Figure 4.3 SDS-PAGE of DEAE sephadex ion exchange fractions



10% SDS-PAGE of ion exchange fractions eluted from DEAE- Sephadex. Gel was run under reducing conditions and stained with Coommassie Brilliant blue. Lane (1). 0.15 M NaCl eluted fraction, Lane (2). 30-50% ammonium sulphate fraction (Load), Lane (3). 0.3 M NaCl eluted fraction.

4.4.3 Gel Filtration Chromatography

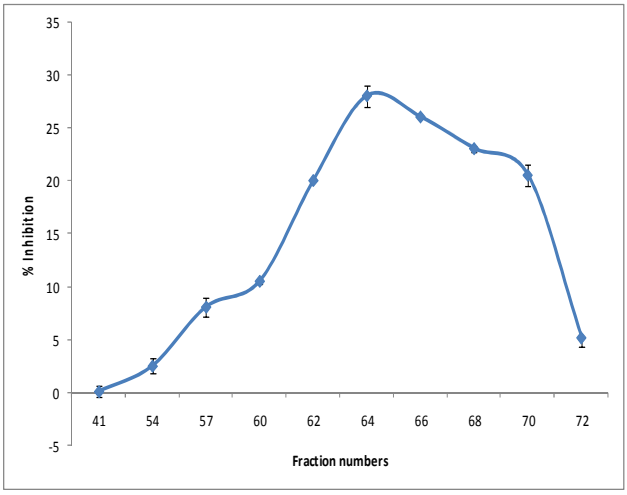
The inhibitory fractions obtained from ion exchange fractions pooled, concentrated and loaded on to Sephadex G-100 gel filtration column (60 x 1.8 cm). Table 4.6 and Figure 4.4 show the elution of inhibitor from the column as judged by protease inhibition assay. Protein profile of the fractions from gel filtration when separated by SDS-PAGE is shown in figure 4.5.

Table 4.6 Inhibition of fractions eluted from gel filtration chromatography

Fractions	Mean % inhibition ± SE
E 41	00.12 ± 0.52
E 54	2.53 ± 0. 74
E 57	8. 10 ± 0. 87
E 60	10.54 ± 0.25
E 62	20.05 ± 0. 05
E 64	28.05 ± 0.98
E 66	26. 05 ± 0.05
E 68	23. 06 ± 0.28
E 70	20.55 ± 0.98
E72	5.20 ± 0.87

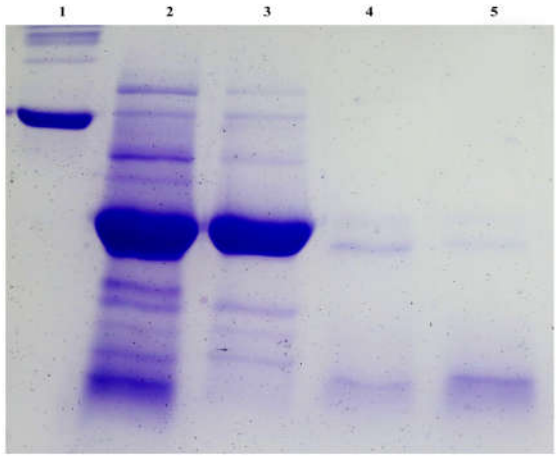
Values are Mean ± Standard Error (n=6)

Figure 4.4 Elution profile of fractions containing inhibitor from gel filtration chromotography



Values are Mean ± Standard Error (n=6)

Figure 4.5 SDS-PAGE of gel filtration fractions from Sephadex G-100 column



10% SDS-PAGE of gel filtration fractions from Sephadex G-100. Gel was run under reducing conditions and stained with Coommassie Brilliant blue. Lane (1). BSA (1 µg), (Lane 2). 0.3 M NaCl fraction from DEAE Sephadex (load) (Lane 3). 41st fraction, Lane (4). 60th fraction, Lane (5). 64th fraction.

Gel filtration chromatography did not resulted in pure protein as judged from SDS-PAGE and the quantity of inhibitor obtained was low (Figure 4.5). Thus for further purification of the inhibitor, trypsin affinity chromatography of the DEAE-Sephadex eluted protein was done.

4.4.4 Trypsin Affinity Chromatography

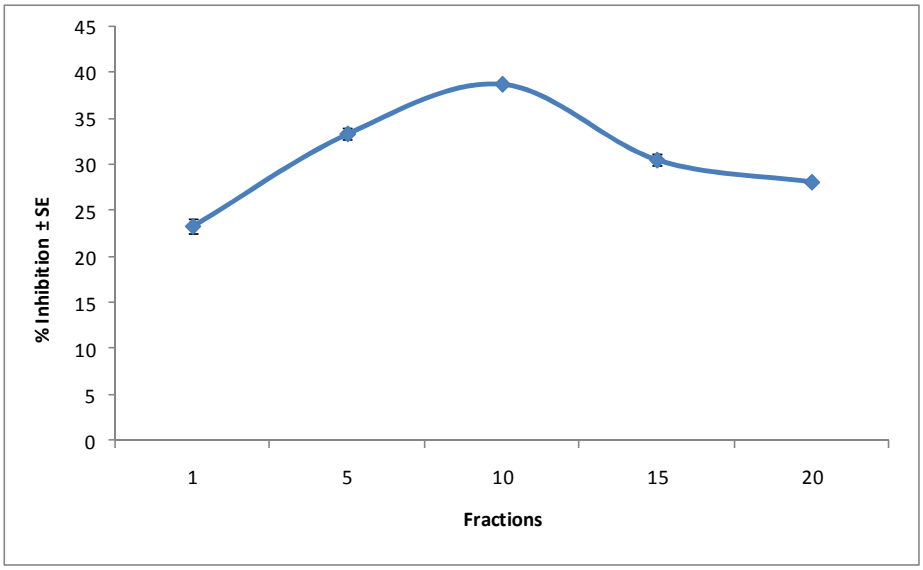
Fractions with inhibition against gut proteases of *S. mauritia* from DEAE-Sephadex ion exchange column pooled and concentrated (15 mg) and loaded on to trypsin affinity chromatography column. The bound inhibitor from affinity column was eluted with 5 mM HCl and immediately neutralized with 0.5 M NaOH. Table 4.7 and Figure 4.6 show the trypsin affinity column eluted fractions with their percentage inhibition.

Table 4.7 Trypsin affinity eluted fractions and their percentage inhibition towards larval gut protease

Fraction	Mean % inhibition ± SE
E1	23.33 ± 0.86
E5	33.38 ± 0.58
E10	38.78 ± 0. 65
E15	30 .54 ± 0.58
E20	28.12 ± 0. 23

Values are Mean ± Standard Error (n=6)

Figure 4.6 Elution profile of fractions containing inhibitor from trypsin affinity chromatography



Values are Mean ± Standard Error (n=6)

The fractions from trypsin affinity chromatography were pooled, dialyzed against Tris buffer pH 8.0 and concentrated. Table 4.8 shows percentage inhibition of concentrated trypsin affinity elute

(1mg/ml) towards larval gut proteases of *S. mauritia* and against trypsin.

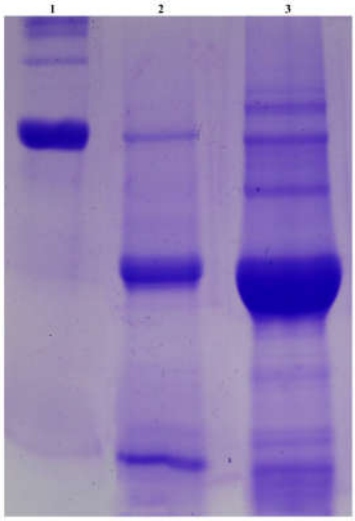
Table 4.8 Inhibition of trypsin and larval gut proteases by elute from trypsin affinity chromatography

Sample	% Inhibition of trypsin	% Inhibition of larval gut protease
Concentrated Trypsin affinity elute (1 mg/ml)	76.56 ± 0.26	52.45 ± 0.87

Values are mean ± SE (n=6)

The inhibitory fractions obtained from trypsin affinity chromatography yielded 4 bands on SDS-PAGE (Figure 4.7).

Figure 4.7 SDS - PAGE of pooled and concentrated fractions from trypsin affinity chromatography



10% SDS-PAGE of of pooled and concentrated fractions from trypsin affinity chromatography. Lane (1). BSA (5 µg), Lane (2). Trypsin affinity elute (10 µg), Lane (3). 0.3 M NaCl elute (50 µg) from ion exchange chromatography (Load).

4.4.5 Immobilized Metal Affinity Chromatography (Ni - IMAC)

In order to remove the contaminating proteins, the eluted inhibitory fraction from trypsin affinity chromatography was loaded on to Ni-IMAC resin. The inhibitor protein came out in the flow through as it did not bind to the resin but the contaminating proteins bound to the resin. The collected flow through was concentrated and used for the protease inhibition assay against both trypsin and the larval gut proteases. Table 4.9 shows the percentage inhibition of trypsin and the larval gut proteases by the inhibitor purified using Ni- IMAC.

Table 4.9 Inhibition of trypsin and larval gut proteases by protein purified using Ni-IMAC column

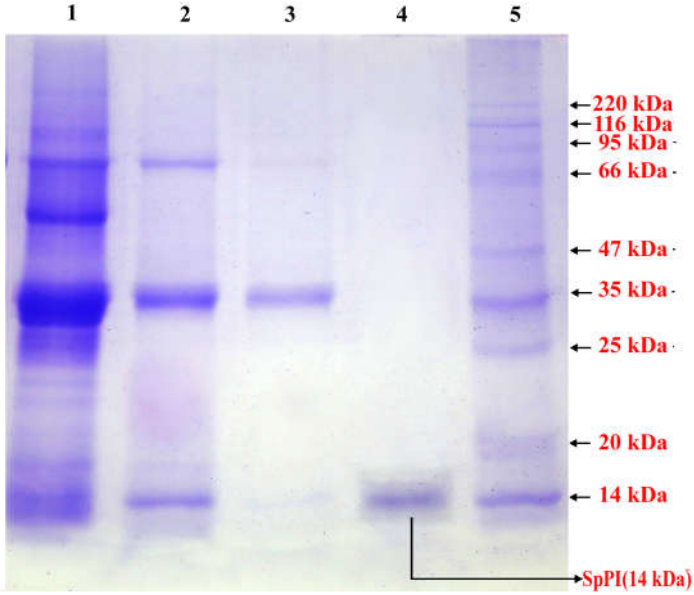
Sample	% Inhibition of trypsin	% Inhibition of larval gut protease
Flow through (pure protein) (0.1 µg/µl)	70.12 ± 0.31	50.00 ± 0.12

Values are Mean ± Standard Error (n=6)

The concentration of purified protease inhibitor from *Spatholobus parviflorus* required for 50% inhibition of larval gut proteases of *S. mauritia* was found to be 0.1 µg/µl. This experiment was done with gut extract protein concentration of 0.6 µg/µl and gave an absorbance of 0.4 in the azocasein assay. This is equivalent to the absorption given by 0.1 µg/µl trypsin. The purified inhibitor inhibited trypsin to the extent of 70.12%. The purified inhibitor obtained from Ni-IMAC resin which inhibited larval gut proteases of *S. mauritia* was subjected to SDS-PAGE and Acid PAGE.

The purity of the inhibitor is checked by SDS-PAGE and the inhibitor appeared as a single protein band confirming its purity and homogeneity (Figure 4.8). The purified protease inhibitor from *Spatholobus parviflorus* is named as *Spatholobus parviflorus* Protease Inhibitor (SpPI).

Figure 4.8 SDS-PAGE of elute from ion exchange, trypsin affinity and purified inhibitor



10% SDS-PAGE of inhibitor eluted from ion exchange column, trypsin affinity column and IMAC column. Gel was run under reducing conditions and stained with Coommassie Brilliant blue. Lane (1). 0.3 M NaCl elute of ion exchange chromatography, Lane (2). Trypsin affinity elute (Load), Lane (3). IMAC Elute, Lane (4). IMAC Flow through, Lane (5). Protein Marker

4.5 CHARACTERIZATION OF *SPATHOLOBUS PARVIFLORUS* PROTEASE INHIBITOR (SpPI)

4.5.1 Electrophoresis

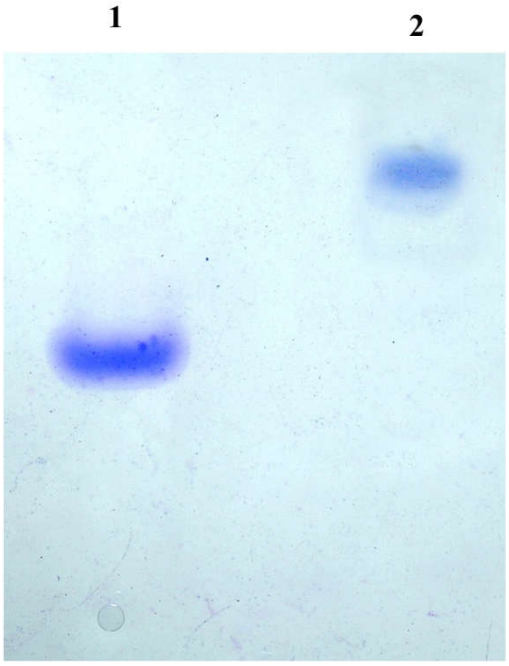
4.5.1.1 Sub unit molecular weight determination of isolated plant protease inhibitor

The sub unit molecular weight of the isolated inhibitor was determined from SDS-PAGE from a plot of log molecular weight of molecular weight marker and their relative mobility and the relative mobility of the inhibitor. The sub unit molecular weight was found to be 14 kDa (Figure 4.8).

4.5.1.2 Native PAGE (Acid PAGE)

Native Acid PAGE was done to know whether the inhibitor moves in the acid PAGE. It is running above trypsin in Acid PAGE indicating that it may be multimer (Figure 4.9). As the yield was low enough protein was not available for determination of native molecular weight by gel preparation. In alkaline PAGE no clear band is observed for SpPI (Data not shown).

Figure 4.9 Acid PAGE of purified inhibitor from *Spatholobus parviflorus* seed extract

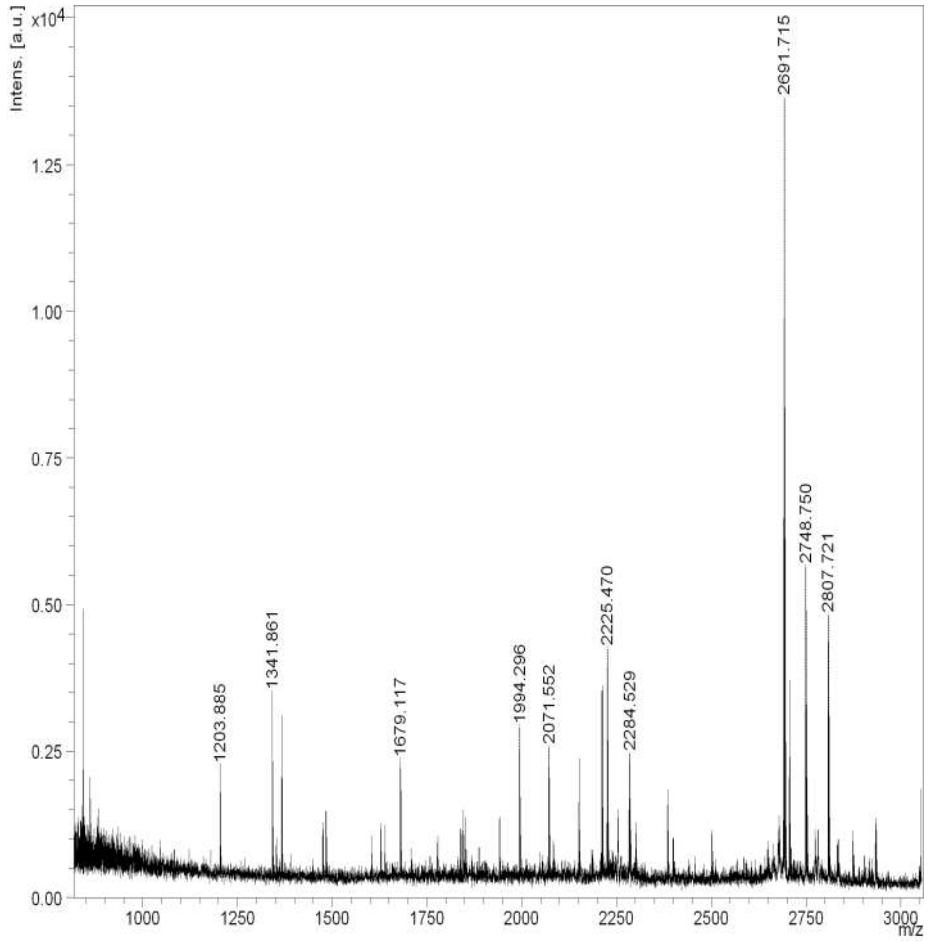


Acid PAGE was done with 7.5% separating gel and fixed and stained. Lane (1). 5 µg trypsin, Lane (2). Purified inhibitor.

4.5.2 LC-MS/MS analysis of purified protein

LC-MS/MS analysis of SDS-PAGE separated protein revealed that it is a new protein as the mass spectrometry data is not matching with data from other reported proteins in the data base. Figure 4.10 (a) and Figure 4.10 (b) show the LC-MS/MS spectrum of the purified inhibitor and table 4.10 shows the mass list of the peptides from the purified inhibitor.

Figure 4.10 (a) LC-MS/MS spectrum of the peptides from the purified inhibitor



LC-MS/MS spectrum of the peptides from the purified inhibitor.

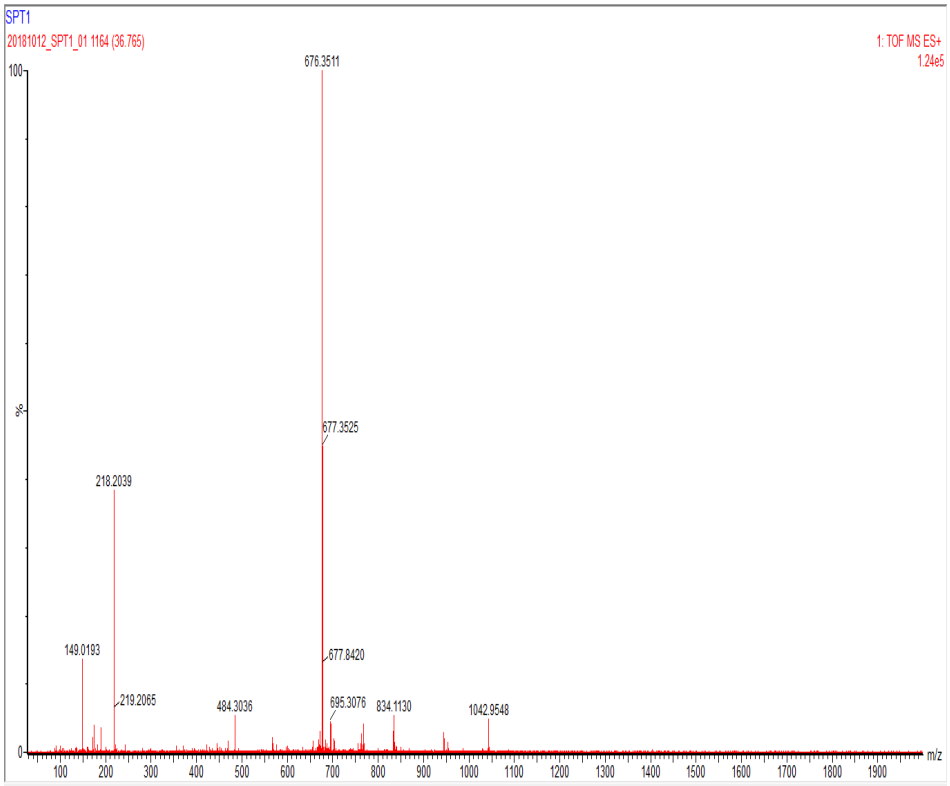
Acquisition Parameter

Date of acquisition	:	2018-09-19T11:42:12.015+05:30
Acquisition method name	:	D:\Methods\flexControlMethods\Specification\ RP_700-3500_Da.par
Aquisition operation mode	:	Reflector
Voltage polarity	:	POS
Number of shots	:	1000

Instrument Info

User	:	BDALDE
Instrument	:	FLEX-PC
Instrument type	:	ultraflexTOF/TOF

Figure 4.10 (b) LC-MS/MS spectrum of the peptides from the purified inhibitor (initial region expanded)



LC-MS/MS spectrum of the peptides from the purified inhibitor (initial region expanded).

Acquisition Parameter

Date of acquisition	:	2018-09-19T11:42:12.015+05:30
Acquisition method name	:	D:\Methods\flexControlMethods\Specification\ RP_700-3500_Da.par
Aquisition operation mode	:	Reflector
Voltage polarity	:	POS
Number of shots	:	1000

Instrument Info

User	:	BDALDE
Instrument	:	FLEX-PC
Instrument type	:	ultraflexTOF/TOF

Table 4.10 LC-MS/MS mass list of peptides from purified inhibitor

m/z	time	Intens.	SN	Quality Fac.	Res.	Area	Rel. Intens.	FWHM	Chi^A2	Bk. Peak
842.675	45945.89	4211.774	12.502	1339.873	10070.455	585.288	0.607	0.084	43469.081	0
1203.885	54833.06	2139.854	7.741	1118.597	12132.373	426.182	0.308	0.099	16967.685	0
1341.861	57865.00	2990.645	12.234	2046.967	13264.108	682.943	0.431	0.101	26668.890	0
1365.975	58378.54	2711.855	11.131	216.206	13094.910	636.371	0.391	0.104	204973.108	0
1679.117	64674.44	2079.614	8.650	1509.187	9058.995	1036.936	0.300	0.185	47169.097	0
1994.296	70439.85	1929.353	8.401	1380.280	9620.938	1205.523	0.278	0.207	63371.049	0
2071.552	71781.72	1871.944	8.450	1901.794	10679.933	1097.658	0.270	0.194	40692.413	0
2211.444	74149.18	2568.847	12.715	1869.238	9657.788	1828.207	0.370	0.229	129116.003	0
2225.470	74382.34	2739.585	13.714	4239.341	11016.438	1751.347	0.395	0.202	94636.905	0
2284.529	75356.16	1503.690	7.405	870.049	7638.037	1353.935	0.217	0.299	43238.880	0
2691.715	81752.37	6940.386	44.509	3363.109	9884.742	6805.012	1.000	0.272	910788.367	0
2693.660	81781.71	3529.016	21.916	266.425	43948.624	765.055	0.508	0.061	526582.195	0
2705.597	81961.55	1547.740	9.022	672.454	9067.740	1484.937	0.223	0.298	78786.789	0
2748.750	82608.42	2984.994	19.014	964.710	9896.106	2979.517	0.430	0.278	248836.276	0

Table 4.11. shows the peptide list from the purified inhibitor. The peptide sequence analysis showed a Kunitz type sequence in the inhibitor indicationg that the SpPI is a Kunitz type serine protease inhibitor.

Table 4.11 LC-MS/MS Data Peptide List

Peptide identifier	Ions used in quantitation	Ions	Deconvoluted peptide ions	Deconvoluted charges	Retention time (min)	Neutral mass	Best identification Score	Sequence	Accession	Description	Maximum CV	Normalized abundance		Spectral counts	
												SPT1		SPT1	
												20181012_SPT1_01	20181012_SPT1_02	20181012_SPT1_01	2018 1012_SPT1_02
38.76_1364.7532n	3	3	#2013,#13,#1251	1,2,3	38.7598333	1364.753228	5.9767	NGGTYYILPVIR	I1KYW5	Uncharacterized protein OS=Glycine max OX=3847 GN=100500599 PE=4 SV=1	4.69573	63242.06685	67586.0637	0	2
38.74_483.3182n	2	2	#184,#864	1,2	38.744425	483.3182495	0	PVIR	I1KYW5	Uncharacterized protein OS=Glycine max OX=3847 GN=100500599 PE=4 SV=1	6.57925	3228.391166	3543.43198	0	2
38.76_596.4005n	1	1	#981	1	38.7598333	596.4004731	0	LPVIR	I1KYW5	Uncharacterized protein OS=Glycine max OX=3847 GN=100500599 PE=4 SV=1	9.78393	324.9102772	373.208057	0	1
36.80_1350.7408n	1	1	#38	2	36.7956167	1350.740808	7.3702	NGGTYYVLPVIR	B1ACD0	Kunitz trypsin inhibitor p20-1-like protein OS=Glycine max OX=3847 PE=2 SV=1	4.19653	25884.03013	27467.1728	1	1
36.80_483.3181n	1	1	#764	1	36.7956167	483.318074	0	PVIR	B1ACD0	Kunitz trypsin inhibitor p20-1-like protein OS=Glycine max OX=3847 PE=2 SV=1	10.5046	515.4512692	598.169881	1	1

Cont…

Continued from page from table 4.11

Peptide identifier	Ions used in quantitation	Ions	Deconvoluted peptide ions	Deconvoluted charges	Retention time (min)	Neutral mass	Best identification Score	Sequence	Accession	Description	Maximum CV	Normalized abundance		Spectral counts	
												SPT1		SPT1	
												20181012_SPT1_01	20181012_SPT1_02	20181012_SPT1_01	20181012_SPT1_02
36.80_754.3318n	1	1	#1694	1	36.7956167	754.3317887	0	NGGTYYV	B1ACD0	Kunitz trypsin inhibitor p20-1-like protein OS=Glycine max OX=3847 PE=2 SV=1	10.9175	168.106187	196.232641	0	1
41.79_1992.9737n	1	1	#148	3	41.7899167	1992.97374	5.1514	HPTAGIDYFEAHVPPQSK	A0A2K3LH66	Uncharacterized protein OS=Trifolium pratense OX=57577 GN=L195_g033858 PE=4 SV=1	26.7743	12431.8308	8473.89474	0	1
34.04_2207.0935n	2	2	#540,#1187	3,4	34.0430167	2207.093467	3.9707	DLVNNYYLFYIVTMHFR	A0A2K3LH66	Uncharacterized protein OS=Trifolium pratense OX=57577 GN=L195_g033858 PE=4 SV=1	0.19253	2338.345283	2331.98702	0	1
37.74_2283.1772n	1	1	#43	3	37.73795	2283.177177	5.6428	VTVNDFVYSGLATAGNTSNIIK	V7AYN3	Uncharacterized protein OS=Phaseolus vulgaris OX=3885 GN=PHAVU_009G227800g PE=3 SV=1	0.08209	93479.80998	93588.3969	1	0

Table 4.12 LC-MS/MS protein data list

Accession	Peptide count	Unique peptides	Confidence score	Mass	Description	Normalized abundance	
						SPT1	
						20181012_SPT1_01	20181012_SPT1_02
A0A2K3LH66	2	2	9.1221	21266.8062	Uncharacterized protein OS=Trifolium pratense OX=57577 GN=L195_g033858 PE=4 SV=1	7385.088	5402.941
B1ACD0;A0A0B2P2H2;C6T586;Q39869;Q9XIS8	3	3	7.3702	22847.8886	Kunitz trypsin inhibitor p20-1-like protein OS=Glycine max OX=3847 PE=2 SV=1	8855.863	9420.525
I1KYW5;A0A0B2P2F7;C6T280	3	3	11.9534	22397.5625	Uncharacterized protein OS=Glycine max OX=3847 GN=100500599 PE=4 SV=1	22265.12	23834.23
V7AYN3	1	1	5.6428	21891.0478	Uncharacterized protein OS=Phaseolus vulgaris OX=3885 GN=PHAVU_009G227800g PE=3 SV=1	93479.81	93588.4

Table 4.12 shows the protein data list and indicates the similarity of SpPI to Kunitz type protease inhibitor from like *Glycine max* even though they differ in molecular weight.

4.5.3 Determination of optimum pH for inhibition

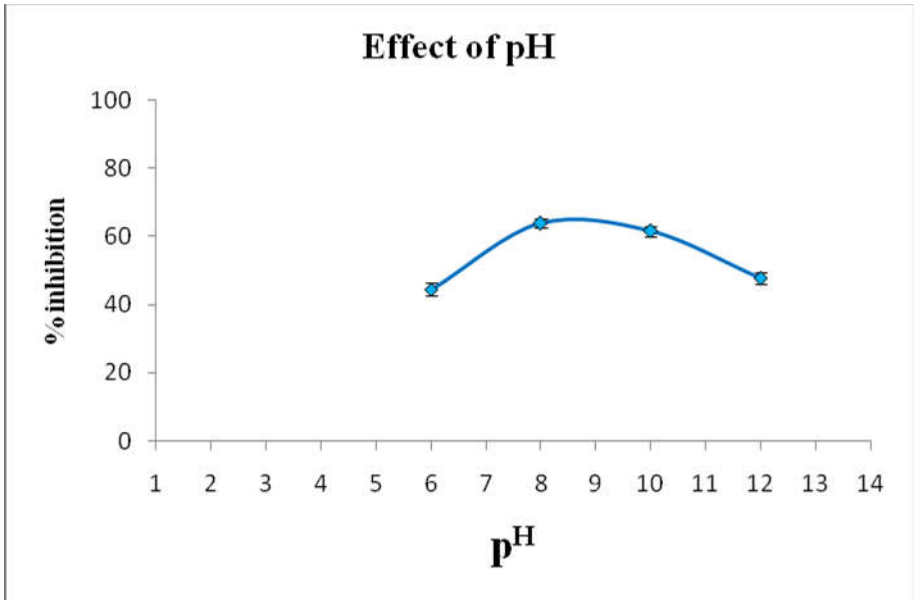
Optimum pH for protease inhibitor for maximal inhibition was determined by evaluating the inhibiton at different pH values, with appropriate controls. Four different pH (6.0, 8.0, 10.0 and 12.0) values were used for the experiment. Table 4.13 and Figure 4.11 show the inhibition at different pH values.

Table 4.13 Effect of different pH on inhibition of gut proteases of *S. mauritia* larvae by the inhibitor

pH	% inhibition Mean ± SE
6.0	44.5 ± 1.8
8.0	63.81 ± 1.4
10.0	61.46 ± 1.6
12.0	47.56 ± 1.7

Values are Mean ± Standard Error (n=6)

Figure: 4.11 Effect of different pH on inhibition of gut proteases of *S. mauritia* larvae by the inhibitor



Values are Mean ± Standard Error (n=6)

It was observed that the optimum pH for maximal percentage inhibition (63.81 ± 1.4%) was at pH 8.0, above and below pH 8.0 the inhibition decreases gradually as shown in figure (4.11).

4.5.4 Thermal stability of the inhibitor

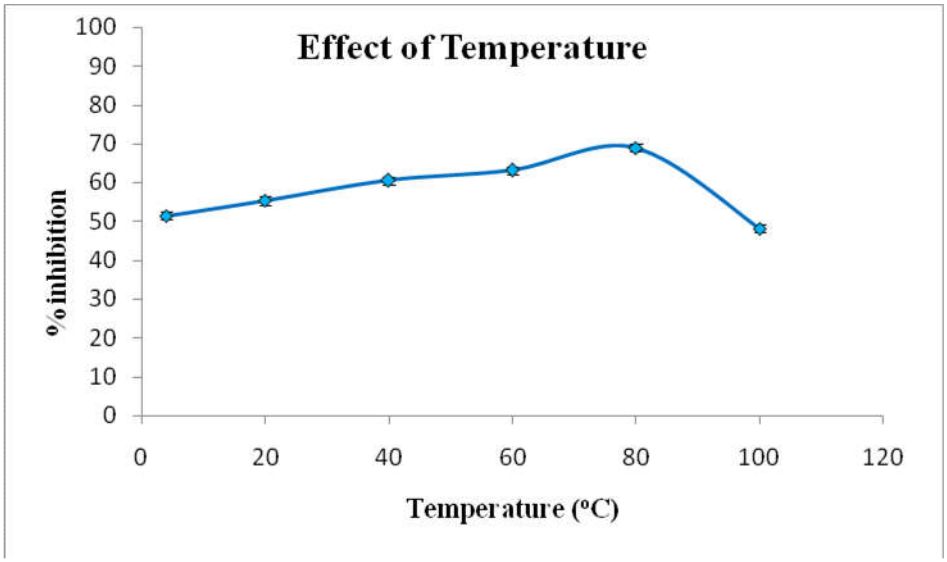
Temperature stability of the inhibitor at different temperatures was evaluated by incubating protease inhibitor at different temperatures ranging from 4 ^{0}C to 100 ºC for 30 minutes. Table 4.14 and Figure 4.12 show the stability of the inhibitor at different temperatures.

Table 4.14 Effect of temperature on stability of the protease inhibitor

Temperature	% inhibition Mean ± SE
4	51.43 ± 1.2
20	55.3 ± 1.18
40	60.5 ± 1.23
60	63.08 ± 1.14
80	68.87 ± 1.3
100	48.08 ± 2.42

Values are Mean ± Standard Error (n=6)

Figure 4.12 Effect of temperature on stability of the protease inhibitor



Values are Mean ± Standard Error (n=6)

The maximum inhibition of 68.87 ± 1.3% was observed at 80^0 C and above and below 80^0 C the inhibition declines gradually. This indicates that the inhibitor is heat stable up to 80^0 C, but retains 48.08 ± 2.42% of inhibition even at 100 ^{0}C.

DISCUSSION

5.1 SCREENING OF PLANT EXTRACTS FOR PROTEASE INHIBITOR AGAINST LARVAL GUT PROTEASES OF *SPODOPTERA MAURITIA* AND PROTEINASE K TREATMENT

In the beginning of our screening we used trypsin for screening of plant extracts to shortlist the plants with trypsin inhibition as a preliminary screening step for screening with gut extract. But we found that in many cases where high trypsin inhibition is found, the same extract inhibited the gut extract to a lesser extent. This may be due to the presence of other enzymes in the gut extract not inhibited by the inhibitor. Thus we continued screening plant extracts directly with the gut extract.

Of the 60 plant extracts screened for inhibition of gut proteases of *Spodoptera mauritia* 15 plants showed greater than 50% inhibition of gut protease activity. The highest inhibition (75.61 ± 0.08%) is shown by the seed extract of *Abelmoschus moschatus* Medik. Four trypsin inhibitors with molecular weights 22.4, 21.2, 20.8 and 20.2 kDa are purified and characterized from this plant (Dokka *et al.,* 2015). The 21.2 kDa protein is found to have antimicrobial activity as well (Dokka *et al.,* 2015). Another species from this genus, *Abelmoschus manihot* (L) also contain inhibitor against gut proteases of *S. mauritia*. Its inhibition (72.57 ± 0.04%) is similar to that of *A. moschatus*. Proteinase K treatment of *A. manihot* indicates that the major

inhibitor/s is proteinaceous in nature. This plant was not selected for purification of the inhibitor as it is likely that it may have similar inhibitors reported from *A. moschatus*. Though there are reports of protease inhibitors from other species of *Abelmoschus*, no protease inhibitor were reported from *A. manihot*. Jain *et al.,* reported wound healing property of methanolic and petroleum extracts of *A. manihot* in Wistar albino rats (Jain *et al.,* 2009).

The other 13 plants having greater than 50% inhibition are *Chrysophyllum cainito* L. (75.56 ± 1.12%), *Areca triandra* Roxb. (73.33 ± 0.04%), *Calophyllum inophyllum* L. (69.62 ± 1.03%), *Baccaurea courtallensis* Müll.Arg. (68.62 ± 0.46%), *Artocarpus heterophyllus* Lam. (68.02 ± 0.91%), *Connarus monocarpus* L (61.96 ± 0.39%), *Spatholobus parviflorus* (DC.) Kuntze (60.36 ± 0.39%), *Acacia intsia* (L.) Willd. (60.02 ± 0.01%), *Samanea saman* (Jacq.) Merr. (58.77 ± 7.88%), *Hibiscus aculeatus* F.Dietr. (55.06 ± 0.92%), *Zapoteca formosa* subsp. *rosei* (Wiggins) (54.56 ± 0.08%), *Mallotus tetracoccus* Kurz (53.00 ± 1.70%) and *Hopea parviflora* Bedd. (52.24 ± 0.92%).

Second highest percentage inhibition was found in the leaf extract of *Chrysophyllum cainito* L. (75.56 ± 1.12%). The inhibitor in this extract may be non proteinacious in nature as its inhibition is unaffected by proteinase K treatment. The possibility of inhibiting proteinase K need to be ruled out. Ethanol extract from the leaves of *Chrysophyllum cainito* have antioxidant activity and gallic acid is one of the major compounds present in the leaf extract (Ningsih *et al.,*

2016, Shailajan and Gurjar, 2014). But no protease inhibitor was reported from this plant.

Areca triandra inhibited gut extract of *S. mauritia* to the extent of 73.33 ± 0.04%. Extract from another species of Areca (*Areca catcheu*) is reported to inhibit gut proteases of *S. mauritia*. (Abhilash and Kannan, 2012). *Calophyllum inophyllum* seed extract gave 69.62 ± 1.03% inhibition towards gut proteases of *S. mauritia*. HIV-1 protease and HIV-1 integrase enzyme inhibitor was reported from the ethanolic and water extract of *C. inophyllum* (Narayan *et al.,* 2011). The chloroform extract of *C. inophyllum* showed a promising larvicidal activity against *Culex quinquefasciatus* (Rana *et al.,* 2017). Antibacterial, antifungal as well as repellent activities were also reported from this plant. No protease inhibitors against larval gut proteases of *S. mauritia* were reported from the extract of *C. inophyllum.* Proteinase K treatment of *A. triandra* and *C. inophyllum* indicates that inhibitor in these plants may be non-proteinaceous in nature.

Beccaurea courtallensis Müll. Arg. extracts inhibits 68.62 ± 0.46% of larval gut protease activity of *S. mauritia.* Methanol and benzene extract of *B. courtallensis* showed sensitivity against *E.coli and Staphylococcus aureus* (Abhishek *et a*l., 2011). Even after proteinase K treatment, the extract from *B. courtallensis* retains its inhibitory activity revealing that the inhibitor may be non-protein in nature. No plant protease inhibitors were reported from this plant. The percentage of inhibition by *Artocarpus heterophyllus* Lam. was 68.02 ± 0.91%. A novel 14.5 kDa cysteine protease inhibitor was isolated

and purified from mature jack fruit (*Artocarpus heterophyllus*) seed and it strongly inhibit midgut proteases of yellow stem borer (*Scipophaga incertulas*) larvae (Shamim and Singh, 2011). A Trypsin/ chymotrypsin inhibitor was isolated from jack fruit seed (*Artocarpus integrifolia*) (Annapurna *et al.,* 1991). A 26 kDa protease inhibitor was isolated by Bhat and Pattabiraman from the jack fruit seed (*Artocarpus integrifolia)* (Bhat and Pattabiraman, 1989). Presence of PIs which inhibits larval gut proteases of *S. mauritia* was reported from the seeds of *Artocarpus integrifolia* by Abhilash and Kannan (Abhilash and Kannan, 2012).

Connarus monocarpus L seed extract showed 61.96 ± 0.39 % inhibition towards the gut proteases of *S. mauritia*. 'Rapanone' and 'Bergenin' were isolated from the root extract of *C. monocarpus* (Aiyar *et al.,* 1964). Costa *et al.,* reported Leishmanicidal and antifungal properties of *C. suberosus* extract (Costa *et al.,* 2014). Proteinase K treatment indicates that the inhibitor from the *C. monocarpus* extract may be non- proteinaceous in nature. Extract from the seeds *of Acacia intsia* (L.) Willd. showed 60.02 ± 0.01% inhibition towards the larval gut enzyme. *A. pennata* were used by some tribes for the treatment of gastrointestinal infections and Lalchhandama observed a significant mortality of the poultry tapeworm compared to the control (Lalchhandama, 2013). Kannan *et al*., reported the hepatoprotective action of the extract from the *A. nilotica* against acetaminophen-induced hepatocellular damage in the Wistar rats (Kannan *et al*.2013). Extract from aerial parts of *A. ferruginea* regulates the inflammatory mediators like TNF-, IL-2, IL-6 etc there by inhibits tumor progression

(Sakthivel and Guruvayoorappan, 2013). Anticancer activities were reported from the *A.nilotica* extract (Sakthivel *et al.,* 2012). No protease inhibitors were reported from *C. monocarpus* and *A. intsia* seed extract. Proteinase K treatment indicates the inhibitor from these two plant extracts were non-proteinaceous in nature.

Hibiscus aculeatus F. Dietr seed extract inhibits larval gut proteases of *S. mauritia* to an extent of 55.06 ± 0.92%. Gastro protective and antioxidant activities of the ethanolic extract from the roots of *H. aculeatus* were reported in rats (Sunilson, *et al.,* 2008).The aqueous extract of *H. rosasinensi*s roots showed highly significant dose-dependent anti-ulcer activity in albino rat (Kumari *et al.,* 2010). Seed extract of *Calliandra rosei* Wiggins inhibits 54.56 ± 0.08 % of larval gut protease activity. Insecticidal activity of *Calliandra* species (*C. angustifolia* and *C. haematocephala*) against *Spodoptera frugiperda* was shown, when ground leaf powders and pure compounds extracted from leaves incorporated into the artificial diet (Romeo, 1984). Shaheen *et al.,* reported the antiviral activity of methanolic extract from *Calliandra haematocephala leaves* (Shaheen *et al.,* 2014). No protease inhibitor against larval gut proteases of *S. mauritia* were reported from the seeds of *Hibiscus aculeatus* and *Calliandra rosei*.

Mallotus tetracoccus Kurz and *Hopea parviflora* Bedd. inhibited the gut enzyme of *S. mauritia* larvae to the extent of 53.00 ± 1.31% and 52.24 ± 0.92% respectively. Antioxidant properties against DPPH (2, 2-diphenyl-1-picrylhydrazyl radical) were reported from the bark of *Mallotus philippinensis* (Arfan, 2009). Bhat *et al.,* reported the

antimicrobial activityof *Hopea parviflora* extract against *Staphylococcus areus* (Bhat *et al.,* 2009). No protease inhibitors were reported from *Mallotus tetracoccus* and *Hopea parviflora*.

Among the plant extracts with greater than 50% inhibition, the inhibitor is proteinaceous in nature and without any reports of presence of protease inhibitor is *Spatholobus parviflorus* seed extract. The extract from *Spatholobus parviflorus* seed inhibited gut protease activity to the extent of 60.36 ± 0.39%. Thus *Spathalobus parviflorus* seed was selected for the purification of the protease inhibitor. The other plant with more than 50% inhibition with proteinaceous inhibitor is *Samanea saman*. But this plant is reported to contain a 17.89 kDa chymotrypsin inhibitor (Maqtari and Saad, 2010).

Spatholobus parviflorus (DC.) Kuntze is a woody climber belonging to the Fabaceae family and is commonly known as "Valliplash" or "Athambuvalli". The alcoholic leaf extract of *Spatholobus parviflorus* have antioxidant, antibacterial and cytotoxic activity (Jesy and Jose, 2017). Nine bioactive compounds were identified from the ethanolic leaf extracts of *S. parviflorus* (Madhavan, 2015). A lectin, named SPL was purified from the seeds of *S. parviflorus* and it was noticed that purified lectin agglutinated human erythrocytes of all ABO blood groups. Antifungal activity against *A. niger* and *Fusarium* sp was also shown by SPL (Geethanandan *et al.,* 2013).

Spatholobus parviflorus (DC.) Kuntze seeds were used for isolation of protease inhibitor.

5.2. PURIFICATION OF PROTEASE INHIBITOR FROM S*PATHOLOBUS PARVIFLORUS*

Even though there are other plants showing higher percentage of inhibition in the screening, *Spatholobus parviflorus* was selected for purification of the inhibitor for the following reasons. In some of the plants (*Chrysophyllum cainito, Beccaurea courtallensis, Areca triandra* and *Calophyllum inophyllum*) the protease inhibitor is non-proteinacious in nature. A proteinacious inhibitor is already reported from *Samanea saman* (Jacq.) Merr.). *Abelmoschus manihot* (L) is excluded from purification as a proteinacious inhibitor is reported from *Abelmoschus moschatus* and it is likely that the inhibitors from *A. manihot* will be similar to the one reported from *A. moschatus*. Purification and characterization of PPIs which are proteinaceous in nature is advantages as its gene cloning and expression in the host plant will be a feasible strategy for managing insect pests. Thus we selected *S. parviflorus* which gave appreciable inhibition of gut protease activity of *S. mauritia* and the inhibitor is a protein.

In order to enrich the fractions with proteinaceous protease inhibitor and to eliminate non-protein components of extract, ammonium sulfate fractionation was done. Proteins in the seed extract were precipitated with three different ammonium sulfate concentrations (0–30%, 30-50% and 50-70%) and the highest inhibition (55.92 ± 0.99%) was obtained for 30-50% fraction followed by 30% fraction, (49.28 ± 1.12%) and 70 % fraction (10.98 ± 0.08%). Among the ammonium sulphate fractions, highest trypsin inhibition at 30-60% ammonium sulphate fraction was reported for tamarind trypsin

inhibitor (TTI) by Araujo *et al* (Araujo *et al*. 2005). For the purification of *Allium sativum* Protease Inhibitor (ASPI), 30-50% saturated fractions of ammonium sulphate was subjected to subsequent purification steps (Shamsi *et al.,* 2016). The protein profile of the ammonium sulfate fraction indicate that there are number of protein bands with some having very high intensity. There is 1.5 fold purification of the inhibitor protein on a protein basis when 30-50% ammonium sulfate fraction is prepared from the crude extract. This is similar to the fold purification of 1.6 times obtained for ammonium sulphate fractionation during the purification of the protease inhibitor from *Antheraea mylitta* (Shrivastava and Ghosh, 2003).

Ammonium sulfate fraction (30-50%) containing 150 mg of protein was loaded on to DEAE ion exchange resin equilibrated in 20 mM Tris buffer pH 8.0. Fractions eluted with 0.3 M NaCl gave more inhibition compared to fractions from 0.15 M and 0.55 M. Thus the majority of the inhibitor is coming off with 0.3 M NaCl. The SDS-PAGE of the 0.3 M NaCl eluted fractions showed around 10 detectable bands. 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 were loaded on to Sephadex G- 100 gel filtration column. The eluted protein on SDS-PAGE gave 3 bands. As the quantity of the inhibitor protein obtained is low in gel filtration, for further purification trypsin affinity chromatography was used.

To further purify the protease inhibitor, the pooled and concentrated fractions from DEAE loaded on to trypsin affinity column and the eluted protein was checked for inhibition, then pooled and concentrated. The concentrated inhibitor inhibits trypsin up to 76.56 ± 0.26% and larval gut enzyme of *S. mauritia* up to 52.45 ± 0.87%. The reduced inhibition towards gut extract is expected as the gut may contain other proteases not inhibited by the inhibitor. On SDS-PAGE, pooled and concentrated fractions from trypsin affinity showed 4 bands indicating that the contaminating proteins are present. Prasad et al, isolated a protease inhibitor of 8 kDa from the seeds of *Vigna mungo* (Black gram) also failed to get pure protein after Trypsin-Sepharose affinity chromatography (Prasad *et al.,* 2010). But in many cases Trypsin-Sepharose affinity purification is enough to obtain pure protein (Mello *et al*., 2001, Rai *et al.,* 2008).

As pure protein was not obtained in trypsin affinity or gel filtration, elute from trypsin affinity was subjected to Ni-IMAC. Inhibitor alone came out in the flow through retaining the contaminants on to the gel. Thus a single pure protein as judged by SDS-PAGE was obtained. Fifty percentage of gut protease activity of *S. mauritia* is inhibited by the pure inhibitor at a concentration of 0.1 µg/µl. At the same concentration the purified protein exhibited 70% inhibiton towards bovine trypsin. Trypsin inhibitor purified from the seed extract of *Inga vera* showed greater than 50% inhibitory activity against five different Lepidopteran pests (*Helicoverpa zea, Heliothis virescens*, *Spodoptera frugiperda*, *Corcyra cephalonica*, and *Anagasta kuehniella*) (da Silva Bezerra *et al.,* 2016). Transgenic rice plants

expressing protease inhibitor gene showed resistance against insect pest. In feeding experiments with potato plant expressing protease inhibitor, oryzacystatin II showed that the larvae of Colorado potato beetle exhibited weight loss of 18% compared to control larvae fed on non-transformed leaves (Cingel *et al*., 2015).

5.3 CHARACTERIZATION OF PLANT PROTEASE INHIBITOR

5.3.1 Determination of sub unit molecular weight of isolated plant protease inhibitor

The sub unit molecular weight of the inhibitor determined from SDS-PAGE was found to be 14 kDa. The native protein on Acid PAGE moves above trypsin indicating that it may be a multimer in the native state. The protein did not move clearly in alkaline PAGE (Data not shown). Most of the PPIs are small molecules with relative molecular masses ranging from 5-25 kDa. (Singh and Rao, 2002).

A 14 kDa serine protease inhibitor was reported from the seeds of *Butea monosperma* L. The inhibitor strongly inhibits the larval gut proteases of *Helicoverpa armigera* as well as bovine pancreatic trypsin (Pandey *et al.,* 2014). Chen *et al.,* reported 14 kDa trypsin inhibitor from the kernals of seven resistant corn genotype and it inhibits the growth of *Aspergillus flavus* (Chen *et al.,* 1998). Another trypsin inhibitor of 14 kDa was purified and characterized from the seed extract of mung bean (*Vigna radiate* (L.) R. Wilczek) (Klomklao *et al.,*2011).

5.3.2 Identification of isolated plant protease inhibitor by LC-MS/MS

Protein sequence analysis was done by mass spectrometry. 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 *Spatholobus parviflorus* is named as *Spatholobus parviflorus* Protease Inhibitor (SpPI). Amino acid sequence analysis of the peptides from mass spectrometry reveals that the inhibitor belongs to the Kunitz type family of protease inhibitor as it contains sequences conserved in Kunitz type protease inhibitors. For example sequence from *Glycine max* [UniProt K.B - Q39869 (Q39869_SOYBN)] contain the same sequence (NGG TYYVLPVIR) obtained for the *Spatholobus parviflorus* inhibitor.

```
10 20 30 40 50
MKSTTSLALF LLCALTSSYQ PSATADIVFD TEGNPIRNGG TYYVLPVIRG
60 70 80 90 100
KGGGIEFAKT ETETCPLTVV QSPFEGLQRG LPLIISSPFK ILDITEGLIL
110 120 130 140 150
SLKFHLCTPL SLNSFSVDRY SQGSARRTPC QTHWLQKHNR CWFRIQRASS
160 170 180 190 200
ESNYYKLVFC TSNDDSSCGD IVAPIDREGN RPLIVTHDQN HPLLVQFQKV
EAYESSTA
```

Amino acid Sequence of Kunitz type inhibitor from *Glycine max* (Soybean) [UniProt K.B - Q39869 (Q39869_SOYBN)].

A 21 kDa Kunitz type protease inhibitor named as OPI (Okra Protease Inhibitor) was purified from the seed extract of *Abelmoschus esculentus* (okra) by Datta *et al.,* 2019). Another 21 kDa Kunitz type protease inhibitor (*Tamarindus* Trypsin Inhibitor, TTI) was isolated

from the seeds of *Tamarindus indica*. The TTI inhibits 87% of larval gut proteases of *Helicoverpa armigera* (Pandey and Jamal, 2014). Karthik *et al.,* purified a low molecular weight serine protease inhibitor from the seed extract of *Cicer arietinum* (L) and it inhibits both trypsin ans chymotrypsin (Karthik *et al*., 2019). A novel protease inhibitor of 10 kDa, having antifungal and antibacterial activity was isolated and characterized from mung bean (*Phaseolus mungo*) (Wang et al., 2006).

5.3.3 Determination of optimum pH for inhibition

Optimum pH for protease inhibitor for maximal inhibition was determined by evaluating the inhibition at different pH values, with appropriate controls. The different pH values (6.0, 8.0, 10.0 and 12.0) were used for the analysis. It was observed that the optimum pH for the maximal percentage inhibition of protease inhibitor was pH 8.0 (63.81 ± 1.4%). Above and below pH 8.0 the inhibition decreases gradually. The inhibitor is likely to work in the gut pH of *S. mauritia* larvae as the gut pH is around 9.0, and the inhibition is not declining much at pH 9.0. Hence, the stability of the purified inhibitor of *S. parviflorus* in alkaline range of pH implies that it is likely to work *in vivo*. The stability over a wide range of pH was reported for the inhibitor from *Solanum aculeatissimum* (Krishnan and Murugan, 2015). A Kunitz type protease inhibitor (PI) of 21 kDa purified from the seed extract of *Pithecellobium dumosum* (Benth) showed activity in a wide range of pH (2.0-12.0) (Rufino *et al.,* 2013). Intra molecular disulfide bridges provide the stability for the PIs over a wide range of pH and temperature (Oliveira *et al*., 2007). Another Kunitz type PI purified from the seed extracts of *Platypodium elegans* and *Igna edulis* were

also stable over a wide range of pH from 2.0 to 12.0 (Ramalho *et al.,* 2019, Dib *et al*., 2019).

5.3.4 Thermal stability of the *Spatholobus parviflorus* inhibitor

Thermal stability of the inhibitor was evaluated by incubating the inhibitor at different temperatures for 30 minutes. The inhibiton did not decrease up to 80 ^{0}C. Thus the inhibitor is heat stable up to 80 ºC and retains 48.08 ± 2.42% of inhibition even at 100 ^{0}C. In general Kunitz type PPIs are heat stable. Many PI isolated from plants under Fabaceae were stable upto a temperature of 80 ^{o}C and lost some of its inhibitory activity above this temperature (Saini, 1989, Godbole *et al.,*1994) and it may be due to the compact disulfide linkages which stabilizes the compact structure of the PPI (Kansal *et al*., 2008). Kunitz type PIs isolated from different plants varies in the pattern of disulfide linkages and polypeptide chains (Dib *et al.,* 2019). Aguirre *et al.,* reported an 8.7 kDa PI from *Hyptis suaveolens* (L.) seeds inhibited proteases of *Prostephanus truncatus* and was stable over a wide range of temperature (04–95 ^{o}C) (Aguirre *et al*., 2004). A heat stable serine PI from the tubers of potato was reported (Kim *et al.,* 2006). Thermal stability of the PIs is important for various biotechnological industries.

The other kinetic parameters of inhibitor was not studied except determining the concentration required for 50% inhibition of the gut enzyme activity of *S. mauritia* as the inhibitor may be inhibiting many proteases in the gut extract. As the yield of SpPI is comparatively low (1 mg from 100 g of seed), the physiological effect of feeding SpPI to the larvae of *S. mauritia* could not be examined. Besides the pesticidal

acivity, the SpPI may have other applications in the pharmaceutical/ food industries, as it is an inhibitor of trypsin.

5.4 FUTURE PROSPECTS

Studying the physiological effect of the inhibitor on *Spodoptera mauritia* larvae and other pests which utilizes mainly serine proteases will be useful for designing better insect control strategies. For getting large quantity of pure SpPI, the gene may be cloned and expressed in a suitable expression system such as bacteria. The purified protein may be used in feeding experiments to study the physiological effect of SpPI on *S. mauritia* larvae. Once physiological effect is confirmed, gene coding for the protein may be cloned and expressed in host plant to study the degree of protection offered from pest attack for commercial exploitation. Also the inhibitor can be used as a serine protease inhibitor to control the activity of serine protease enzymes in pharmaceutical, food industry and other applications.

SUMMARY AND CONCLUSIONS

Spodoptera mauritia (Boisduval) (Lepidoptera: Noctuidae) sometimes cause considerable damage to the crops. Plant protease inhibitors (PPIs) inhibit proteases and are effective in preventing the growth of larvae of many pests by inhibiting their gut protease activity. In this study we screened plant extracts to identify extracts containing PPIs against gut proteases of *S. mauritia* and purified and characterized a protease inhibitor from the seeds of *Spatholobus parviflorus.* Of the sixty plants screened for protease inhibitor against larval gut proteases of *S. mauritia*, 15 plant extracts showed greater than 50% inhibition of gut protease activity. The inhibition ranged from zero to 75.00% with four plants showing greater than 70.00% inhibition. Of the fifteen plants with greater than 50% inhibition, ten plant extracts were treated with proteinase K to check the nature of the inhibitor and found that in seven of them the major inhibitor is non- protein in nature. The protease inhibitor was purified and characterized from *Spatholobus parviflorus* as there is no report of protease inhibitor from this plant and it gave higher inhibition among proteinacious inhibitors identified in this study. Proteinaceous inhibitors are better suited as they can be expressed in host plants to protect from pest attack. The inhibitor was purified from the seed extract using ammonium sulfate fractionation, DEAE-Spehadex ion exchange chromatography, gel filtration, Trypsin–Sepharose affinity chromatography and Ni- affinity chromatography. The concentration of the inhibitor required for 50% inhibition of the get protease activity of *Spodptera mauritia* is

0.1 μg/μl and at this concentration it inhibited trypsin to the extend of 70%. The purified inhibitor is a serine protease inhibitor with a subunit molecular weight of 14 kDa on SDS-PAGE. The inhibitor is heat stable up to 80 ^{0}C and the maximum inhibition is at pH 8.0. LC-MS/MS analysis of the inhibitor revealed that this is a new inhibitor as peptides from the inhibitor are not matching with the peptides of the proteins in the database. Also the presence of conserved sequence motif of Kunitz type inhibitor indicates that this new inhibitor is a Kunitz type serine protease inhibitor which is supported by our experimental data. Studying the physiological effect of this inhibitor on *Spodoptera mauritia* larvae and other pests which utilizes mainly serine proteases will be useful for designing better insect control strategies. One approach in this direction will be to clone the gene coding for the inhibitor and express in the host plant. Also the inhibitor can be used as a serine protease inhibitor to control the activity of serine protease enzymes in pharmaceutical, food industry etc. and other applications.

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