DISCUSSION

Protease inhibitor are referring to a group of protein that regulate 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 defence mechanism and nowadays become great interest to many reseracher 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 this plant were analysed 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 reasearcher 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 percipitation 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 are 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. This method 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, its 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 inhibitory 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 was narrowed to choose the two most highest inhibition percentage toward trypsin. Since the most 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 this 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 protese inhibitor in both sample extracts selected.

The SDS-PAGE with 17% resolving gel cencentration 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 improvision 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 these protease inhibitor will stop the protein degradation by trypsin. The presence of these protease inhibitor were proven by

the presense of the band on both gel 1 and gel 2 with trypsin incubation and with out 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 sample 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 eluents 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 inhibitory activity toward untreated protease namely trypsin that was obtained from *Chrysomya megacephala.* These blowflies are also known as the oriental latrine fly belond to the Calliphoridae family. It is known to spread disease to human and also cause myiasis in human where it invade 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* extacted protease.

Eventhough 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 inhibitory 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 againts 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 bovine trypsin compared to *Senna surattensis* leaves.

The next test done on *Senna surattensis* leaves lyophilized extract was thermostability 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 which claimed to contain subtilisins which is 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 ranging 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).