3.0 Methodology

3.1 List of Equipments

1. Centrifuge machine, Eppendorf 5842R
2. Centrifuge machine, Hettich ZENTRIFUGE MICRO 20
3. Spectrophotometer, GENESYS 10UV
4. Microplate reader, ASYS UMV340
5. Freeze drier, Labconco FreeZone[6]
6. Balance, Mettler Toledo EL3002
7. Agarose gel set, Mini gel set, HU6
8. Illuminator, AlphaImage Mini
9. Incubator, RS-biotech GALAXY R+
10. Vertical laminar flow cabinet, ESCO Airstream
11. Autoclave machine, TOMY high-pressure steam sterilizer ES-315
12. Microscope, MOTIC AE31
13. Reswelling tray, GE Healthcare
14. Ettan IPGphor, Ammersham biosciences
15. Ettan DALTsix gel caster, GE Healthcare
16. Ettan DALTsix electrophoresis unit, GE Healthcare
17. Orbital shaker, PROTECH
18. ImageMaster 2D Platinum v7.0, GE Healthcare
19. ABI 4800 plus MALDI TOF/TOF Analyzer, Applied Biosystems
20. Speedvac machine, Thermo Scientific SAVANT DNA120 Speedvac Concentrator
21. Step One Plus Real-time PCR system, Applied Biosystem
22. Centrifuge machine, Hettich ZENTRIFUGE MICRO 20
23. pH meter, pH211 microprocessor
24. Water bath, Memmert

25. Grinder

26. Hot plate

27. Hemocytometer

28. Vortex

3.2 List of chemicals and materials

1. Plant samples were purchased from Delto Medicama Plantation (M) Sdn Bhd, Sabak Bernam, Selangor. Each of the plant samples has been identified by the Herbarium, ISB, UM with each sample assigned with its own serial number. Large type leaves (LL) and fruits (BF): KLU 046470; medium type leaves (ML): KLU 046469; small type leaves (SL) and fruits (SF): KLU 046467.
2. Ammonium sulfate, R&M
3. Snakeskin dialysis tube and BCA kit were purchased from Thermo Scientific.
4. All chemicals for antioxidant and anticancer activities were purchased from Sigma Aldrich expect DMSO which was from Merck.
5. All chemicals for proteomic work flow were purchased from Merck except TEMED and CHCA which were from Sigma.
6. Immobiline drystrips were purchased from GE Healthcare.
7. ZipTip was purchased from Millipore.
8. All RT-PCR kits were purchased from Applied Biosystems.
9. FBS, penicillin/streptomycin, Amphotericin B and accutase were purchased from PAA.
10. Carbon dioxide and liquid nitrogen were purchased from MOX.

3.3 Plant sample extraction

3.3.1 Crude sample extraction

Different types of *F. deltoidea's* dried plant samples were ground into powder with a grinder. A total of 100 g ground powder was boiled with distilled water in the ratio of 1:10 for two hours. The same amount of distilled water was subsequently added into the boiled samples and continuously boiled for another two hours. The boiled samples were then concentrated at 50˚C to approximately 60% volume on a hot plate. This was followed by centrifugation at 4500 × g, filtration (to remove sediment with filter paper). The resulting sample was then lyophilized with a freeze drier.

3.3.2 Semi-purification of samples

In the semi-purification or fractionation of samples, ammonium sulfate precipitation was performed. In this method, 1 g of freeze dried sample was dissolved in 100 ml of distilled water. Ammonium sulfate was then added into the solution in increments of 10% saturation for each fraction from 0% to 100% saturation based on table 3.1. For example, in 100 ml of sample solution, 5.6 g of ammonium sulfate was added to the solution to form a 10% saturated ammonium sulfate solution. After the salt was fully dissolved, the sample solution was left to stabilize for 10 minutes. The solution was then centrifuged at 13,000 × g for 10 minutes. The resulting pellet was re-dissolved in distilled water while the supernatant underwent another round of precipitation to reach 20% saturation. In order to reach 20% saturation, 5.7 g of ammonium sulfate was added into the solution. These steps were repeated until the solution reached 100% saturation. All the precipitated samples were then dialyzed at 4℃ for 48 hours (distilled water was changed every 12 hours with the volume of 2 L each time) to remove the salt in the solution. The dialysates were then freeze dried.

Table 3.1: Nomogram for ammonium sulfate saturation for 1 L at 25˚C, 4.1 M

Initial concentration / Percentage of saturation	Final concentration / Percentage of saturation ammonuim sulfate																			
	%	**10**	**15**	**20**	**25**	**30**	**33**	**35**	**40**	**45**	**50**	**55**	**60**	**65**	**70**	**75**	**80**	**85**	**90**	**95**	**100**
	0	56	84	114	144	176	196	209	243	277	313	351	390	430	472	516	561	610	662	713	767
	10		28	57	86	118	137	190	183	216	251	288	326	365	406	449	494	540	592	640	694
	15			28	57	88	107	120	153	185	220	256	294	333	373	415	459	506	556	605	657
	20				29	59	78	91	123	155	189	225	262	300	340	382	424	471	520	569	619
	25					30	49	61	93	125	158	193	230	267	307	348	390	436	485	533	583
	30						19	30	62	94	127	162	198	235	273	314	356	401	449	496	546
	33							12	43	74	107	142	177	214	252	292	333	378	426	472	522
	35								31	63	94	129	164	200	238	278	319	364	411	457	506
	40									31	63	97	132	168	205	245	285	328	375	420	469
	45										32	65	99	134	171	210	250	293	339	383	431
	50											33	66	101	137	176	214	256	302	345	392
	55												33	67	103	141	179	220	264	307	353
	60													34	69	105	143	183	227	269	314
	65														34	70	107	147	190	232	275
	70															35	72	110	153	194	237
	75																36	74	115	155	198
	80																	38	77	117	157
	85																		39	77	118
	90																			38	77
	95																				39

Source: Ammonium Sulfate Solution, 4.1 M, Sigma.

3.3.3 Protein estimation

Protein estimation was then performed according to the BCA kit protocol in order to determine the amount of protein present after different percentiles of ammonium sulfate precipitation. Based on the graph obtained, the samples were pooled into three fractions which were: 0-30%, 31-60% and 61-90% precipitation. From this point forward, these samples will be known as semi purified fraction 30, 60 and 90 respectively.

3.4 Preliminary antioxidative studies

Three assays were conducted in the preliminary studies of *F.deltoidea's* properties. The assays were: total phenolic content assay, DPPH assay and lipid peroxidation. These assays were performed on all crude extracts and semi purified fractions.

3.4.1 Total phenolic content assay

3.4.1.1 Preparation of reagents and gallic acid standard

a) 10% Folin-Ciocalteu reagent

   For 10% Folin-Ciocalteu reagent, 10 ml of Folin reagent was mixed with 90 ml of distilled water.

b) 10% sodium carbonate

   For 10% sodium carbonate solution, 10 g of sodium carbonate was dissolved in distilled water. The final volume of the solution was 100 ml.

c) Gallic acid standard

   Sample concentration ranged from 0-100 µg/ml was prepared by dissolving gallic acid in distilled water.

3.4.1.2 Assay protocol

In the determination of total phenolic content, the Folin-Ciocalteu method (Hinneburg *et al.*, 2006) was employed. Firstly, 250 µl of 1mg/ml sample was mixed with 250 µl of 10% Folin-Ciocalteu reagent and the mixture was incubated in the dark for three minutes. Then, 500 µl of 10% sodium carbonate was added into the mixture and further incubated for an hour. Finally, absorbance was read at 750 nm. The phenolic content will be quantified based on the gallic acid standard.

3.4.2 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

3.4.2.1 Preparation of reagents

a) The DPPH stock solution was prepared by dissolving 7.8 mg of DPPH in 20 ml of methanol. To obtain DPPH reagent, 3 ml of DPPH stock solution was mixed with 47 ml of methanol.

b) Ascorbic acid (positive control)

   Sample concentration ranged from 12.5-200 µg/ml was prepared by dissolving ascorbic acid in distilled water.

3.4.2.2 Assay protocol

DPPH radical scavenging activity was measured by the method of Cotelle *et al* (Cotelle *et al.*, 1996) with some modification. The control for this assay was the absorbance of DPPH reagent measured in the absence of sample. For sample absorbance, 100 µl of sample (sample concentration ranged from 0.1 mg/ml to 20 mg/ml) was mixed with 1.9 ml DPPH reagent and incubated for 30 minutes in the dark before the absorbance was taken. The control and sample absorbance was read at 515 nm. Ascorbic acid was selected as the positive control in DPPH assay. The inhibition activity was calculated by the equation:

$$Percentage\ of\ inhibition = \frac{\text{Absorbance(blank)} - \text{Absorbance(sample)}}{\text{Absorbance(blank)}} \times 100\%$$

The inhibition concentrations (IC_{50}) of the samples were obtained based on the estimation of plotted inhibition graph.

3.4.3 Lipid peroxidation

3.4.3.1 Preparation of reagents

a) 0.1 M phosphate buffer, pH 7.4

   For 0.1 M phosphate buffer, pH 7.4, 3.12 g of monosodium phosphate monohydrate and 20.75 g of disodium phosphate heptahydrate were dissolved in 800 ml of distilled water. The pH was then adjusted to pH 7.4 with 0.1 M of NaOH. Distilled water was added into the solution until the final volume reached 1 L.

b) Buffered egg yolk

   For buffered egg yolk, 0.5 g of egg yolk was homogenized in 20 ml of phosphate buffer.

c) 0.03% Ferrous sulfate

   For 0.03% ferrous sulfate, 30 mg of ferrous sulfate was dissolved in distilled water. The final volume of the solution was 100 ml.

d) 15% trichloroacetic acid (TCA)

   For 15% TCA, 15 g of TCA was dissolved in distilled water. The final volume of the solution was 100 ml.

e) 1% thiobarbituric acid (TBA)

   For 1% TBA, 1 g of TBA was dissolved in distilled water. The final volume of the solution was 100 ml.

f) Ascorbic acid (positive control)

   Sample concentration ranged from 0.1-10 mg/ml was prepared by dissolving ascorbic acid in distilled water.

3.4.3.2 Assay protocol

In lipid peroxidation assay, Daker *et al.* (Daker *et al.*, 2008) method was performed. In this assay, egg yolk was used as the source of polyunsaturated fatty acids. Firstly, 0.5 ml of buffered egg yolk was mixed with 50 µl of sample (sample concentration ranged from 2.5 mg/ml to 40 mg/ml) and 50 µl of 0.03% ferrous sulfate. The mixture was then incubated for one hour at 37℃. This was followed by the addition of 0.25 ml of 15% TCA and 0.5 ml of 1% TBA into the mixture which was then boiled for 10 minutes. The mixture was then cooled down to room temperature before it was centrifuged at 3000× g for 10 minutes. The supernatant was then collected and absorbance was measured at 532 nm. In lipid peroxidation assay, ascorbic acid was used as the positive control. The inhibition activity was calculated by the equation:

$$Percentage\ of\ inhibition = \frac{\text{Absorbance(blank)} - \text{Absorbance(sample)}}{\text{Absorbance(blank)}} \times 100\%$$

The inhibition concentrations (IC_{50}) of the samples were obtained based on the estimation of plotted inhibition graph.

3.5 Cell culture

All cells were cultured in an incubator at 37˚C, 5% carbon dioxide. CaSki cells were cultivated in RPMI-1640 medium while HepG2 and Chang Liver cells were grown in DMEM medium.

3.5.1 Preparation of media, buffer and sample solution

(a) Basic medium

In basic medium preparation, 10.4 g of medium powder, 2 g of sodium bicarbonate and 0.52 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were added into 1 L of autoclaved distilled water. After the solutes were fully dissolved, the solution was filtered with 0.20 µm cellulose acetate syringe filter and kept at 4˚C.

(b) 10% medium

For 10% medium, 90 ml of basic media was added with 10 ml of deactivated foetus bovine serum (FBS) and 1ml of 250 µg/ml Amphotericin B and 2ml of 100X penicillin/streptomycin. The medium was then filtered with 0.20 µm cellulose acetate syringe filter and kept at 4˚C.

(c) Phosphate buffer saline (PBS)

Phosphate buffer saline (PBS) was prepared by adding one tablet of PBS in 200 ml of autoclaved distilled water. PBS was then filtered with 0.20 µm cellulose acetate syringe filter and kept at 4˚C.

(d) Cryopreserving solution

Cryopreserving solution was prepared by mixing 9 ml of FBS and 1 ml of dimethylsulfoxide (DMSO). The mixture was then filtered with 0.20 µm cellulose acetate syringe filter.

(e) 0.4% trypan blue solution

To prepare 0.4% trypan blue, 0.4 g of trypan blue was dissolved 20 ml of distilled water. The volume was then brought up to total volume of 100 ml with distilled water.

(f) Preparation of sample solution

For MTT assay, all crude extracts and semi purified fractions were prepared by dissolving 0.5 mg of sample in 1 ml of 10% medium followed by five serial dilutions (1:2). In endogenous antioxidative enzymes assays, treatment was given at IC_{50} values for SF samples based on the result of MTT assay. In DNA fragmentation assay, the sample was dissolved in 10% medium with concentration ranged from 0.125 mg/ml to 1 mg/ml. For proteomics and RT-PCR, the SF semi purified fractions were prepared at the IC_{50} values based on MTT assay.

3.5.2 Cell culture procedure

(a) Cell revival procedure

A tube of cryopreserved cells was removed from liquid nitrogen and thawed at 37˚C. This was followed by the addition of 1ml 10% medium. The cells were then centrifuged at 180× g for 5 minutes. The pellet was then re-suspended in 20% medium and transferred into 25 cm^2 culture flasks.

(b) Trypsinization procedure

Medium was removed from the culture flask and the cells were washed twice with PBS. Cells were then trypsinized by the addition of 1 ml of accutase into PBS. Detached cells were collected by centrifugation at 180× g for five minutes. The pellet was then re-suspended in medium.

(c) Cryopreservation procedure

All cells were preserved in cryopreserving solution. The cells were transferred into a cryotube and kept at 4˚C for 30 minutes. Then, the tube was kept at -20˚C for 2 hours, -80˚C for at least 4 hours and then finally into liquid nitrogen for long term storage.

(d) Cell count

100 µl of re-suspended cells was added into 900 µl of 0.4% trypan blue solution. 20 µl of the mixture was then pipetted onto a hemocytometer. Viable cells were calculated and the total cell number in the solution was estimated.

3.6 MTT assay

3.6.1 Preparation of MTT solution

In order to prepare 5 mg/ml MTT solution, 5 mg of MTT was dissolved in 1 ml of phosphate buffer saline.

3.6.2 Assay protocol

A total of 5 x 10^3 cells (100 µl) were placed into 96-well plates and 80 µl of 10% medium was added into each well. The cells were left for 24 hours prior to being treated with 20 µl of different concentrations of samples and doxorubicin (positive control). After treatment, the cells were incubated for another 72 hours. The medium with samples were removed prior to addition of MTT to avoid contact. This was replaced with 180 µl of fresh 10% medium. Then, 20 µl of 5 mg/ml MTT solution was added and incubated for four hours. Finally, medium with MTT was removed and 150 µl of DMSO was added into each well and incubated for 10 minutes in order to dissolve the purple color formazan. The plate was then read at 570 nm using a microplate reader. Percentage of cells viability was calculated using the following equation while the IC_{50} values were estimated through the plotted graph:

$$Cell\ viability = \frac{\text{Absorbance(untreated)} - \text{Absorbance(sample)}}{\text{Absorbance(untreated)}} \times 100\%$$

3.7 Endogenous antioxidative enzymes

The action of antioxidative enzymes of the cell after being treated with small type fruit crude extract and its semi purified fractions were determine through catalase, glutathione peroxidase and superoxide dismutase assays. Half a millions of cells were plated to the 6 cm culture dish. Following 24 hours incubation, the cells were treated at IC_{50} values for doxorubicin (positive control) and samples and incubated for a further 72 hours. The cells were then lysed in 200 µl of 0.1% NP-40 by freeze-thawing. Supernatant was collected following centrifugation at 20,000 × g for 10 minutes at 4˚C.

3.7.1 Catalase

3.7.1.1 Preparation of reagents

All the reagents were prepared fresh prior to use.

a) 50 mM hydrogen peroxide

   For 50 mM hydrogen peroxide preparation, 85 µl of 30% hydrogen peroxide was added into distilled water to form a solution with a final volume of 15 ml.

b) 2 M sulfuric acid

   For 2 M of sulfuric acid preparation, 1 volume of 18 M sulfuric acid was added with 8 volumes of distilled water.

c) 20 mM potassium permanganate

   For 20 mM potassium permanganate preparation, 47.4 mg of potassium permanganate was dissolved in 15 ml of distilled water.

d) Catalase standard

   Sample concentration ranged from 0-0.35 µg/ml was prepared by dissolving catalase in 0.1% NP-40.

3.7.1.2 Assay protocol

10 µl of cell lysate was mixed with 50 µl of 50 mM hydrogen peroxide. The mixture was incubated for three minutes. Then, 50 µl of 2 M of sulfuric acid was added into the mixture followed by 50 µl of 20 mM potassium permanganate. The absorbance was read at 490 nm and catalase concentration was quantified based on catalase standard.

3.7.2 Glutathione peroxidase

3.7.2.1 Preparation of reagents

a) 3 mM NADPH

   For 3 mM NADPH preparation, 2.5 g of NADPH was dissolved in 1 L of distilled water.

b) 10 mM reduced glutathione

   For 10 mM reduced glutathione preparation, 3.07 g of reduced glutathione was dissolved in 1 L of distilled water.

c) 0.5 U glutathione reductase

   For 0.5 U glutathione reductase preparation, 1 µl of 500 U/ml glutathione reductase was added into each reaction.

d) 12 mM tert-butyl hydroperoxide

   For 12 mM tert-butyl hydroperoxide preparation, 1.15 µl of 30% hydrogen peroxide was added into distilled water to form a solution with a final volume of 1ml.

3.7.2.2 Assay protocol

Glutathione peroxidase assay was performed based on manufacturer's protocols with some modification. 10 µl of cell lysate was mixed with 120 µl of phosphate buffer and 50 µl of mixture which consist of 3 mM NADPH, 10 mM reduced glutathione and 0.5 U glutathione reductase. The mixture was equilibrated for five minutes. Then, 20 µl of 12 mM tert-butyl hydroperoxide was added and a kinetic study was conducted for 10 cycles of 45 seconds interval at 340 nm. The GPx activity was calculated based on the equation:

$$Activity\ (U/ml) = \frac{\frac{Initial\ absorbance - Final\ absorbance}{Time\ taken}}{extinction\ coefficient} \times 20$$

3.7.3 Superoxide dismutase

3.7.3.1 Preparation of reagents

a) 2 mM ethylenediaminetetraacetic acid (EDTA)

   For 2 mM EDTA preparation, 0.74 g of EDTA was dissolved in 1 L of distilled water.

b) 1 mM nitroblue tetrazolium (NBT)

   For 1 mM NBT preparation, 0.82 g of NBT was dissolved in 1 L of distilled water.

c) 1 mM xanthine

   For 1 mM xanthine preparation, 0.15 g of xanthine was dissolved in 1 L of 0.01 M sodium hydroxide solution. For 0.01 M sodium hydroxide solution, 40 mg of sodium hydroxide was dissolved in 1 L of distilled water.

d) 50 mU of xanthine oxidase

   For 50 mU of xanthine oxidase preparation, 1 µl of 50 U/ml glutathione reductase was added into each reaction.

3.7.3.2 Assay protocol

Superoxide dismutase assay was performed based on manufacturer's protocol with some modification. 20 µl of cell lysate was mixed with 160 µl of reaction mixture which consist of phosphate buffer, 2 mM EDTA, 1 mM NBT and 1 mM xanthine. The mixture was equilibrated for one minute. Then, 20 µl of 50 mU of xanthine oxidase was added into the mixture and read at 550 nm for 9 cycles at 90 seconds interval. The inhibition percentage of NBT was calculated based on the equation:

$$Percentage\ of\ inhibition = \frac{\text{Absorbance(blank)} - \text{Absorbance(sample)}}{\text{Absorbance(blank)}} \times 100\%$$

3.8 DNA fragmentation

1 x 10^6 cells were grown in 6 cm culture dish. Following 24 hours incubation, the cells were treated with different concentrations of doxorubicin (positive control) and samples. The medium was collected after 24 hours of treatment for DNA fragmentation analysis.

3.8.1 Preparation of reagents

a) TE/Triton buffer

   In order to prepare TE/Triton buffer, 2 ml of Triton X-100, 1.21 g of Tris base and 0.37 g of EDTA were dissolved in distilled water until the final volume reaches 1 L.

b) 10% SDS

   In order to prepare 10% SDS solution, 10 g of SDS was dissolved in distilled water until the final volume reaches 100 ml.

c) 5 M sodium chloride

   In order to prepare 5 M of sodium chloride solution, 292.5 g of sodium chloride was dissolved in distilled water until the final volume reaches 1 L.

d) TE buffer

   In order to prepare TE buffer, 1.21 g of Tris base and 0.37 g of EDTA were dissolved in distilled water until the final volume reaches 1 L.

e) 5X TBE buffer

   In order to prepare 5X TBE buffer, 53 g of Tris base, 27.5 g of boric acid and 3.72 g of EDTA were dissolved in distilled water until the final volume reaches 1 L.

f) 2% agarose gel

   In order to prepare 2% agarose gel, 2 g of agarose was dissolved in 100 ml of 1X TBE buffer and dissolved by heating in microwave.

3.8.2 Assay protocol

Collected medium was centrifuged at 2,000x g for 5 minutes. The resulting pellet was re-suspended and lysed in 250 µl of TE/Triton buffer on ice for 10 minutes. The lysed sample was then centrifuged at 13,000× g for 15 minutes at 4˚C. Supernatant (200 µl) was collected and 1.5 µl of 10 mg/ml RNase A added. The mixture was then incubated for one hour at 37˚C. This was followed by the addition of 12.5 µl 10% SDS and 8 µl of 5 mg/ml proteinase K. The mixture was then incubated for another hour at 50˚C. After that, 0.1 volume of 5 M sodium chloride and one volume of ice-cold isopropanol were added into the mixture. Following 10 minutes of incubation, the sample was centrifuged at 13,000× g at 4˚C for 15 minutes. The resulting pellet was dissolved in 10 µl of TE buffer and 5 µl of the sample was subjected to electrophoresis in 2% agarose gel at 60V. The gel was then stained in ethidium bromide and visualized under UV illuminator.

3.9 Proteomics

3.9.1 Sample extraction

3.9.1.1 Preparation of lysis buffer

For 25 ml of lysis buffer preparation, 0.25 g of SB3-10, 0.25 g CHAPS, 7.51 g urea and 3.81 g of thiourea were dissolved in distilled water until the final volume reaches 25 ml.

3.9.1.2 Extraction procedure

Cultures which were treated with semi-purified fractions and doxorubicin were extracted with the use of freeze-thaw method. Cells were collected into a 1.5 ml eppendorf tube. After washing once with PBS, 200 µl of lysis buffer was added to the sample. The sample was then vortexed on ice for 10 minutes followed by freezing on dry ice for five minutes. The procedure was repeated for another two times until all the cells were lysed. Bradford protein estimation was then conducted to estimate the protein content of the sample. The sample was then aliquot into 0.5 ml eppendorf tubes with a content of 110 µg per tube and stored in -80˚C.

3.9.2 Two-dimensional (2D) gel electrophoresis

3.9.2.1 IEF

3.9.2.1.1 Preparation of buffers

a) Urea rehydration stock solution

In order to prepare urea rehydration stock solution, 12 g of urea, 0.5 g of CHAPS, 125 µl of IPG buffer and 50 µl of 1% bromophenol blue solution were dissolved in distilled water until the final volume reaches 25 ml. The solution was stored in 1 ml aliquots at -20 ℃. Prior to use, 2.8 mg DTT was added into the 1 ml aliquots.

b) Sample buffer

In order to prepare sample buffer, 4.8 g of urea, 0.4 g of CHAPS, 48.44 mg of Tris base, 0.1 g of DTT and 10 µl of 1% bromophenol blue solution were dissolved in distilled water until the final volume reaches 10 ml. The solution was stored in 1 ml aliquots at -20 ℃.

3.9.2.1.2 IEF procedure

A 24 cm immobiline drystip was rehydrated with 450 µl urea rehydration stock solution in reswelling tray. The strip was covered with mineral oil to prevent dehydration of the strip. Following 18 hours rehydration, IEF was conducted using cup-loading method. The strip was placed into a strip holder and both ends of the strip were attached to electrodes. Loading cup was then placed at the positive electrode. Sample which consists of 110 µg protein per tube was mixed with sample buffer to make a 110 µl mixture. Sample equivalent to 100 µg protein (100 µl) was loaded into loading cup. IEF was performed using IPGphor for 75 kVh. The steps for IEF are shown in Table 3.2. After IEF, the strip can be either continued with second dimension electrophoresis or stored in -20 ℃ freezer.

Table 3.2: IEF steps

Step	Method	Voltage (V)	Time (hr)
1	Step and hold	100	1.5
2	Gradient	500	1
3	Gradient	1000	1
4	Gradient	8000	3
5	Step and hold	8000	Until total Vhr reached 75,000

3.9.2.2 2-D PAGE

3.9.2.2.1 Preparation of buffers

a) SDS equilibration buffer solution

In order to prepare SDS equilibration buffer solution, 72.10 g of urea, 10 ml of pH 8.8 Tris-HCl, 69 ml of 87% (w/w) glycerol, 4 g of SDS and 400 µl of 1% bromophenol blue solution were dissolved in distilled water until the final volume reaches 200 ml. The solution was stored at -20 ℃.

- Reducing buffer: 0.10 g of DTT was dissolved in 10 ml SDS equilibration buffer solution
- Alkylating buffer: 0.25 g of iodoacetamide was dissolved in 10 ml SDS equilibration buffer solution

b) 10X Laemmli SDS electrophoresis buffer

In order to prepare 10X Laemmli SDS electrophoresis buffer, 30.3 g of Tris base, 144.1 g of glycine and 10 g of SDS were dissolved in distilled water until the final volume reaches 1 L.

c) Monomer stock solution

In order to prepare 30% T, 2.6% C monomer stock solution, 300 g of acrylamide, and 8 g of N,N'-methylenebisacrylamide were dissolved in distilled water until the final volume reaches 1 L.

d) 4X resolving gel buffer solution, pH 8.8

4X resolving gel buffer solution was prepared by dissolving 181.7 g of Tris base in 800 ml of distilled water. The pH was then adjusted to pH 8.8 and distilled water was added until the final volume reaches 1 L.

e) 10% ammonium persulfate solution

10% ammonium persulfate solution was prepared prior to use. 0.1 g of ammonium persulfate was dissolved in 1 ml of distilled water.

f) Agarose sealing solution

0.5 g of agarose was heated and dissolved in 100 ml of 1X Laemmli SDS electrophoresis buffer. Later, 0.2 ml of 1% bromophenol blue was added into the solution.

g) Bromophenol blue stock solution

0.01 g of bromophenol blue and 6 mg of Tris base were dissolved in 1 ml of distilled water.

h) Saturated butanol

50 ml of butanol was mixed with 5 ml distilled water.

i) Gel storage buffer

250 ml of 4X resolving gel buffer, 10 ml of 10% SDS and 740 ml of distilled water were mixed together for the preparation of gel storage buffer.

3.9.2.2.2 Preparation of 11% electrophoresis gel

The gel caster was set up and 11% gel (Table 3.3) was poured into the gel caster. The mixture was overlaid with saturated butanol. Once the gel polymerized, it was removed from the gel caster and kept in gel storage buffer until used.

Table 3.3: 11% gel recipe

Chemicals	Volume (ml)	Volume (ml)	Volume (ml)
Acrylamide	22.2	66.6	133.2
4X resolving gel buffer	15	45	90
ddH_2O	21.9	65.7	131.4
10% APS	0.3	0.9	1.8
10% SDS	0.6	1.8	3.6
TEMED	0.02	0.06	0.12
Total volume	60	180	360

3.9.2.2.3 2-D PAGE procedure

Prior to gel electrophoresis, focused IEF strips were equilibrated in SDS equilibration buffer solutions. The first equilibration was performed for 10 minutes with the addition of DTT. This was followed by a second 10 minutes equilibration with the addition of iodoacetamide. The strip was then placed onto the 11% gel and sealed with agarose sealing solution. The second dimension was performed in 1X Laemmli SDS electrophoresis buffer using Ettan DALTsix electrophoresis unit. The run was stopped when the dye front reached 1 cm above the gel edge. The electrophoresis was divided into two phases as shown in Table 3.4.

Table 3.4: Gel electrophoresis steps

Phase	Voltage (V)	Current (mA / gel)	Watt (W / gel)	Time (hr)
1	80	10	1	1
2	500	40	13	5

3.9.2.3 Silver staining (Yan *et al.*, 2000)

3.9.2.3.1 Preparation of staining solution

a) Fixing solution

For 1 L fixing solution, 400 ml of ethanol and 100 ml of glacial acetic acid were added into 500 ml of distilled water.

b) Sensitizing solution

For sensitizing solution, 300 ml of ethanol, 5 ml of 25% glutaraldehyde, 2.0 g of sodium thiosulfate and 68 g of sodium acetate were dissolved in distilled water. The final volume of the solution was 1 L.

c) Silver solution

For silver solution, 2.5 g of silver nitrate and 0.4 ml of 37% formaldehyde were dissolved in distilled water. The final volume of the solution was 1 L.

d) Developing solution

For developing solution, 25 g of sodium carbonate, 0.4 ml of 37% formaldehyde, and 28 µl of 5% sodium thiosulfate were dissolved in distilled water. The final volume of the solution was 1 L.

e) Stopping solution

For stopping solution, 14.6 g of EDTA was dissolved in distilled water. The final volume of the solution was 1 L.

3.9.2.3.2 Silver staining procedure

The silver staining protocol used was compatible with mass spectrum anaylsis. The 2D gels were fixed in fixing solution for at least 30 minutes. Gels were then sensitized in sensitizing solution for 30 minutes. After that, the gels were washed thrice with distilled water for five minutes per wash. Then, the gels were placed in silver solution for 20 minutes. Before the gels were developed, it was washed twice with distilled water for one minute each wash. The gels were then placed in developing solution and allowed to develop until the spots were visualized. Finally, the reaction was stopped using stopping solution. For preparative gels, the method was modified whereby glutaraldehyde and formaldehyde was eliminated respectively from the sensitizing and silver solutions.

3.9.3 Image Master analysis

After obtaining at least seven replicates of reproducible gels, the gels were analyzed using ImageMaster 2D Platinum version 7.0 software. Spots which were up- or down regulated by at least two folds were considered as being differentially expressed. They were processed for protein identification by MALDI.

3.9.4 Matrix assisted laser desorption/ionization (MALDI)

3.9.4.1 Preparation of solutions

a) 100 mM ammonium bicarbonate

   To prepare 100 mM ammonium bicarbonate, 7.91 g of ammonium bicarbonate was dissolved in distilled water until the volume reaches 1 L.

b) Destaining solution

   For destaining solution preparation, 0.05 g of potassium ferricyanide and 0.12 g of sodium thiosulfate were dissolved in distilled water until the volume reaches 10 ml.

c) Reducing solution

For reducing solution preparation, 7.7 mg of DTT was dissolved in 5 ml of 100 mM ammonium bicarbonate.

d) Alkylating solution

For alkylating solution preparation, 50.86 mg of iodoacetamide was dissolved in 5 ml 100 mM ammonium bicarbonate.

e) Washing solution

For washing solution preparation, equal volumes of acetonitrile (ACN) and 100 mM ammonium bicarbonate were mixed.

f) Dehydrating solution

100% ACN was used as the dehydrating solution

g) 50% ACN

50% ACN was prepared by mixing equal volumes of 100% ACN and distilled water.

h) Equilibration/washing solution

The equilibration solution was 0.1% trifluoroacetic acid (TFA). For 0.1% TFA preparation, 0.1 ml of TFA (99%) was added into distilled water until the final volume reaches 100 ml.

i) 50% ACN in 0.1% TFA

1 µl of TFA (99%) was added in 1 ml of 50% ACN

j) 10 mg matrix solution

10 mg of α-cyano-4-hydroxy cinnamic acid (CHCA) was dissolved in 400 µl of buffer A and 600 µl of buffer B. The mixture was then centrifuged and the supernatant was used as the matrix solution.

- Buffer A: Mixture of 979 µl distilled water, 20 µl ACN and 1 µl of TFA (99%).
- Buffer B: Mixture of 19 µl distilled water, 980 µl ACN and 1 µl of TFA (99%).

3.9.4.2 Sample preparation

Gel plugs were destained in 100 µl of destaining solution for 30 minutes with constant mixing using vortex. The destaining solution was then replaced with 150 µl of reducing solution and incubated at 60 ℃ for 30 minutes. The gel plugs were then alkylated using 150 µl of alkylating solution for 20 minutes in the dark. This was followed by washing the gel plugs thrice with 500 µl of washing solution for 20 minutes each by vortex. Finally, the gel plugs were dehydrated in 50 µl of dehydrating solution with constant vortex for 15 minutes and dried using speedvac for 15 minutes. The gel plugs were then digested overnight with 25 µl of 7 ng/µl trypsin in 50 mM ammonium bicarbonate at 37 ℃. The digested peptides were extracted with 50 µl of 50% ACN followed by 50 µl of 100% ACN. Extracted peptides were pooled in a new tube. The pooled samples were dried using speedvac. Dried peptides were re-suspended in 10 µl of equilibration buffer.

3.9.4.3 Sample clean up procedure

Zip tip is an eppendorf tip with C-18 microcolumn used in order to purify and concentrate digested peptides. First, 10 µl of 100% ACN was drawn for three times to activate the column. The tip was then equilibrated five times with 10 µl of 0.1% TFA. Samples were bound to the column by aspirating the solution up and down for 10 times. The unbound components were removed by washing with 10 µl of 0.1% TFA for three times. Samples were eluted with 1.5 µl of 50% ACN in 0.1% TFA.

3.9.4.4 Sample spotting and mass spectrometry analysis

1.5 µl of matrix solution was added into the eluted sample. 0.7 µl of the mixture was then spotted onto a MALDI sample plate (384 Opti-TOF). Once dried, the plate was subjected to tandem mass spectrometry analysis for protein identification. Peptide mass spectra were obtained by MALDI-TOF/TOF mass spectrometer in the positive ion reflector mode. For precursor ion selection, all fractions were measured in single MS before MS/MS was performed. For MS/MS spectra, the peaks were calibrated by default. The 20 most abundant precursor ions *per* sample were selected for subsequent fragmentation by high-energy CID. The collision energy was set to 1 keV and air was used as the collision gas. The criterion for precursor selection was a minimum S/N of 5. The mass accuracy was within 50 ppm for the mass measurement and within 0.1 Da for CID experiments. The other parameters for searching were of trypsin, 1 missed cleavage, variable modification of carbamidomethyl and oxidation of methionine, peptide charge of 1+, and monoisotopic. For database searches, known contamination peaks such as keratin and autoproteolysis peaks for trypsin were removed before searching. Spectra were processed and analyzed by the Global Protein Server Explorer 3.6 software (Applied Biosystems). This uses an internal MASCOT (Matrix Science, UK) program for matching MS and MS/MS data against database information. The data obtained were screened against human database (August 2008) downloaded from the Swiss-Prot/TrEMBL homepage (http://www.expasy.ch/sprot).

3.10 Real-time quantitative reverse transcription-PCR (Real time RT-PCR)

3.10.1 RNA extraction (Appendix A)

The RNA were extracted according to the protocol given in RNAqueous-4PCR kit (Appendix A1). The quantity and purity of the RNA were determined prior to conversion to cDNA.

3.10.2 Reverse transcription of RNA to cDNA

1 µg of RNA were mixed with high capacity RNA-to-cDNA master mix. Transcription was performed according to the manufacturer's protocol (Appendix A2).

3.10.3 Gene expression analysis

The probe and primers designed by Applied Biosystem were coated on the 96-well plate. Prior to analysis, TaqMan gene expression master mix and cDNA were added into the wells. The gene expression assay was conducted according to the protocol (Appendix A3) using StepOnePlus PCR machine. The results were analyzed using StepOne software v2.1 (Applied biosystem). The fold changes or relative quantification (RQ) of a specific gene was calculated using the equation $RQ=2^{-\Delta\Delta Ct}$.

Ct= threshold cycle; ΔCt= Ct of specific gene - Ct of endogenous control; ΔΔCt=ΔCt of treated sample - ΔCt of untreated sample.