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

Various 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 filatrate 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 disard 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 were left incubated at dark room at room tempearture 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 assays 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 were 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 againts 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 were 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 samples 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 observed 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 futher 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 were 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 thermostability. 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 were monitores 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.