SCREENING AND PARTIAL PURIFICATION OF PROTEASE INHIBITORS FROM *SENNA SURATTENSIS* LEAVES

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ABSTRACT

This study was done to determine the inhibitory activity that were exhibited by different parts of the local plants in Malaysia selected from the 4 families which are Leguminosae, Rubiaceae, Apocynaceae and Euphorbiaceae. The screening methods used in this study are Bradford Assay and Trypsin Inhibitory Assay. Both these assays revealed that *Senna surattensis* leaves showed the highest inhibitory activity of 83 % compared to other 41 plant samples studied in this study. SDS-PAGE and Tricine SDS on this sample extract showed the presence of this protease inhibitor through the formation of band. From these band also, the molecular weight for *Senna surattensis* leaves was determined to be 27.93 kDa. From the mode of inhibition study carried on *Senna surattensis* leaves, it was found out that this plant belongs to the competetive inhibitor group with K_i value of 8.89 $x10^{-5}$ mM. Thermostability test reavealed that *Senna surattensis* leaves extract can only work best at temperature below 60˚C and achieve its optimum inhibitory temperature at 45°C with 87.35 % of inhibitory activity. *Senna surattensis* leaves extract also showed the ability to inhibit the protein extracted from *Chrysomya megacephala* through the study performed on the crude *Chrysomya megacephala* protein extract. The IC_{50} value of *Senna surattensis* leaves extract was determined to be 0.0174 µg/µl. Although with all of these promising result, further test need to be done to confirm it.

ABSTRAK

Kajian ini dijalankan untuk menentukan aktiviti-aktiviti perencatan yang ditunjukkan oleh beberapa jenis bahagian tumbuh-tumbuhan tempatan di Malaysia yang terdiri daripada 4 famili iaitu Leguminosae, Rubiaceae, Apocynaceae and Euphorbiaceae. Kaedah penyaringan ujian Bradford dan ujian perencatan tripsin menunjukkan bahawa daun *Senna surattensis* mempunyai kebolehan perencatan yang paling tinggi berbanding 41 sampel tmbuh-tumbuhan yang lain di dalam kajian ini iaitu sebanyak 83 %. Ujian SDS-PAGE dan Ujian Tricine SDS terhadap sampel ini menunjukkan kehadiran protin perencat melalui jalur yang terhasil. Melalui jalur ini juga, berat molekular bagi daun *Senna surattensis* dianggarkan sebanyak 27.93 kDa. Daripada penentuan Mod perencatan ke atas ekstrak daun *Senna surattensis*, didapati ianya tergolong dalam kumpulan perencatan kompetetif dengan nilai K_i sebanyak of 8.89 $x10^{-5}$ mM. Ujian kestabilan suhu yang dijalankan menunjukkan bahawa ekstrak daun *Senna surattensis* hanya mampu berfungsi di bawah suhu 60˚C dan mencapai suhu perencatan optimum pada 45ºC dengan 87.35 % aktiviti perencatan. Ekstrak daun *Senna surattensis* juga mampu merencat protein yang diekstrak daripada *Chrysomya megacephala* melalui ujian yang dijalankan ke atas ekstrak protin mentah *Chrysomya megacephala*. Nilai IC_{50} yang diperolehi untuk ekstrak daun *Senna surattensis* adalah 0.0174 μg/μl.

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LIST OF SYMBOLS AND ABBREVIATIONS:

BapNA	*N*-alpha-benzoyl-dl-arg-*p*-nitroanilide
BBI	Bowman-Birk inhibitor
BSA	Bovine Serum Albumin
Bt	*Bacillus thuringiensis*
BTI	Barley trypsin inhibitor
cm	centimetre
CpTi	Cowpea trypsin inhibitor
DMSO	Dimethy Sulfoxide
DPPH	1,1-Diphenyl-2-picrylhydrazyl
g	Gram
HCl	Hydrochloric Acid
HMW	High Molecular Weight
KDa	Kilo Dalton
Ki	Inhibition constant
LMW	Low Molecular Weight
M	Molar
mA	mili Ampere
MAP	Mitogen-Activated protein
MCF7	Michigan Cancer Foundation-7
mg	miligram
ml	Mililiter
NaOH	Sodium Hydroxide
nm	nanometer

PIs	Protease inhibitors
PVY	Potato Virus Y
rpm/min	Revolution per minute
SDS	Sodium dodecyl Sulfate
SDS PAGE	Sodium dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SPIs	Serine Protease inhibitors
TEMED	N,N,N'N' –tetramethylenediamine
TEV	Tobacco Etch Virus
TPCK	L-1-tosylamido-2-phenylethyl chloromethyl ketone
UV	ultra violet
v/v	volume per volume
w/v	weight per volume
xg	Gravity
α	Alpha
β	Beta
γ	Gamma
°C	Degree Celcius
%	Percent

CHAPTER 1: INTRODUCTION

1.1 Introduction

Proteases have been described in large numbers naturally in animals, plants and microorganisms and have been extensively studied in order to their structures and functional properties (Laskowski and Kato, 1980; Hibbetts *et al.*, 1999). Proteases are known as proteinases, peptidases or proteolytic enzymes and are involved in many beneficial functions. Many biological functions including food digestion, lysosom degradation, and signaling cascades rely on proteases (Fear *et al.*, 2006). The proteases are the enzymes which *in vivo* catalyzed the hydrolytic breakdown of proteins into specific peptide fractions and amino acids (Otlewski *et al.*, 1999; Fear *et.al*, 2006; Habib H. and Khalid M.F., 2007).

Proteases are divided into endoproteases and exoproteases where function as regulators to control endogenous enzymes that regulatory activity of proteolytic, whether the target enzymes are of exogenous or endogenous origin in the presence of the active enzyme (Barret, 1987; Ryan, 1990; Kato, 2002). There are classified according to their mechanism of catalysis and the amino acid essential for its activity, as cysteine proteases with a cysteine, aspartic proteases with an aspartate group, metalloproteases with a metal ions (Zn^{2+}, Ca^{2+} or Mn^{2+}) and serine proteases with serine and histidine (Neurath, 1984; Carlini and Grossi-de-Sa, 2001).

Hans Neurath (1984) was among the first scientist recognized that protease act not only as digestive enzymes, but also fulfill numerous other functions for many biological organism including food digestion, lysosome degradation, and signaling cascades rely on proteases and also functional for others (Neurath, 1984; Laskowski and Qasim, 1999; Fear *et al.*, 2006). Hans Neurath also recognized that proteinases are highly beneficial and must be

extremely controlled by the respective cell or organism because they are potentially hazardous to their natural environment because may lead high pressure on the environment and proteases can be responsible for serious diseases when uncontrolled (Neurath, 1984; Laskowski and Qasim, 1999; Carlini and Grossi-de-Sa, 2001).

The control of proteases is normally achieved by regulated expression, secretion or activation of proproteinases. Proteases also controlled by degradation of mature enzymes and by inhibition of their proteolytic activity (Otlewski *et al.*, 1999). A huge number of inhibitors has been detected, they were isolated from various cells, tissues and organisms (Otlewski *et al.*, 1999). From the experimented, inhibition of proteases by proteins itself has been adaptation to overcome the potential risk of proteolysis and develop specificity of recognition (Otlewski *et al.*, 1999).

The most intensively studied group of protease inhibitors (PI) are serine protease inhibitors such as trypsin, chymotrypsin, elastase and subtilisin (Otlewski *et al.*, 1999). Serine protease inhibitors are divided into subfamilies based on their amino acid sequences (Bode and Huber, 1992). The Kunitz-type and Bowman-Birk families are the best characterized of the serine protease inhibitors.

Trypsin is the largest and diverse serine proteases families that are found in the digestive system of many vertebrates. Once the protease has been activated, trypsins are used as a major form to control on its. In human, trypsin is produced in an inactive form by the pancreases as the inactive proenzyme trypsinogen, where it acts to hydrolyze peptides into their smaller building blocks. These peptides are the result of the enzyme pepsin breaking down the proteins in the stomach. Trypsin enzyme acts to degrade protein and it is often referred to as a proteolytic enzyme or proteinase.

Trypsins are bind with inhibitors which as a competitive substrate analog to form an inactive complex, inactive the digestive enzymes against insect digestive enzymes. The proteolytic activity of the serine protease stops by trypsin inhibitors when its function is no longer necessary. This inhibitor is cause depression of growth, nutritional disorders and pancreatic hypertrophy or hyperplasia (Liener and Kakade, 1980).

The enzymatic mechanism is similar to other serine proteases that contain three residues that is histidine-57, aspartate-102, and serine-195 (Rawling and Barrett, 1994) form a charge relay which serves to make the active site serine nucleophilic. This is achieved by modifying the electrostatic environment of the serine. The enzymatic reaction that trypsins catalyze is thermodynamically favorable but requires significant activation energy. Trypsin also contains an "oxyanion hole" formed by the backbone amide hydrogen atoms of Gly-193 and Ser-195 which serves to stabilize the developing negative charge on the carbonyl oxygen atom of the cleaved amide. In the catalytic pocket (S1) of trypsins are located the aspartate residue (Asp 189) which responsible for attracting and stabilizing positively-charged lysine or arginine, and is thus responsible for the specificity of the enzyme.

Therefore, in this study research is focused on the function of serine proteases inhibitors by using potential of local plants that are important to control insecticides and thus make them beneficial in agronomical and health relevance. This research study will be involving a through screening, isolation, identification and characterization of protease inhibitors.

CHAPTER 2: LITERATURE REVIEW

2.0 Bioactive peptides and its classification

Peptides with biological activities generally contain three to twenty amino acid units, which are proteins synthesized in the form of large prepropeptides in the cell. Bioactive peptides in plants supposedly involved in defense mechanisms that confer resistance against phytophagous predators and infection by viruses, bacteria, fungi, nematodes and other organisms (Carlini and Grossi-de-Sa, 2001). The best known plant proteins are lectins, ribosome-inactivating proteins (RIPs), arcelins, alpha-amylase canatoxin and protease inhibitors.

2.0.1 Lectins

Lectins are class of a protein of non-immune origin that possess at least one non-catalytic domain that specifically and reversibly binds to mono or oligosaccharide (Lis and Sharon, 1986; Peumans and Van Damme, 1995; Carlini and Grossi-de-Sa, 2001). The seeds of the *Leguminoseae* are rich sources of lectins, but the same lectin and homologs are also found in other parts of the plant, such as the bark, stem, and leaves. This family includes lectins such as ConA, soybean agglutinin, and lentil lectin. Two other smaller families of plants whose lectins have been characterized are the Gramineae; cereals, such as wheat germ and Solanaceae; potatoes and tomatoes.

The function of lectins in plants is still controversies with their biological roles in the parent organisms (Carlini and Grossi-de-Sa, 2001). The binding site for carbohydrate in some lectins involves a combination of hydrophobic interactions and van der Waals contacts to which small plant growth regulators such as adenine can bind to (Roberts and Goldstein, 1983; Carlini and Grossi-de-Sa, 2001). Although plant lectins have an ability to bind

carbohydrate, evidence exists that these proteins may have additional activities. For example, some lectins, such as *Dolichus biflorus*, bind adenine residues with high affinity and specificity in regions of the protein outside the common carbohydrate-binding site.

Some plant lectins have shown entomotoxic effects when fed to insects from Coleoptera, Homoptera, and Lepidoptera orders (Peumans and Van Damme, 1995; Carlini and Grossi-de-Sa, 2001). Transgenic expression of the gene encoding *G. nivalis* agglutinin in rice plants decreases survival and fecundity of insects attacking the transgenic plants (Schuler *et al.,* 1998). Therefore this class of protein should be studied more for their potential.

2.0.2 Ribosome-inactivating proteins (RIPs)

Ribosome-inactivating proteins or RIPs are the proteins that are capable of inactivating ribosomes in many plants (Peumans *et al.,* 2001). RIPs are a group of cytotoxic N-glycosidases that specifically cleave nucleotide N-C glycosidic bonds. RIPs were first identified more than 100 years ago. Their biological functions are determined to play a role in plant defence mechanism.

RIPS have been described into three types, types I is composed of a single polypeptide chain, whereas type II is a heterodimer consisting of a chain, which is attached to a sugar-binding B chain that functionally equivalent to a type I while type III is unknown function that forms single chain containing an extended carboxyl-terminal domain (Park *et al.,* 2004).

The effect of ribosome show differential sensitivity to RIPs in plant cells and isolated ribosomes, while ribosomes of protozoans and fungi seem to be highly sensitive. Ricin, the toxic principle of castor bean was identifried as a protein at the 19th century was shown to be ineffective to a variety of insects of different orders, although it was able to inhibit

protein synthesis by insect ribosomes in cell-free preparations (Gatehouse *et al*., 1990; Carlini and Grossi-de-Sa, 2001). Since the isolation and characterization of ricin, many structurally and functionally related proteins have been identified in a wide variety of plants (Peumans *et al.,* 2001).

2.0.3 Alpha-Amylase inhibitors

Alpha-amylases are found in microorganisms, animals and plants, whixh catalyze the initial hydrolyses of alpha-1,4-linked sugar polymers into shorter oligosaccharides, an important step towards transforming sugar polymers into single units that can be assimilated by the organism. The first alpha-amylase inhibitor characterised was that of the monomeric 13 kD known as 0.31 form, from wheat (Carlini and Grossi-de-Sa, 2001).

This endosperm protein is relatively abundant in seeds that suggesting a role as a storage or reserve protein, as regulators of endogenous enzyme or as defensive mechanisms against the attacks of animal predators and insect or microbial pests. These inhibitors were also relevant in several aspects of human health such as to control of diabetes and obesity, diagnosis of pancreatic hyperamylasemia disorders and nutritional and toxicological aspects of foods (Turcotte *et al.,* 1994; Bisschoff *et al.,* 1994; Carlini and Grossi-de-Sa, 2001). The amylase inhibitors present in seeds used as food present some toxicological significance in the diets of infants who have a lower production of pancreatic alpha-amylase than adults and for patients with impaired peptic or gastric function (Richardson, 1991).

Alpha-amylase inhibitors also show great interest as potentially important tools of natural and engineer resistance of crop plant against pests that are improved through the use of transgenic technology (Gatehouse and Gatehouse, 1998, Carlini and Grossi-de-Sa, 2001).

Focused on lectin-like inhibitors present in common bean *Proteus vulgaris* seeds have been shown that toxic effects to several insect pests (Ishimoto and Chrispeels, 1996). The effect was well determined not only in different oraganisms by enzymatic activity but also in feeding assay experiments (Ishimoto *et al.,* 1996).

Transgenic pea and azuki bean seeds expressing the inhibitor, Alpha-amylase-1, of the domesticated common bean *Proteus vulgaris* was completed resistance against bruchids (Ishimoto *et al.,* 1996; Morton *et al.,* 2000). Therefore these proteins can be safely introduced into food plants because the transgenic grains showed minimal effects on mammalian digestion system (Pusztai *et al.,* 1999; Carlini and Grossi-de-Sa, 2001).

2.0.4 Arcelins

Arcelins are lectin-related proteins detected only in wild beans (*Phaseolus vulgaris*) and exists in six electrophoretic variants which supposedly involved in defence mechanisms to confer resistance against predators such as bruchid beetles (Chrispeels and Raikhel, 1991; Carlini and Grossi-de-Sa, 2001), although the precise mechanism behind the toxicity of arcelin is as yet unknown.

Arcelins can be divided into three subgroups,group one are arcelin 1, arcelin 2 and arcelin 6, group two is arcelin 4 and group three are arcelin 5a and arcelin 5b. Sequence data of arcelin 6 was indicated that this protein is closely related to arcelin 1 and arcelin 2. Biochemical data indicate that arcelin 3 belongs to the same subgroup of arcelin 4.

Arcelin 5 has been reported to consist of a mixture of two major protein fractions, termed arcelin 5a, Arc5a, 32.2 kDa and arcelin 5b, Arc5b with 31.5 kDa (Goossens *et al.,* 1994).

Arc5b and Arc5a contain one and two glycans, respectively, while Arc5c is not glycosylated. Two different arcelin-5 cDNA sequences were reported (Goossens *et al.,*

1994), called *arc5-I* and *arc5-II*. They encode two polypeptides of 240 amino acids (26.8 and 27.0 kDa) with a high identity (96.9%, a difference of 8 residues in the N-terminal part of the chain).

The *arc5-I*-encoded protein contains three potential glycosylation sites, while the*arc5-II* encoded protein contains only two. Arc5a and Arc5b are encoded by *arc5-I* and *arc5-II*, respectively, while Arc5c could be encoded by *arc5-II* or by a third copy of the *arc5* gene with a much lower rate of expression. Arcelins are thought to provide resistance against the bean bruchid pest *Zabrotus subfasciatus* (Osborn *et al.,* 1988). Among the arcelin variants, Arc1 and Arc5 appear to be the most promising in conferring insect resistance (Kornegay *et al.,* 1993) discovery of putative novel enzymatic (Goossens *et al.,* 1994).

2.0.5 Protease inhibitors

Protease inhibitors (PIs) are found in animals, plants and microorganisms (Laskowski and Kato, 1980). Protease inhibitors adopt many different structures, ranging in size from mini-proteins to large macromolecular structures, much larger than the target enzyme (Otlewski *et al*., 1999). PIs are classified into two large groups based on their structural dichotomy which include the low molecular weight peptidomimetic inhibitors and protein protease inhibitors (Fear *et al.,* 2007).

PIs are divided into five groups of serine, cysteine, threonine, aspartyl and metalloprotease inhibitors according to the mechanism employed at the active site of proteases they inhibit (Fear *et al.,* 2007). Two mechanisms can occured prroteolytic inhibition by protease inhibitors by irreversible trapping reactions and reversible tightbinding reactions (Rawlings *et al.,* 2004).

From previous studies, plants seed are widely distributed and a rich source of protease (Richardson, 1991; Mello and Silva-Filho, 2002; Chaudhary *et al.,* 2008). Protease inhibitors (PIs) have evolved to inhibit proteolytic enzymes. They are classified according to their types of enzyme they inhibit (Mosolov, 1998; Otlewski *et al.,* 1999; Carlini and Grossi-de-Sa, 2001). The molecular mass of these inhibitors can vary from 4 to 85 kDa, with majority in the range of 8 to 20 kDa (Hung e*t al.,* 2003).

PIs play different roles in their action as storage proteins (Xavier-Filho, 1992), as regulators of endogenous proteolytic activity (Ryan, 1990), as participants in program cell death, or as components with extraordinary properties that protective naturally to defense against pathogens and pests attack such as viral, bacterial, fungal and others, or play regulatory roles during plant development, involve as markers in studies of plant diversity and evolution in relation to host co-evolution and other properties (Ryan, 1990; Lu *et al.,* 1998). PIs also are known to be involved in clinical studies, such as blood coagulation, immune regulation, platelet aggregation and anti-carcinogenesis (Kennedy, 1998; Chaudhary *et al.,* 2008).

In the past decade, PIs are used as therapeutic agents for the treatment of human immunodeficiency virus (HIV) and hypertension. Research done by Hilder et al. In 1987 shown the first success experiment of using the stable genes that encoded the stable inhibitors was transferred to plants to improve their resistance to pests or fungi (Hilder, 1987; Ryan, 1990). They transferred trypsin inhibitor gene from *Vigna unguiculata* to tobacco, which conferred resistance to wide range of insect pests including Lepidopterans, Coleopterans and Orthoptera to protect the plants.

Since proteinase inhibitor genes are primary gene products, they are excellent candidates for engineering pest-resistance into plants and extremely overcome the potential risk of proteolysis. From the latest studies show that PIs also influence insect development by causing amino acid deficiency, reducing the ability of essential amino acids for the production of the other proteins and remain active under different gut pH (Pompermayer *et al.,* 2001; Bhattacharya *et al.,* 2007).

2.1 Classification of protease inhibitors

They are classified according to their mechanism of catalysis and the amino acid present in the active center such as cysteine proteinases, with a cysteine, aspartic proteinases, with an aspartate group, metalloproteinases, with a metallic ion such as Zn^{2+}, Ca^{2+} and $Mn^{2+}$and serine proteinases, with serine and histidine (Neurath, 1984; Carlini and Grossi-de-Sa, 2001). Attachment of the proteases to a certain group depends on the structure of catalytic site and the amino acid essential for its activity.

2.1.1 Cysteine protease inhibitors

Cysteine protease inhibitors or cystatin are known as phytocystatins in plants. The cysteine protease class includes papain, calpain and lysosomal cathepsins and have been recognized in maize, soybean, and potato and in many variety of plant (Gruden *et al.,* 1997; Ryan *et al.,* 1998). Phytocystatins are 5 to 87 kDa proteins, usually found in cystatins subfamilies I and II (Arai *et al.,* 1998; Carlini and Grossi-de-Sa, 2001). One group of phytocystatins contains a single domain and second group has multiple domains that are found in sunflower seeds and potato tubers (Walsh and Strickland, 1993; Pernas *et al.,* 1999; Carlini and Grossi-de-Sa, 2001).

The primary and tertiary structures of cysteine protease inhibitors have been determined (Kauzuma *et al.,* 2000; Carlini and Grossi-de-Sa, 2001) Homology of cysteine protease inhibitors are similar with serine protease inhibitors, such as Kunitz-type trypsin inhibitor family that belong to potato tuber phytocystatins (Ishikawa *et al.,* 1994). The phytocystatins are displaying high inhibitory activity toward insect gut proteinases making them attractive to control insect pests (Bode and Huber, 1992; Gatehouse and Gatehouse, 1998; Carlini and Grossi-de-Sa, 2001).

Cysteine protease inhibitors were reduced fecundity, increased motarlity, decreased weight and severe deformations when fed to lepidoptera and coleopteran species (Elden, 2000). Cysteine proteases have been best characterized in the Bruchidae in the Coleoptera, it also occurs in the Curculionoideae, Meloidae and Silphidae (Xavier-Filho, 1992).

Therefore, in this study research are focusing on the function of serine proteases inhibitors by using potential of local plants that are important to control insecticides and thus make them beneficial in agronomical and health relevance.This research study will be involving a thorough screening, isolation, identification and characterization of bi-functional inhibitor of α-amylase and protease inhibitors.

2.1.2 Aspartic protease inhibitors

Aspartic proteases are a family of protease enzymes which inhibits the catalytic activity of an aspartyl protease, a class of proteases that contains active site aspartate residue (Asp). Aspartic proteases include pepsin and rennin. Members of the aspartic protease family have been characterised in humans, plants, fungal and retroviruses. Eukaryotic aspartic proteases include pepsins, cathepsins, and renins.

Aspartic proteases were also involved in defence mechanism. Protease inhibitors active against serine, cysteine and metallocarboxy-proteases are ubiquitous, while inhibitors active towards aspartic proteases have not been detected in seeds (Valueva and Mosolov, 1999). Research by Dash and friends in 2001 were recognized that the kinetic studies have revealed the bifunctional characteristics of a novel bifunctional inhibitor (ATBI) from an extremophilic *Bacillus* sp., as it was found to inhibit xylanase and aspartic protease. This report had shown a novel class of antifungal peptide, exhibiting bifunctional inhibitory activity (Dash *et al.,* 2001).

2.1.3 Metalloproteinases inhibitors

Tissue inhibitors of metalloproteinases (TIMPs) are the major cellular inhibitors of the matrix metalloproteinase (MMP). Matrix metalloproteinases are a class of enzymes involved in degradation of extracellular matrix that can break down proteins, such as collagen and gelatin. Metalloproteinases include thermolysin and carboxypeptidase A. As they inhibit cell migration they have antiangiogenic effects. They may be both endogenous and exogenous. Exogenous matrix metalloproteinases inhibitors include batimastat and marimastat. The most notorious endogenous metalloproteinases are tissue inhibitor of metalloproteinases (TIMP). There are also cartilage-derived angiogenesis inhibitors.

2.1.4 Serine protease inhibitors

Serine protease inhibitors and their binding to cognate proteinases have been extremely well characterized over the years (Bode and Huber, 1992). Serine protease inhibitors are one of the most diverse families of macromolecules that achieve similar biological functions with entirely different scaffolds. Serine protease inhibitors have been classified

about 20 subfamilies, based on amino acid sequence and mechanism of interaction including a-helical, b-sheet and a/b proteins, as well as small disulfide rich proteins. Based on their mechanisms of action, three types of serine protease inhibitors are now recognized as canonical inhibitors, non-canonical inhibitors and serpins (Laskowski and Kato, 1980).

They are the group of proteolytic enzymes which are characterized by a catalytically active serine residue in their active site. Several serine protease inhibitors are effective protect host plants by against various insect enzymes and therefore have been studied as an alternative approach to pest control (Reckel *et al.,* 1997; Leo *et al.,* 2001). Serine protease inhibitors such as trypsin, chymotrypsin and elastase are the most intensively studied (Otlewski *et al.,* 1999). All three enzymes are synthesized by the pancreatic acinar cells, secreted in the small intestine, and are responsible for catalyzing the hydrolysis of peptide bonds. These enzymes are shown similar in structure through their X-ray structures.

Each of these digestive serine proteases targets different regions of polypeptide chain, based upon the side chains of the amino acid residues surroundinsg the site of cleavage. Following a positively-charged amino acid residue, trypsin is responsible for cleaving peptide bonds. Instead of having the hydrophobic pocket of the chymotrypsin, there exists an aspartic acid residue at the base of the pocket. This can then interact with positively-charged residues such as arginine and lysine on the substrate peptide to be cleaved.

Chymotrypsin is responsible for cleaving peptide bonds following a bulky hydrophobic amino acid residue. Preferred residues include phenylalanine, tryptophan, and tyrosine, which fit into a snug hydrophobic pocket. Elastase is responsible for cleaving peptide bonds following a small neutral amino acid residue, such as Alanine, glycine, and valine. These amino acid residues form much of the connective tissues in meat. The pocket that is

in "trypsin" and "chymotrypsin" is now partially filled with valine and threonine, rendering it a mere depression, which can accommodate these smaller amino acid residues.

Serine proteases have been isolated from various seeds that have been isolated and characterized from *Leguminosae, Cucur bitaceae*, *Solanaceae Graminae* and *Rutaceae* families (Garcia-Olmedo *et.al.,* 1987; Oliva *et al.,* 2000; Mello *et al.,* 2002; Oliveira *et al.,* 2002; Shee and Sharma, 2007) and their physiological roles are extensively studied including the regulation of endogenous proteases during seed dormancy, the reserve protein mobilization, the protection against the proteolytic enzymes of parasites and insects and also as storage or reserve proteins.

Most serine PIs is low-molecular mass molecules from 3 to 25 kDa that inhibit trypsin and/or chymotrypsin. Kunitz-type inhibitors are proteins of ~20 kDa, with low cysteine content and a single reactive site, whereas the Bowman–Birk type inhibitors have ~8 to 10 kDa as well as high cysteine content and two reactive sites (Richardson, 1991; Bhattacharyya and Babu, 2007). Kunitz and Bowman–Birk inhibitors are vary in their mode of stability but lack a-helix structurally (Bhattacharyya and Babu, 2007). The linkages of disulfide in the Bowman–Birk inhibitors minimize their conformational entropy and enhance their stability whereas Kunitz inhibitors are stabilized chiefly by hydrophobic interactions of short stretches of hydrogen bonded sheets (Sweet *et al.,* 1974; Ramasarma *et al.,* 1995; Bhattacharyya and Babu, 2007).

The distributions of these two families of inhibitors are in the seeds of *Leguminosae* that contain high amounts of protein that suppress proteolytic activities *in vivo* and *in vitro* by forming stable stoichiometric complexes (Richardson, 1991; Bhattacharyya and Babu, 2007). Kunitz type inhibitors are more common in the seeds of highly primitive Mimosoideae and primitive Caesalpinioideae, in comparison to the recently evolved

Papilionoideae which frequently shows the presence of Bowman-Birk inhibitors (Macedo *et al.,* 2002).

2.1.4.1 Bowman-Birk Inhibitor (BBI)

The Bowman-Birk Inhibitor (BBI) is a polypeptide that has ability to inhibit both trypsin and chymotrypsin at independent binding sites. It is characterized by content of high cystine and the absence of glycine. BBI is a soy-derived protease inhibitor with anticarcinogenic and anti-inflammatory properties, has been currently shown to be well tolerated in clinical trials as a human cancer-preventive agent for pre cancerous conditions, such as oral leukoplakia and the inflammatory disease, ulcerative colitis (Gran *et.al,* 2006).

In 1963, Bowman and Birk were the earliest scientists that identify and characterise a member of this family from soybean (*Glycine max*) (Bowman 1946; Birk *et al.,* 1963). The most well studied member of this family is the soybean inhibitor (Habib and Fazili, 2007). BBI can be found in many plant seeds. From the recent researches the inhibitors have been found in cereals and legumes (Tanaka *et al.,* 1997; Laing and McManus, 2002). The inhibitors of this family are generally found in seeds, but are also wound-inducible in leaves (Eckelkamp, 1993) and in the grass family Poaceae (Odani *et al.,* 1986; Habib and Fazili, 2007). A small cyclic inhibitor has been identified in sunflower (*Helianthus annuus*) called sunflower trypsin inhibitor 1 (SFTI-1) (Habib and Fazili, 2007).

BBIs have been classified according to their structural features and inhibitor characteristics. The first reactive site in these inhibitors is usually specific for trypsin, chymotrypsin and elastase. The inhibitors have molecular weights ranging from 7000 to 8000, and these inhibitors are stabilized by the presence of disulfide bridges (Chen *et al.,* 1992; Lin *et al.,* 1993). The 14 half-cystine residues are conserved in all BBIs and help to maintain their

active conformation, All BBI molecules have two regions of tandem homology and each has a reactive site. Thus, BBIs can inhibit two proteinases simultaneously and independently and are considered as “double-headed” inhibitors (Chen *et al.,* 1992).

The inhibitors from dicotyledonous plants consist of a single polypeptide chain have a molecular mass of approximately 8 kDa and are double-headed, with two homologous domains each bearing a separate reactive site for the cognate proteases (Birk, 1985). These inhibitors interact independently, but simultaneously, with two proteases, which may be same or different (Raj *et al.,* 2002; Birk, 1985). Two types of the inhibitors can be found from monocotyledonous plants. One group consists of a single polypeptide chain with a molecular mass of about 8 kDa. They have a single reactive site. Another group has approximately 16 kDa with two reactive sites (Odani *et al.,* 1986; Tashiro *et al.,* 1987).

The main sturucture of BBI are single polypeptides and comprise a binary arrangement of two sub-domains with a conserved array of seven disulfide bonds. The BBI family of protease inhibitors contains a unique of two disulfide-linked nine-residue reactive site loops that adopts a characteristic canonical conformation (Bode and Huber, 1992) and the positions of the P1 residues are indicated (Odani and Ikenaka, 1976). The loop is called protease-binding loop and binds the protease in a substrate-like manner (Lee and Lin, 1995; Habib and Fazili, 2007).

BBIs are cysteine-rich proteins with inhibitory activity against proteases that are widely distributed in monocot and dicot species (Lin *et al.,* 2006). Recent studies shown that proteinase inhibitors of certain types are anticarcinogenic.The soybean derived BBI with a well-characterized ability to inhibit trypsin and chymotrypsin is particularly effective in suppressing carcinogenesis in a variety of *in vivo* and *in vitro* systems (Kennedy, 1998).

The anticarcinogenic compounds have the ability to reduce the forming of oxygen radicals, to suppress the growth of chemical-induced colon and anal gland tumors, lung tumor in mice, and breast tumor in rat to suppress the chemicalor radiation-induced cell transformation, to reduce spontaneous chromosome abnormality (Kennedy, 1998) and to prevent tumor invasion and metastasis.

2.1.4.2 Kunitz-type inhibitors

Kunitz-type inhibitors are a type of protein which functions as a protease inhibitor (Rawlings, 2004) and mostly active against serine proteases, but may also inhibit other proteases (Ritonja *et al.,* 1990; Laing and McManus, 2002). Kunitz-type inhibitors are usually in plants and widespread in soybean, legumes seeds, cereals and in solanaceous species (Laskowski and Kato, 1980; Ishikawa *et al.,* 1994). Kunitz-type PIs have been found in potato tubers (*S. tuberosum*) (Plunkett *et al.,* 1982; Park *et al.,* 2005). The inhibitors with antifungal activity have been located in the roots of punce ginseng (*Pseudostellaria heterophylla*) (Wang and Ng, 2006).

Kunitz-type inhibitors usually have molecular weight approximately 18 to 20 kDa proteins, usually made of two disulfide bridges or contain from 170 to 200 amino acid residues in one polypeptide chain in their single reactive site with low cysteine content. This family have been shown to inhibit trypsin, chymotrypsin and subtilisin (Laing and McManus, 2002; Park et al., 2005) and they also inhibit other proteases. Structurally, Kunitz inhibitors lack a-helix, but vary in their mode of stability. Kunitz inhibitors are stabilized chiefly by hydrophobic interactions of short stretches of hydrogen bonded sheets (soybean Kunitz trypsin inhibitor) whereas the disulfide linkages in the Bowman–Birk inhibitors minimize

their conformational entropy and enhance their stability (Sweet *et al.,* 1974; Ramasarma et al., 1995).

Joubert and others scientist found the source of Kunitz-type typsin inhibitor from *Erythrina* seeds to check abilities of inhibition. From the results, the proteins inhibited trypsin strongly and they were poor inhibitors of chymotrypsin (Joubert *et al.,* 1987). Kunitz trypsin inhibitors also effectively inhibit the activity of proteolytic of lepidopterans, such as black cutworm, tobacco, budworm and others.

2.2 Application of serine protease inhibitors

According to research by Bhattacharyya *et al.,* 2007, studied on trypsin and chymotrypsin inhibitor from *Caesalpinia bonduc* (CbTI) seeds. They also studied on the effects of CbTI on insect gut proteases reflect that CbTI is a powerful antifendant of insect herbivores. The deleterious effects of CbTI on larval gut proteinases of *S. litura* were similar to previously observed results with proteinase inhibitors from other leguminous plant (Gomes *et al.,* 2005).

Another research by Bhattacharyya *et al.,* 2007, researched on the roles of serine proteases involved in the digestion mechanism of the cutworm Spodoptera litura (Lepidoptera: Noctuidae) were examined (in vitro and in vivo) following feeding of plant protease inhibitors. A trypsin inhibitor from Archidendron ellipticum (AeTI) was purified by ammonium sulfate fractionation, size-exclusion chromatography (HPLC) and ion-exchange chromatography and its bioinsecticidal properties against S. litura were compared with Soybean Kunitz trypsin inhibitor (SBTI). AeTI inhibited the trypsin-like activities of the midgut proteases of fifth instar larvae of S. litura by over 70%. Dixon plot analysis revealed

competitive inhibition of larval midgut trypsin and chymotrypsin by AeTI, with an inhibition constant (Ki) of 3.5×10−9 M and 1.5×10−9 M, respectively.

However, inhibitor kinetics using double reciprocal plots for both trypsin and chymotrypsin inhibitions demonstrated a mixed inhibition pattern. Feeding experiments conducted on different (neonate to ultimate) instars suggested a dose-dependent decrease for both the larval body weight as well as % survival of larva fed on diet containing 50, 100 and 150 μM AeTI. Influence of AeTI on the larval gut physiology indicated a 7-fold decrease of trypsin-like protease activity and a 5-fold increase of chymotrypsin-like protease activity, after being fed with a diet supplemented with 150 μM AeTI. This study suggests that although the early (1st to 3rd) larval instars of S. litura are susceptible to the trypsin inhibitory action of AeTI, the later instars may facilitate the development of new serine proteases, insensitive to the inhibitor (Bhattacharyya *et al.,* 2007).

Research on purification and characterization of a highly stable and potent trypsin inhibitor was purified to homogeneity from the seeds of Putranjiva roxburhii belonging to tree of tropical India by acid precipitation, cation-exchange and anion-exchange chromatography (Chaudhary *et al.,* 2008). SDS page analyses showed that protein consist of a single polypeptide chain with molecular mass of approximately 34 kDa when under reducing conditions. From the report, the structural stability of inhibitor complete at the high temperatures and the complete loss of inhibitory activity were observed above 90^{o}C. N-terminal amino acid sequence of 10 residues did not show any similarities to known serine protease inhibitors, however, two peptides obtained by internal partial sequencing showed significant resemblance to Kunitz-type inhibitors.

In another case, research done by Kim and friends in 2005 from Republic of Korea were described the purification and characterization of the antimicrobial peptide potamin-1 (PT-1) from potato. A 5.6 kDa trypsin-chymotrypsin protease inhibitor obtained and isolated from tubers of the potato by extraction of the water-soluble fraction, dialysis, ultrafiltration, and C18 reversed-phase high performance liquid chromatography. PT-1 strongly inhibited pathogenic microbial strains, including *Candida albicans*, *Rhizoctonial solani*, *Clavibacter michiganense subsp. Michiganinse* (Kim *et.al.,* 2005).

The sequence of PT-1 had 62% homology with serine protease inhibitor belonging to the Kunitz family and the peptide inhibited chymotyrpsin, trypsin and papain. These protease inhibitors were composed of polypeptide chains joined by disulfide bridges and reduced PT-1 almost completely lost its activity against fungi. The results suggested that PT-1 is an excellent candidate as a lead compound for the development of novel oral or other anti-infective agents (Kim *et.al.,* 2005).

Serine protease inhibitors have been proposed as a strategy against insect pests. Macedo et al. 2004 have been studied that transgenic grains which expresses gene encoding protease inhibitors may prevent seed damage against Lepidoptera and Coleopteran species without side effects which include reduced fecundity, increased mortality, decreased weight gain and severe deformation though out their developmental phases (Macedo *et al.,* 2002). The potential of these inhibitors that expressed in transgenic plants showed a higher resistance to various insects.

Broadway (1995) were researched on six species of Lepidoptera have also been evaluated for their susceptibility to serine proteinase inhibitors from cabbage. The serine proteinase activity in the midguts of larval *PZutella xylostella* was moderately inhibited (40-50%), and *Trichoplusia ni, Lymantria dispar,* and *Helicoverpa zea* were substantially inhibited (55-

85%) by cabbage proteinase inhibitors, while Trypsin and chymotrypsin activity from larval *Pieris rapae* and *Pieris napi* were not significantly inhibited (0-18%), *in vitro,* by cabbage proteinase inhibitors.

These results shown that the growth and development of the latter three species should be reduced following ingestion of these inhibitors but chronic ingestion of cabbage proteinase inhibitors only reduced the growth and development of *T. ni.* A shift in the relative proportion of digestive enzymes was responded to ingestion of proteinase inhibitors because lack of biological activity of the proteinase inhibitors against the other two species. Following ingestion of cabbage proteinase inhibitors, the trypsin(s) in *T. ni* was moderately susceptible (37% inhibited), while the predominant trypsin-like enzyme(s) in the midgut of larval *L. dispar* and *H. zea* were resistant to inhibition by cabbage trypsin inhibitors (13-18% inhibited). These results were confirmed for *H. zea* and *T. ni* feeding on proteinase inhibitors in tomato foliage (Broadway *et al.,* 1995).

Atkitson and friends (1993) were reported that protease inhibitors may function to protect the reproductive tissue against potential pathogens. Their research had shown the isolation of cDNA clone encoding a protein with sequence similarity to a protease inhibitors type II of potato and tomato. These protease inhibitors were expressed in ornamental tobacco, *Nicotiana alata* stigmas that derived from a precursor protein which is isolated peptide inhibitors with five homologous inhibitors to six regions of the amino acid sequence deduced from the cDNA clone (Atkinson *et al.,* 1993).

Brown and Ryan (1984) shown that serine protease inhibitors in leaves of plants are response to wounding. In leaves of tomato, potato tubers and legume seeds contain inhibitors that 10% or more of the stored proteins (Green and Ryan, 1972; Richardson, 1977; Atkinson *et al.,* 1993). PIs can accumulate to 2% of the soluble protein within 48

hours of insect attack or other types of wounding (Brown and Ryan, 1984; Graham *et al.,* 1986).

The main objective of this research was:

1) To screen plant extracts with inhibitory activities
2) To purify to homogeneity proteins with inhibitory activities
3) To characterise the active proteins

CHAPTER 3: MATERIALS AND METHODS

3.1 MATERIALS

All chemicals and materials employed in this study were of the highset grade obtainable, unless it were otherwise described.

1. **PROMEGA**-Madison, Wisconsin, USA

   -Tris-Base

2. **R&M CHEMICAL**-Washington, New Jersey

   -Dimethy Sulfoxide (DMSO)

3. **FISHER SCIENTIFIC**-Hampton, New Hampshire

   -Triton X-100

4. **VIVASCIENCE**-Gottingen, Germany

   -Centrifugal concentrator

5. **SYSTERM-**Selangor, Malayisa

   -Dichloromethane, 95% Ethanol, Glycerol, Sodium Hydroxide (NaOH), Methanol, Ammonium Sulfate, 85% Phosphoric Acid, Acetone

6. **SIGMA Chemical Corporation**-Saint Louis, USA

   -Coomassie Brilliant Blue G, Trypsin from bovine pancreas TPCK Treated, Bovine Serum Albumin, Bromsulfalein

7. **BIORAD Laboratories**-Hercules, CA, USA

   -N-alpha-benzoyl-dl-arg-*p*-nitroanilide (BapNA), Ammonium Persulfate, 2.5% and 10% Sodium Dodecyl Sulphate, Phenol Red, 10x Tris/Glycine/SDS, N,N,N'N' – tetramethylenediamine (TEMED), 30% Acrylamide/Bisacrylamide, 1.5M Tris-HCL pH 8.8, 0.5M Tris-HCL pH 6.8, 10x Tricine SDS Running Buffer, Tricine buffer, 25mM Tris/HCL buffer pH8.0

8. **INVITROGEN**-USA

-Novex® 10-12% Tricine Gel (1.0mm, 12 well), Novex® Tris-Glycine SDS Sample Buffer (2X), Pre-cast Tricine gel

3.2 EQUIPMENTS

1. Shaking incubator (SI-900R)
2. Jusco 6300 spectrophotometer
3. Hi Trap G-25 chromatography
4. Rotary evaporator
5. Fumehood
6. Spectrophotometer

3.3 METHODS

3.3.1 Preparation of Plant Extract

Several of plants from the families Leguminosae, Rubiaceae, Apocynaceae and Euphorbiaceae were collected from University of Malaya campus ground and the species were identified according to Boo *et al.,* (2006). All part of the plants such as the leaves, trees, roots and fruits were subjected to oven drying at temperature less than 55˚C according to Chen *et al.,* (2005). The dried plants were ground into powder form where then 20g of these plant were weigh and homogenized with 200ml of dicholoromethane. These plants mixture were left overnight at room temperature while being shaked on a shaking incubator (SI-900R) at 100 rpm/min. The homogenate was then filtered to removed the dichloromathane and the resulted precipitate obtained was dried overnight at room temperature in fumehood. The dried precipitate were then macerated in 50% (v/v) ethanol

with 200ml of 95% ethanol: distilled water (1:1). These mixture were shaken overnight on a shaking incubator (SI-900R) at 100 rpm/min. The filtrate were collected through filtration method and subjected to dicholoromethane partitioning for purification. The purified filtrates were concentrated using rotary evaporator at 38°C by removing the ethanol solvent in it. The concentrated extract was centrifuged at 5,000 xg, 4°C for 15 minutes and cotton plug were used to discard the plant debris. Supernatant collected were subjected to 90% ammonium sulphate precipitation and further re-centrifuged at 5,000xg, 4°C for 15 minutes. The supernatant were again re-collected and freeze dried for further analysis.

3.3.2 Protein content determination

Bradford assay (Bradford, 1976) were used to determine the total protein content of the dried extract samples using bovine serum albumin as the stock standard protein. The resulted protein standard curve determination method and graph result was shown in Appendix 1. For every plant tested, 1 mg of dried plants ethanolic extracts were diluted in 100µl of distilled water before 5ml of bradford reagent (preparation refer Appendix 2) were added to the mixture. The mixture was left incubated at dark room at room temperature for 10 minutes before the absorbance was measured at 595 nm using spectrophotometer. The amount of proteins present in each dried plants ethonolic extracts were estimated from the standard curve in Appendix 1.

3.3.3 Trypsin Inhibitory Assay

The dried plants extracts were subjected to Trypsin inhibitory assay as described by Jennings *et al.,* (2001) and Heath *et al.,* (1995). This assay is done in duplicates to screen the presence of inhibitory activity in each sample towards commercial trypsin. Each dried plants extracts were added with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) trypsin treated from bovine pancreas with concentration of 0.5g in 1 ml 50 mM Tris-HCL buffer at pH 8.0 (Appendix 4). The mixture was incubated in water bath at 30°C for 10 minutes before 30 µl of N-alpha-benzoyl-dl-arg-*p*-nitroanilide (BapNA) (0.0435g/ml) was added into the mixture making the final volume to 3ml. The reactions were monitored for 10 minutes against blank solution containing substrate solution in the same buffer used using Jusco 6300 spectrophotometer.

3.3.4 Trypsin inhibitory Activity detection in SDS-polyacrylamide gel electrophoresis and Tricine SDS

The detection of inhibitor of the dried plants ethanolic extracts were done using the SDS-PAGE with Laemmli buffer system (Lemmli, 1970) as described by Hanspal *et al.,* (1982). The electrophoresis was done at room temperature on 17% separating minigel containing 0.1% (w/v) gelatin. Two types of samples were used in this assay, the dried plants ethanolic extracts samples and the eluent from Hi Trap G-25 chromatography samples (refer Appendix 5 for preparation). Each type of samples were prepared differently before tested where 20mg of the dried plants ethanolic extracts samples were diluted with 20 µl aqueous solution while 1 mg of the G-25 chromatography eluent samples were diluted in 20 µl aqueous solution from this point each samples were treated the same way where each

samples was dissloved in aqueous solution of 2.5% (w/v) SDS, 10% (v/v) glycerol and phenol red (4 mg/ml) (Appendix 7).

Electrophoresis for each sample was carried out at at constant current of 20mA with the presence of running buffer at room temperature. The electrophoresis was stopped once the tracking dye reached approximately 1 cm from the bottom of the gel.

The resolving gels were washed 3 times with 300ml of 2.5% (w/v) Triton X-100 for 45 minutes each before finally washed with 300ml of ultrapure water for 45 minutes with gentle rocking on the shaker.

The washed gels were then incubated in 450ml of 0.1M glycine-NaOH buffer pH 8.3 for 16 hours with the addition of 6.4 mg of trypsin at the temperature of 37°C to observe the trypsin effect on the samples.

The sample for the Tricine- Sodium dodecyl sulfate (SDS) were prepared by mixing 20 mg of dried plants ethonolic extracts sample with 120 µl pre-made buffer (refer Appendix 8). 10µl of each samples prepared and 5 µl of benchmark protein ladder were loaded into the pre-cast gel (invitrogen) with 4-20% acrylamide concentration; Tricine SDS gels respective well and elctrophoresis followed by staining were carried out as mentioned above.

3.3.5 Preparation of *Chrysomya megacephala* crude protease extracts

Adult *Chrysomya megacephala* were crushed in the ratio of 1 :500 µl ultrapure water in an eppendorf tube. The extracts were centrifuged and the supernatant was collected and kept in -20°C for further analysis.

3.3.6 Mode of inhibition and K_i value determination

The trypsin inhibitory assay was again applied in determining the plant samples K_i value as described by Jennings *et al*., (2001) and Heath *et al*., (1995) in section 3.2.3. the plants samples were prepared by mixing 1 µg of the dried plant ethanolic extract in 150 µl of ultrapure water. Two concentration of substrate were used in this assay which are 1 mM and 5 mM of BapNA in DMSO. The concentration of plant sample used was 5.0×10^{-3} µg/µl, 2.5×10^{-3} µg/µl, 1.25×10^{-3} µg/µl, 1.0×10^{-3} µg/µl, 6.25×10^{-4} µg/µl, 5.0×10^{-4} µg/µl, 5.0×10^{-5} µg/µl and 5.0×10^{-6} µg/µl. The assays were done in duplicates for each samples and a control without enzymes for both substrates concentration.

3.2.7 Thermostability determination

The trypsin inhibitory assay as described by Jennings *et al*., (2001) and Heath *et al*., (1995) in section 3.2.3 was again used in determining the lyophilized samples extract thermo stability. The samples were prepared by diluting lyophilized samples extract in 250µl of ultrapure water and subjected to the assay with varying incubation period ranging from 15°C-90°C for 10 minutes. 30 µl of BapNA in DMSO were added into the mixture before the reaction was monitors with Jusco 6300 spectrophotometer. The experiments were repeated with two replicates and control without enzymes.

3.2.8 IC_{50} estimation

The dried plant ethanolic extract IC_{50} value were estimated by determining the concentration value at the intersection between percentage of activity and percentage of

inhibition of trypsin by the dried plants ethanolic extracts versus its concentration that were plotted on the same graph. The readings for the percentage of activity and percentage of inhibition of trypsin by the dried plants ethanolic extracts were taken from the trypsin inhibitory assay as described by Jennings *et al*., (2001) and Heath *et al*., (1995). The concentration of plants samples used were 4.0×10^{-3} µg/µl, 6.0×10^{-3} µg/µl, 8.0×10^{-3} µg/µl, 1.0×10^{-2} µg/µl, 1.2×10^{-2} µg/µl, 1.4×10^{-2} µg/µl, 1.6×10^{-2} µg/µl, 1.8×10^{-2} µg/µl and 2.0×10^{-2} µg/µl.

CHAPTER 4: RESULTS

4.1 Screening of trypsin inhibitors

Table 1: Shows various plants sample percentage inhibitory of trypsin activity

Plants families	Plants name		Percentage of Reduction Activity
Leguminosae	*Erythrina fusca*	Leaves	44 %
		Flower	13 %
	Cassia floribunda	Leaves	17 %
		Fruits	8 %
	Delonex regia	Leaves	nil
		Fruits	2 %
	Senna surattensis *	Leaves	83 % *
	Acacia mangium	Leaves	6 %
	Caesalpinia pulcherrima	Leaves	12 %
	Clitoria ternatea	Flower and Fruits	7 %
	Cassia alata	Leaves	nil
		Fruits	nil
	Bauhinia blakeana	Leaves	21 %
		Fruits	nil
	Andira inermis	Leaves	7 %
		Fruits	nil
	Mimosa diplotricha *	Fruits	92 % *
	Pterocarpus indica	Leaves	59 %
		Fruits	nil
	Adenanthera pavomina	Leaves	52 %
	Acacia auriculiformis	Leaves	13 %

		Fruits	Nil
Rubiaceae	*Ixora finlaysoniana*	Leaves	nil
	Mussaenda erytrophylla	Leaves	18 %
		Flower	9 %
	Uncaria spp	Leaves	10 %
		Flower	nil
	Euclinia longiflora	Leaves	7 %
	Porterandia anisophylla	Leaves	34 %
	Morinda elliptica	Leaves	nil
	Gardenia carinata	Leaves	27 %
	Mussaenda philippica 'Queen Sirikit'	Leaves	10 %
	Mussaenda phillipica	Leaves	5 %
		Flower	21 %
	Morinda citrifolia	Fruits	19 %
Apocynaceae	*Allamanda oenotherafolia*	Flower	36 %
	Allamanda cathartica	Leaves	nil
	Plumeria rubra cultivars	Leaves	nil
		Flower	24 %
Euphorbiaceae	*Ricinus communis*	Leaves	8 %
		Fruits	23 %
	Jatropha gossypfolia	Leaves	27 %
		Fruits	30 %
	Macaranga tanarius	Flower	75 %

(*) Selected for further analysis for showing higher percentage of trypsin inhibitory activity

Table 1 listed total of 44 lyophilized plant sample extract from the families Leguminosae, Rubiaceae, Apocynaceae, Euphorbiaceae. From this table, only two plants were selected as the subject for this experiment for the significantly high reduction activity shown. The plants were *Senna surattensis* leaves with 83 % reduction activity and *Mimosa diplotricha* fruits with 92% reduction activity.

Almost from each family, there were some plants extract that were labeled as 'nil'. These refering to the plants extract that showed no trypsin content reduction activity. Total of twelve plants extracts were labeled as 'nil' from the table 1 which comprise of seven plants extracts form the Leguminosae family, three plants extracts from Rubiaceae family and two plants extracts from Apocynaceae family.

Others plants extracts showe varies reduction activity percentage ranging from 5 % to 75 % such as Mussaenda *phillipica* leaves from Apocynaceae family and *Macaranga tanarius* from Euphorbiaceae family respectively.

4.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) result of sample extracts

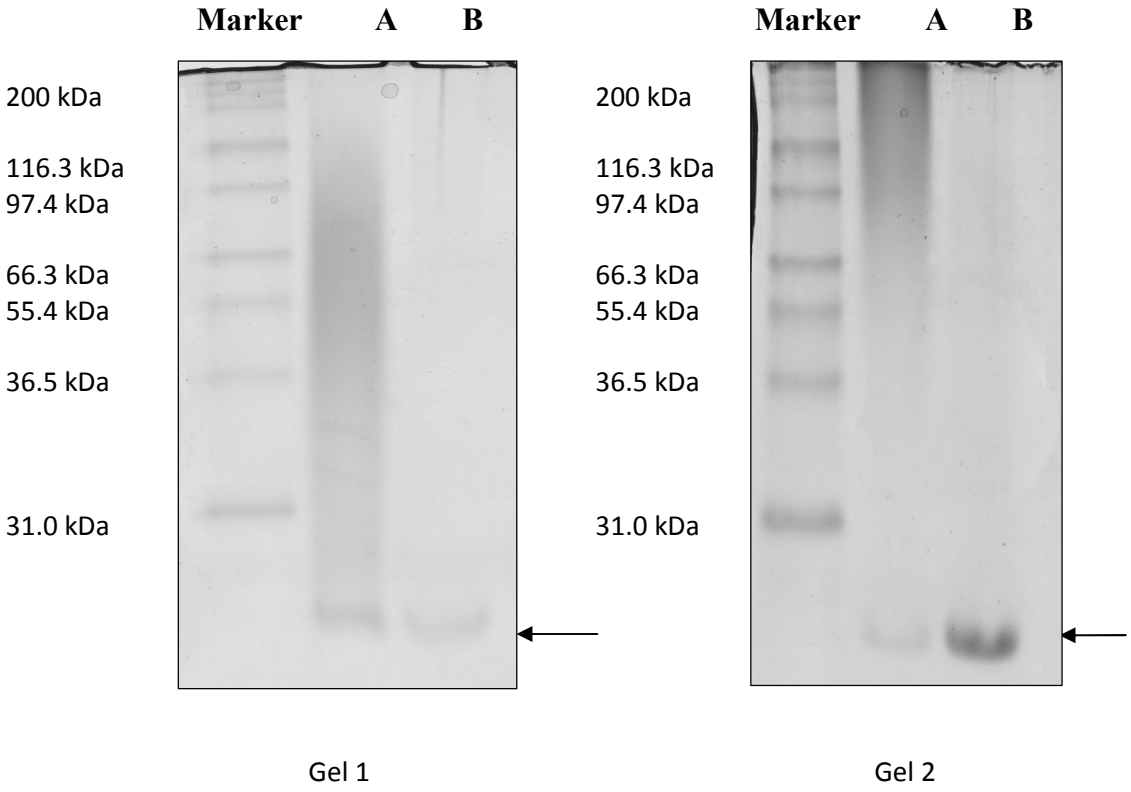

Figure 1: shows SDS-Page gel result of lyophilized sample extracts of *Senna surattensis leaves* (A) and *Mimosa diplotricha* fruit (B) on 17% acrylamide gel under two different conditions which is with trypsin incubation and without trypsin incubation. Markers used in these gels were Mark 12 from invitrogen and these gels were stained with Coomassie Brilliant Blue staining.

Gel 1 shows the results with the presence of trypsin incubation while gel 2 shows the results without the presence of trypsin incubation. From Figure 1, In both gels, there were protein band present (shown by arrow) which indicated no lysis of protein in the band region due to the the presence of protein inhibitor from *Senna surattensis leaves* (A) and *Mimosa diplotricha* fruit (B).

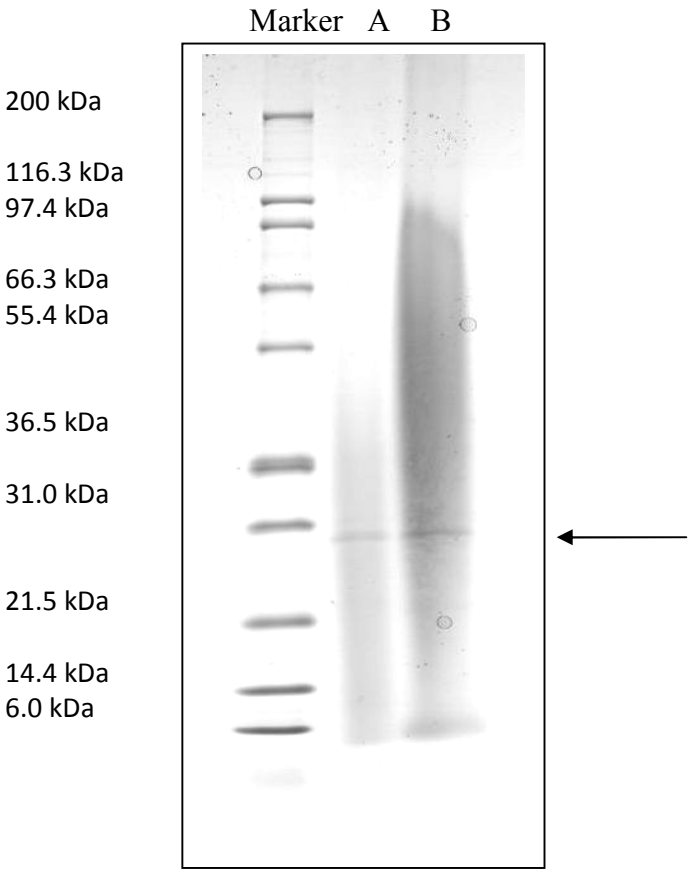

Figure 2: shows the bands of lyophilized sample extracts of *Senna surattensis leaves* (A) and *Mimosa diplotricha* fruit (B) on Pre-cast (invitrogen) Tricine SDS gel at 4-20% acrylamide concentration. Marker used in this gels were Mark 12 from invitrogen and these gels were stained with Coomassie Brilliant Blue staining.

Figure 2 showed a bands for each sample; *Senna surattensis leaves* (A) and *Mimosa diplotricha* fruit (B). The band formation proven the presence of inhibitor in both samples. According to band formation and sample migration, the molecular weight for both sample was estimated to be around 26.0 kDa to 31.0 kDa.

Next, both *Senna surattensis* leaves (A) and *Mimosa diplotricha* fruit (B) was subjected to G-25 chromatography for desalting purpose. The protein was then separated on Tricine SDS (invitrogen). Based on figure 3, the molecular weight of *Senna surattensis* leaves (A) was determine to be 27.93 kDa while *Mimosa diplotricha* fruit (B) was unable to produce any band after desalting proses (result not shown here), thus the sample was ommited from further anylysis.

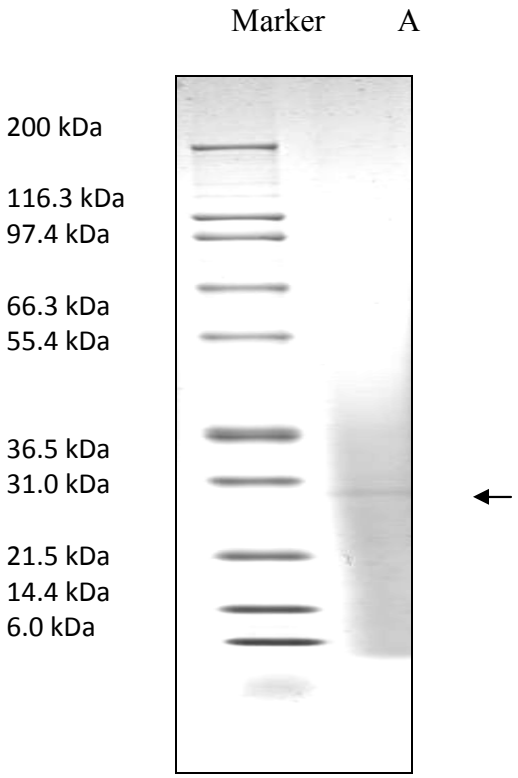

Figure 3: Separation of G-25 chromatography eluent of *Senna surattensis* leaves (A) on Tricine SDS gel. Marker used in these gels was Mark 12 from invitrogen and these gels were stained with Coomassie Brilliant Blue staining.

4.3 Determination of *Senna surattensis*'s leaves Mode of inhibition and K_i value

Table 2: The reduction of trypsin activity percentage at two different concentration of substrate

Initial concentration of *Senna surattensis*'s leaves	**Concentration of substrate (BapNA)**	**Concentration of inhibitor (mg/µl)**	**Concentration of inhibitor (mM)**	**1/v (mol/min/ml)$^{-1}$**
6.7 x 10-3 mg/µl	1mM	5.0×10^{-3}	1.8×10^{-3}	0.6270
		2.5×10^{-3}	9.0×10^{-4}	0.3729
		1.3×10^{-3}	4.5×10^{-4}	0.2220
		1.0×10^{-3}	3.6×10^{-4}	0.1971
		6.3×10^{-4}	2.2×10^{-4}	0.1290
		5.0×10^{-4}	1.8×10^{-4}	0.0875
		5.0×10^{-5}	1.8×10^{-5}	0.0490
		5.0×10^{-6}	1.8×10^{-6}	0.0398
	5mM	5.0×10^{-3}	1.8×10^{-3}	0.2379
		2.5×10^{-3}	9.0×10^{-4}	0.1087
		1.3×10^{-3}	4.5×10^{-4}	0.0673
		1.0×10^{-3}	3.6×10^{-4}	0.0611
		6.3×10^{-4}	2.2×10^{-4}	0.0597
		5.0×10^{-4}	1.8×10^{-4}	0.0572
		5.0×10^{-5}	1.8×10^{-5}	0.0488
		5.0×10^{-6}	1.8×10^{-6}	0.0438

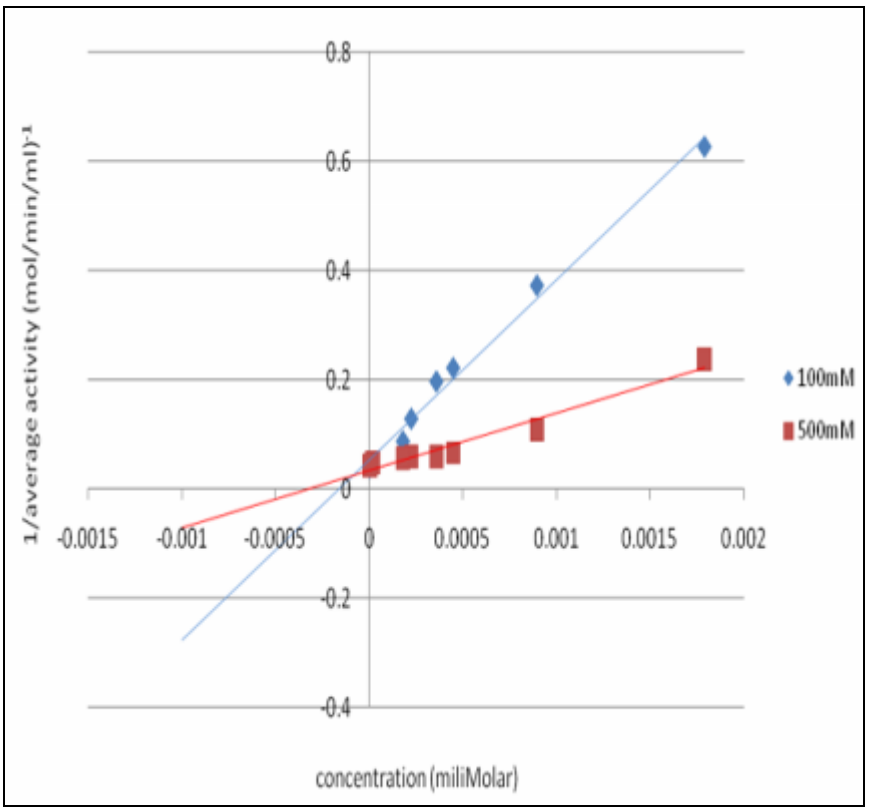

Figure 4: The dixon plot of concentration versus 1/v sample extract of *Senna surattensis*'s leaves in the presence of 1mM and 5mM BapNA in DMSO.

4.4 Determination of *Senna surattensis*'s leaves thermostability

Table 3: Percentage of reduction in trypsin inhibitory activity *Senna surattensis*'s leaves extract in various incubation temperature.

Temperature	Percentage of Reduction
15°C	70.44
30°C	83.52
45°C	87.35
60°C	81.33
75°C	80.13
90°C	79.32

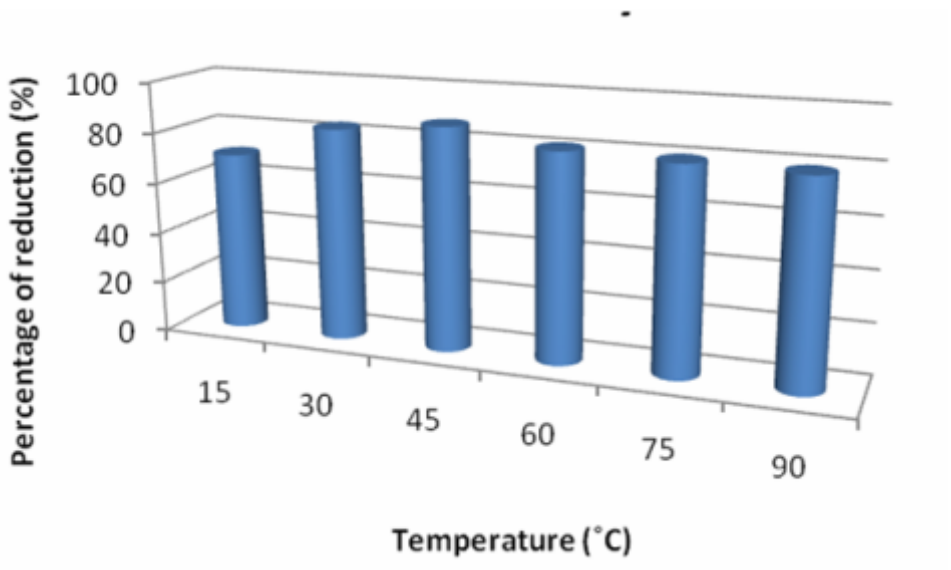

Figure 5: The bar chart plotted of *Senna surattensis*'s leaves extract temperature versus its reduction of activity percentage.

Based on Table 3, the percentage of reduction of the inhibitory activity of lyophilized *Senna surattensis*'s leaves ethanolic extract towards trypsin show a constant increase at the incubation temperature ranging from 15°C to 45°C. 45°C is shown to be the optimal temperature for *Senna surattensis*'s leaves ethanolic extract with 87.35 % reduction of the inhibitory activity towards trypsin. At temperature more than 45°C, a constant decrease of percentage reduction of the inhibitory activity towards trypsin can be seen.

4.5 Estimation of *Senna surattensis*'s leaves IC_{50} value

Table 4: Percentage of reduction in activity of crude insect's trypsin when subjected to different concentration of *Senna surattensis's* leaves proteinaceous extract

Concentration (µg/µl)	Percentage of reduction in activity (%)
0.004	11.9
0.006	12.8
0.008	16.4
0.010	26.4
0.012	34.0
0.014	37.3
0.016	40.1
0.018	59.3
0.020	66.6

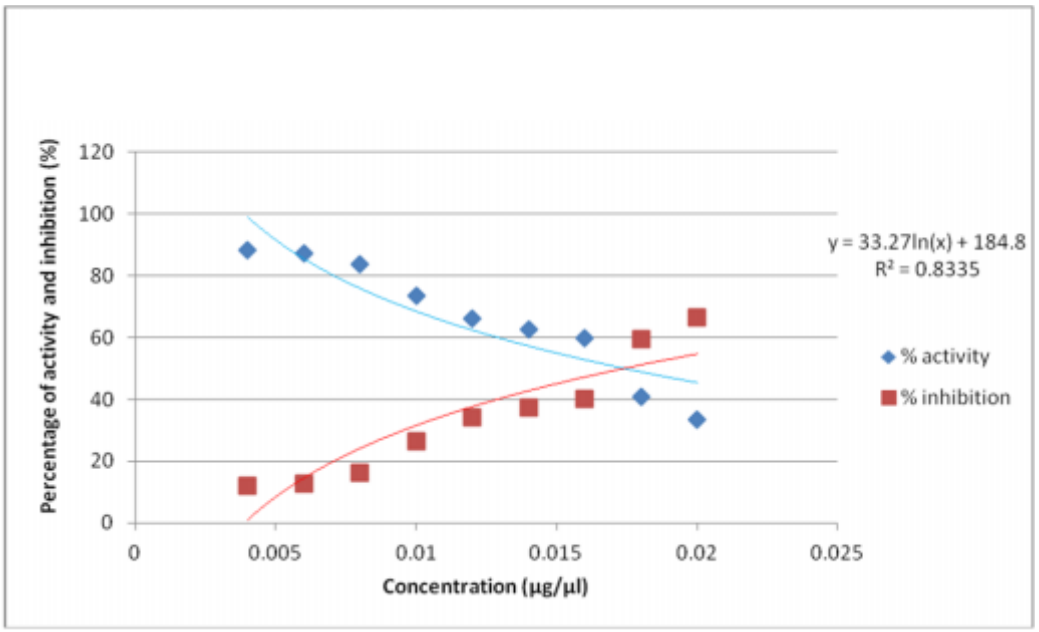

Figure 6: shows the *Senna surattensis*'s leaves percentage of inhibition and activity of trypsin concentration.

DISCUSSION

Protease inhibitors are referring to a group of protein that regulates proteolysis. These inhibitors have been found in numerous sources, including plant such as soybean, human pancreas and hemolymph of several arthropods and insect such as *Bombyx mori* and *Drosophila melanogaster* (Cherqui *et al.,* 2001).

However, in this study, we only focus on plant derived protease inhibitor. Protease inhibitors derived from plants have the ability to reduce insect's digestive capacity through specific inhibition of digestive proteases, resulting in insect growth and development arrest thus can be used as an alternative strategy to control insect pests (Aguirre *et al.,* 2009).

30 different plants were collected in order to study the presence of serine protease inhibitor and to purify them. All of these plants were from the families of Leguminosae, Rubiaceae, Apocynaceae and Euphorbiaceae and the species of these plants were determined according to Boo *et al.,* (2006). All of the plants samples were randomly collected from the University of Malaya campus ground.

Different parts of the plant such as fruits, trees, leaves and flowers were used in this study. There were about 43 plants samples obtained from these 30 different plant species. Leguminosae, Rubiaceae, Apocynaceae and Euphorbiaceae contribute about 21 samples, 13 samples, 4 samples and 5 samples respectively.

As suggested by previous study, the seed and vegetative parts of plant contain various proteinaceous inhibitors of insect, fungal, mammalian and endogenous proteinases. This inhibitor mainly involve in plant defense mechanism and nowadays become great interest

to many researcher as the gene coding for this inhibitor can be genetically transfered to other plants to improve their resistance to pests or fungi (Konarev *et al.,* 2002).

In the screening process, all of these plant were analyzed according to method as described in section 3.2.1. which comprised of dichloromethane fractionation and sample concentration using rotary evaporator. This method are widely used by many researcher for plant crude extracts fractionation such as the fractionation of seven plant materials; fenugreek seeds, harmal seeds, garlic cloves, cinnamon bark, sticky fleabane leaves, and nightshade leaves and fruit for detecting the In vitro Antifungal Activities (Kanan and Al-Najar, 2009).

At the initial stage, some of these plants were treated with ammonium precipitation to concentrate the samples. Aguirre *et al.,* (2009) has also adopted this method in their research where the crude extract of their samples; larvae of *Prostephanus truncatus* was precipitated with ammonium sulfate at 70% saturation for purification. However during this step, we figured out that ammonium precipitated samples were most likely to produce an unknown solution on top of the samples that we later find out as lipid-like solution.

Since, the objective of this study is to screen and purify serine protease inhibitor, we were more interested in hydrophilic protein, thus we decided to discard the lipid-like solution; the hydrophobic solution. But in doing so, we found out that the final volume of the extraction were greatly reduced thus limited our samples extracts for further analysis.

We also feared that by discarding the lipid-like solution will most likely to induce more contamination to our sample extracts. We finally found a better method which is to freeze dried our samples. This method was found to be the best method so far for our samples since it can preserve the quality and quantity of our samples. The powder produced from

the freeze drying methods were stored in -80˚C for long term storage and -20˚C for short term storage and reconstitute with ultrapure water or appropriate buffer for later use in any assays.

After all of this plants had been extracted, the protein content for each plant extracts were determined using the Bradford Assay (Bradford, 1976). The results of this assay were presented in Table 5 in Appendix 3. The estimated protein content obtained is ranged from 0.24 µg/ml to 34.46 µg/ml.

After the protein content estimation, all of the plant extracts were further tested with Trypsin Inhibitory Assay to determined the presence of inhibition towards protease namely trypsin. These methods have been used in determining the total trypsin inhibitor activity of raw soy milk using N-benzoyl-DL-arginine-*p*-nitroanilidehydrochloride (BAPNA) as the substrate (Guerrero-Beltran *et al.,* 2009).

From the results shown in Table 1, it is shown that from the 43 plant samples, about 32 samples shows inhibition towards trypsin activity. The remaining 11 samples did not shows any inhibition towards trypsin activity thus labelled as 'nil'. They comprised of 6 'nil' samples, 3 'nil' samples and 2 'nil' samples from Leguminosae, Rubiaceae, families respectively. These 'nil' labeled samples are most likely resulted from plants that have no protease inhibitor activity ability or loss the protein for protease inhibition ability during the initial ammonium sulphate precipitation and the lipid-like solution removal.

In the Leguminosae family, the plant samples inhibition towards trypsin activity are ranging from as low as 2 % to as high as 92 %, which were *Delonex regia* fruits and *Mimosa diplotricha* fruits respectively. A previous study have reported that Kunitz-type trypsin inhibitors have been isolated from the seeds of *Delonex regia* (Lin and Ng 2008). 10

samples out of 13 samples in Rubiaceae family shows inhibition towards trypsin activity are ranging from 7 % to 34 %.

Only two samples in Apocynaceae family shows inhibition towards trypsin activity which are *Plumeria rubra cultivars* flowers with 24 % and *Allamanda oenotherafolia* flower with 36 %. The last family; Euphorbiaceae samples shows inhibition towards trypsin activity in the range of 8 % to 75 % with no samples labeled as 'nil'.

From the results presented in Table 1, all the results were narrowed to choose the two highest inhibition percentages toward trypsin. Since the highest readings for inhibition towards trypsin activity were from the Leguminosae family, which were *Mimosa diplotricha* fruits and *Senna surattensis* leaves with 92 % and 83 % reaspectively. Both of these plants were selected for further analysis in this study.

This result is agreeable to many previous studies finding that claimed most serine proteinase inhibitors have been isolated and characterized from Leguminosae. Other family that have been also reported to have protease inhibitor as such as Cucurbitaceae, Solanaceae,Graminae, Rutaceae and Euphorbiaceae (Chaudhary *et al.,* 2008).

Then, the lyophilized plants extract samples for both *Mimosa diplotricha* fruits and *Senna surattensis* leaves were electrophorised on Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Figure 1) and Pre-cast (invitrogen) Tricine SDS gel (Figure 2) to shows the presence of protease inhibitor in both sample extracts selected.

The SDS-PAGE with 17% resolving gel concentration and 4% stacking gel concentration were used in this test. We also improvised the SDS-PAGE method by adding an extra steps after the electrophoresis was done. We added trypsin enzyme into 0.1M glycine-NaOH

buffer pH 8.3 and left the gel in the buffer-trypsin mixture for 16 hours at room temperature 37°C.

The improvising was carried out base on our understanding that the trypsin enzyme will degrade all of the protein presence in the gel. But the degradation will not occur if there were any protease inhibitor in the plants extract as this protease inhibitor will stop the protein degradation by trypsin. The presence of these protease inhibitor were proven by the presence of the band on both gel 1 and gel 2 with trypsin incubation and without trypsin incubation respectively as shown in Figure 1.

Both sample extracts were then electrophorised on Tricine Pre-cast gel from invitrogen with 4-20 % acrylamide concentration. We decided to test both sample extracts on tricine gel to further separate the protein that are less than 30 kDa because the previous Leammli SDS-PAGE done can only separate protein that are more than 30 kDa in size.

Results in Figure 2 in section 4.2 shows one band respectively for each sample extracts lane; *Senna surattensis leaves* (A) and *Mimosa diplotricha* fruit (B) below the 31.0 kDa size protein marker.This gel indicates that the protease inhbitor presence may be of low molecular weight proteins. The molecular weight for both samples was estimated to be around 26.0 kDa to 31.0 kDa.

The selected *Mimosa diplotricha* fruits and *Senna surattensis* leaves were then subjected to Hi-Trap Sephadex G-25 chromatography as mentioned in appendix 6 to remove the excessive salt in the plant extracts. The desalted eluent were also freeze dried and kept in -20˚C to minimize the protein degradation thus, preserve the eluent quality.

The freeze dried samples eluents was then again electrophorised on tricine SDS-PAGE. The result was shown in Figure 3 where it showed one clear band on the *Senna surattensis leaves* (A). From this gel and gel in Figure 2, the molecular weight of *Senna surattensis* leaves were calculated and was determined to be 27.93 kDa.

However, the *Mimosa diplotricha* fruit did not manage to produce any band in all the attempt done. It will either shows no band formation on the lane if the the extracts concentration used is low or a thick smear on the samples extract lane if higher concentration were used (results is not shown here). The smearing may have derived from sample overloading or simply because of too concentrated protein loaded into the gel. Thus the *Mimosa diplotricha* fruit were omited from further analysis and the molecular weight of *Mimosa diplotricha* fruit were estimated as to be 29.17 kDa from the Figure 2.

We did further tested the purified protein from *Senna surattensis* leaves protease inhibitor activity toward untreated protease namely trypsin that was obtained from *Chrysomya megacephala.* These blowflies are also known as the oriental latrine fly belong to the Calliphoridae family. It is known to spread disease to human and also cause myiasis in human where it invades an open wound (Sukontason *et al.,* 2005).

The *Chrysomya megacephala* were crushed and the supernatant collected were tested with *Senna surattensis* leaves fortrypsin inhibitory assay. Result are shown in Table 8 in Appendix 10 where *Senna surattensis* leaves extract showed 83.52 % inhibitory activity towards commercial trypsin and 61.74 % inhibitory activity towards *Chrysomya megacephala* extracted protease.

Even though the reading for inhibitory activity towards *Chrysomya megacephala* extracted protease was lower when compared to commercial trypsin, the results still shows that this *Senna surattensis* leaves indeed has protease inhibitor ability towards any source of protease, be it crude or purified protease. The ability of *Senna surattensis* leaves to inhibit protease in *Chrysomya megacephala* has opened a path to do more research for insecticide purpose on this plant extract.

The next test carried out was the study of mode of activity of this *Senna surattensis* leaves. This study is done to determine the plant Ki value using two concentration of substrate. The substrate used in this assay was still (BAρNA) but at two different concentrations of 1 mM and 5 mM respectively. The Ki value of *Senna surattensis* leaves was determined from the dixon plot as shown in Figure 4 in section 4.3 with the reciprocal velocity (1/v) is plotted against concentration of inhibitors for both substrate concentration.

The Ki value of *Senna surattensis* leaves determined is 8.89 $x10^{-5}$ mM. The analysis of Dixon plot showed that *Senna surattensis* leaves is a competitive inhibitor because two lines corresponding to each substrate intersect above the x-axis. A previous study on *Putranjiva roxburghii* belonging to Euphorbiaceae family showed that this plant has ki value of 1.4x 10^{-11} (Chaudhary *et al.,* 2008). By comparing the Ki value in this study with the *Putranjiva roxburghii* Ki value, it shows that *Putranjiva roxburghii* is highly potent inhibitor of bovin trypsin compared to *Senna surattensis* leaves.

The next test done on *Senna surattensis* leaves lyophilized extract was thermo stability determination. In this test, the trypsin inhibitory activity of the lyophilized extract was examined at different temperatures ranging from 15°C-90°C in the water bath. This test

was carried out using Trypsin Inhibitory Assay with N-alpha-benzoyl-dl-arg-*p*-nitroanilide (BapNA) as the substrate.

As result shown in Table 3, it shows that the *Senna surattensis* leaves lyophilized extract inhibitory activity increased as the temperature increased from 15°C-45°C. At 15°C, the extracts showed 70.44 % of inhibitory activity and at 30°C, the extract showed 83.52 % of inhibitory activity. The extract reached its optimum inhibitory temperature at 45°C with 87.35 % of inhibitory activity.

When the reaction temperature was further increased more than 45˚C, the inhibitory activity started to decreased. At 60°C, the inhibitory activity of the extract decreased to 81.33 %, and further decrease to 80.13 % and 79.32% at 75˚C and 90˚C respectively. This is due to the sample extract protein thermal degradation at temperature more than 45˚C.

Research done by Asif-Ullah *et al.,* (2006) on *Cucumisin* claimed to contain subtilisins which are the second largest class of serine proteases. It is found out through the thermostablity study that this plant protease increase as the reaction temperature increased from 20˚C to 70˚C and reach the higher than that of the activities at 37˚C. Due to thermal denaturation of the protein, the activity rapidly decreased as the reaction reached 80˚C and higher. By comparing it with our results, it shows that *Senna surattensis* leaves have limited range of temperature to works in compared to Cucumisin.

The final test done on *Senna surattensis* leaves lyophilized extract was IC_{50} value determination using trypsin inhibiory assay. Nine concentration of this purified protein from *Senna surattensis* leaves were used in this experiment ranged from 0.004 µg/µl to 0.020 µg/µl. Figure 4 shows the effect of *Senna surattensis* leaves lyophilized extract with

different concentration against insect's (*Chrysomia megacephala*) trypsin. The IC_{50} value obtained in this study was 0.0174 µg/µl.

These method adopted to determine the IC_{50} value was also used by other researchers in many previous study in determining few plant with protease inhibitor such as dull black soybean that are found out as to contains Kunitz-type trypsin inhibitor (Lin and Ng 2008). While another study adopted this method towards cinnamon, garlic or sticky fleabane resulted in IC_{50} values for these fractions were found to be in the range of 11.2-24; 30.25-31.50; 25.0-36.0 µg/ml, respectively (Kanan and Al-Najar 2009).

CONCLUSION

This study shows that protease inhibitor that derived from *Senna surattensis* leaves have a wide potential to be explore in many field especially in producing insecticide. This is due to the fact that Serine proteinase inhibitors is thought to have phytochemical defenses against herbivorous insects (Broadway 1995) apart from its other usage in other filed; biological functions, such as blood coagulation, platelet aggregation and anti-carcinogenesis (Chaudhary *et al.,* 2008).

The molecular weight of obtained for *Senna surattensis* leaves were calculated and was determined to be 27.93 kDa while the molecular weight of *Mimosa diplotricha* fruit were estimated as to be 29.17 kDa (Table 7 in Appendix 9). Both plants have the potential serine protease inhibitor that needs to be explored more. The Ki value of *Senna surattensis* leaves determined is 8.89 $x10^{-5}$ mM with the mode of activity determined as competitive inhibitor.

Even though *Senna surattensis* leaves showed stability at a narrow range of temperature, which is only until 60 ˚C, but it is still should be given a fair chance to be studied even more because the efficacy of inhibitor is not only dependent on the temperature it react also dependent on the compatibility of the plant protease inhibitor reactive site and the substrate binding site in the target organism (Broadway 1995).

Current literature review only support the general hypothesis of the serine proteinase inhibitors ability in protecting plants against herbivorous insects (Broadway 1995). Thus, future research should also include the adverse effect of protease inhibitor usage and on compatibility of the protease inhibitor reactive site with other target binding site to produce the most effective insecticide toward wide range of insects.

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APPENDIX 1

DETERMINATION OF PROTEIN STANDARD CURVE

Bradford assay using bovine serum albumin as the stock standard protein was adopted in determining the total protein content as described by Bradford (1976). 1 mg/ml of Bovine serum albumine, BSA was diluted to 20-100 µg and 5 ml of Bradford reagent was added to 0.1 ml of standard BSA. 0.9ml of Bradford reagent added to 0.1 ml of distilled water with the absent of BSA was used as blank. Reactions were monitored at 595 nm after 10 minutes of incubation at room temperature. A standard curve was plotted from the data gathered and this standard curve was used to determine the protein concentration in the plant extracts.

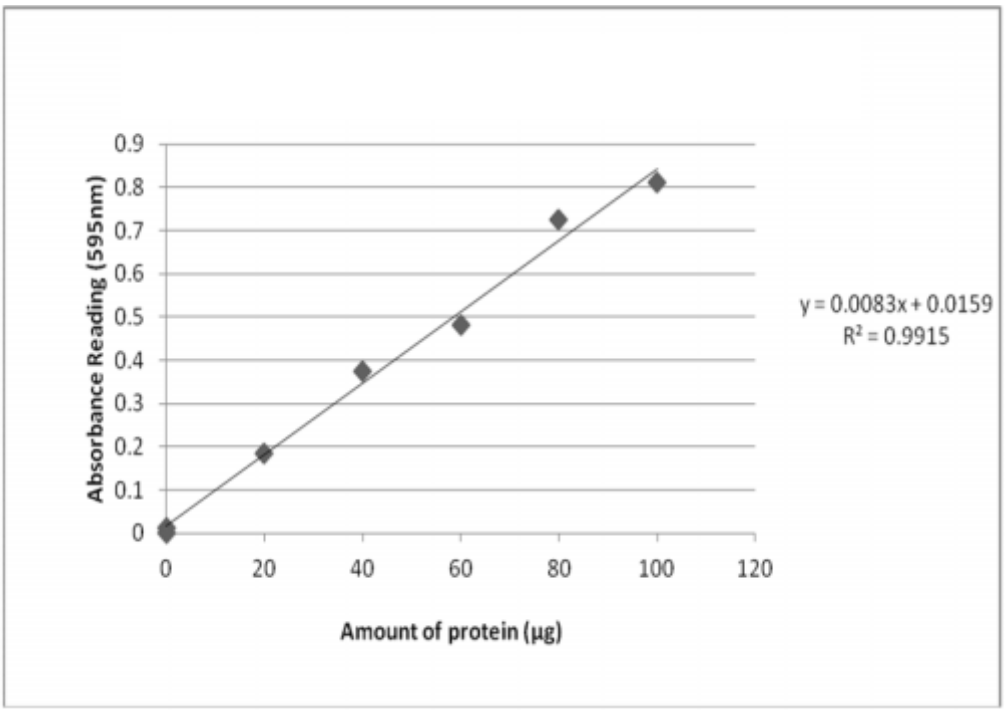

Figure 7: Bovine serum albumin standard curve (µg/ml)

APPENDIX 2

PREPARATION OF BRADFORD REAGENT (Bradford et al., 1976)

100mg Coomasive Brilliant Blue G-250 was dissolved in 50 ml 95% ethanol and further added with100ml 85% (w/v) phosphoric acid and finally diluted to 1 liter with distilled water. Complete dissolved mixture was obtained by overnight stirring using magnetic stirrer before and the reagents were filtered through Whatman no.1 paper just before use

APPENDIX 3

DETERMINATION OF PLANTS SAMPLE PROTEIN CONTENT

Table 5: Protein content in 20g of freeze dried ethanolic plants extract

Plants families	**Plants name**		**Protein Estimation (µg/ml)**
Leguminosae	*Erythrina fusca*	Leaves	0.25
		Flower	12.72
	Cassia floribunda	Leaves	5.52
		Fruits	12.77
	Delonex regia	Leaves	7.62
		Fruits	3.22
	Senna surattensis *	Leaves	34.46
	Acacia mangium	Leaves	21.94
	Caesalpinia pulcherrima	Leaves	7.41
	Clitoria ternatea	Flower + Fruits	5.18
	Cassia alata	Leaves	1.41
		Fruits	3.41
	Bauhinia blakeana	Leaves	6.44

		Fruits	0.24
	Andira inermis	Leaves	16.91
		Fruits	4.84
	Mimosa diplotricha *	Fruits	9.86
	Pterocarpus indica	Leaves	9.88
		Fruits	1.08
	Adenanthera pavomina	Leaves	30.90
	Acacia auriculiformis	Leaves	13.83
		Fruits	18.33
Rubiaceae	*Ixora finlaysoniana*	Leaves	1.03
	Mussaenda erytrophylla	Leaves	4.81
		Flower	11.31
	Uncaria spp	Leaves	6.01
		Flower	8.21
	Euclinia longiflora	Leaves	2.53
	Porterandia anisophylla	Leaves	1.06
	Morinda elliptica	Leaves	1.63
	Gardenia carinata	Leaves	0.63
	Mussaenda philippica ‘Queen Sirikit'	Leaves	2.39
	Mussaenda phillipica	Leaves	5.21
		Flower	0.25
	Morinda citrifolia	Fruits	4.42
Apocynaceae	*Allamanda oenotherafolia*	Flower	11.44
	Allamanda cathartica	Leaves	6.16
	Plumeria rubra cultivars	Leaves	18.28
		Flower	16.93

Euphorbiaceae	*Ricinus communis*	Leaves	4.76
		Fruits	19.63
	Jatropha gossypfolia	Leaves	18.30
		Fruits	2.52
	Macaranga tanarius	Flower	29.44

(*) indicates higher percentage of trypsin inhibitory activity and selected for further analysis in this study.

APPENDIX 4

PREPARATION OF TRYPSIN INHIBITORY ASSAY BUFFER

6.057g of Tris-Base was dissolved into 400 ml of ultrapure water to produce 50 mM Tris-HCl buffer. The buffer was then adjusted the pH to 8.0 using 1M HCl and later added with ultrapure water making the final volume to 1 Liter.

APPENDIX 5

PREPARATION OF HI-TRAP G-25 CHROMATOGRAPHY BUFFER

6.057g of Tris-Base was dissolved into 400 ml of ultrapure water to produce 25 mM Tris-HCl buffer. The buffer was then adjusted the pH to 8.0 using 1M HCl and later added with ultrapure water making the final volume to 2 Liter.

APPENDIX 6

HI-TRAP G-25 CHROMATOGRAPHY

700µl 25mM Tris–HCl buffer, pH 8.0 (refer Appendix 5) was used to resuspend 5mg of freeze dried crude samples. The mixture was then injected into AktaPrime Plus by

Amersham Bioscinece. Sephadex G-25 column equilibrated with 25mM Tris–HCl, pH 8.0. was used the the akta prime for the mixture dasaltation purpose. The mixture was desalted at the flow rate of 2.0 ml/minutes. Eluents for each peak were collected and subjected to freeze dried. The freeze dried extract were reconstituting with buffer or ultrapure water before used in SDS PAGE.

APPENDIX 7

REAGENTS FOR SDS-PAGE PREPARATION

1. 10% Sodium Dodecyl Sulphate

   Dissolving 10 g of SDS in 100 ml of ultrapure water.

2. 10 ml of aqueous solution stock

   was prepared by mixing 0.25ml of 2.5% SDS, 1 ml of 10% gylcerol and 40 mg/ml of phenol red were mixed until completely dissolved and topped up with ultrapure water to 10 ml.

3. 0.1M glycine-NaOH buffer pH 8.3

   7.507g of glycine was dissolved in 400ml of ultratpure water. The pH was adjusted to 8.3 using 1M NaOH prior the solution was topped up with ultratpure water to 1 liter.

4. SDS-PAGE running buffer

   900 ml of ultrapure water was added to 100 ml of BIORAD 10x Tris/Glycine/SDS buffer to make up total volume to 1 liter.

5. Coomassie brilliant blue staining

   100 g of ammonium sulphate was dissolved in 400 ml of ultrapure water in a 1L beaker. 1g of Coomassie brilliant blue G-250 was dissolved in 2ml of ultrapure water in another beaker. Then, 11.8 ml of phosphoric acid was added to the ammonium sulphate solution

prepared in the first before the Coomassie brilliant blue solution prepared from the second beaker was added into it gradually to make sure it is well mixed. Lastly, ultrapure water was used to top up the solution to 1 liter.

APPENDIX 8

BUFFER FOR TRICINE-SDS GEL PREPARATION

900ml of ultrapure water was added to 100ml of BIORAD 10x Tricine-SDS buffer to make up total volume to 1 liter.

APENDIX 9

STANDARD CURVE OF LOG MW AGAIINST RELATIVE MOBILITY DETERMINATION

The distance of each marker band from the gel and the distance traveled by the dye front from the well to its final location on the gel were measured in millimeter. The R_f value of the each marker band were calculated from the data gathered and a standard curve of Log MW against Relative mobility (Rf) scatter graph was plotted using the Microsoft Excel with R_f value as the x- axis and log molecular weight as the y-axis.

R_f value for each plants samples were calculated to determine the molecular weight of the samples by measuring the distance that traveled by the samples. Plants samples molecular weight were calculated from the equation obtained from the curve of Log MW against Relative mobility (Rf) scatter graph that was plotted before.

Table 6: The logarithm molecular weight and its corresponding relative mobility (Rf)

Relative mobility (Rf)	Log MW
0.597015	0.544
0.402985	1.3324
0.298507	1.4914
0.238806	1.5623
0.164179	1.7435
0.104478	1.8215

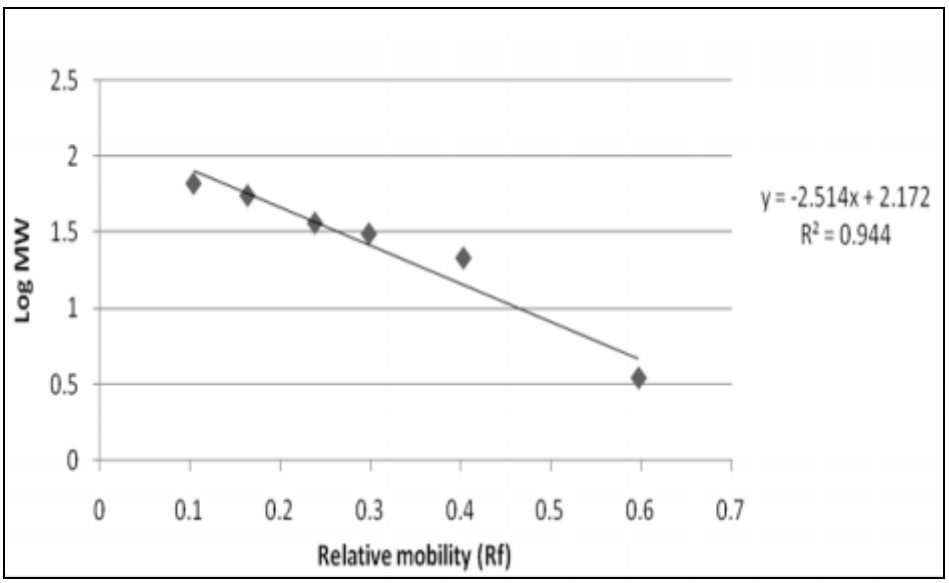

Figure 8: Standard curve of Log MW against Relative mobility (Rf)

Table 7: The molecular weight Senna *surattensis* leaves and *Mimosa diplotricha* flower determination

	Relative mobility (Rf)	**Log MW**	**MW (kDa)**
***Senna surattensis* leaves**	0.2888	1.446	27.93
***Mimosa diplotricha* fruits**	0.2813	1.465	29.17

APPENDIX 10:

TRYPSIN INHIBITORY ASSAY USING *CHRYSOMYA MEGACEPHALA* EXTRACTED PROTEASE

Table 8: Comparison of inhibitory activity percentage obtained between commercial trypsin and *Chrysomya megacephala* extracted protease.

Plant sample	**Commercial trypsin**	***Chrysomya megacephala* extacted protease**
***Senna surattensis* leaves**	83.52 %	61.74 %