CHAPTER 3

MATERIALS AND METHODS

3.1 TONGKAT ALI SAMPLES

A total of 29 different *E. longifolia* (Tongkat Ali) capsule samples were evaluated in this study. They were obtained from various pharmacies and drug stores around peninsular Malaysia. Among the 29 samples 23 of them were approved products (MON-A01- MON-A23)with their company registration number stated on the products. And the other six were non-approved (MON-X01 - MON-X06) by MOH (also not registered with the Registrar Of Companies (ROC), Malaysia). Two standards (CFPR-JV1 and MON-JV1) containing only *E. longifolia* root extract were prepared just prior to the study, CFPR-JV1 was a standardized extract obtained from FRIM, while MON-JV1 was extracted from Tongkat Ali in our laboratory. A Tongkat Ali free aphrodisiac product (MON-NC01) served as a negative control. The name, brand and manufacturer of the capsules are not shown for reasons of anonymity of products.

For products received in the form of capsules, the shells were physically removed and the powdery content was stored in labeled 1.5 µl centrifuge tubes at -20 °C. One sample (MON-X06) was in the form of spherical capsules. The tablets were crushed using mortar and pestle until they became powdery, and the crushed product was stored in labeled 1.5 µl centrifuge tubes at -20 °C.

3.2 BRADFORD PROTEIN QUANTITATION ASSAY

Protein samples were weighed (1, 5 or 10 mg) and placed into 1.5 µl centrifuge tubes. One ml of ddH_2O was added to samples and the solutions were vortex gently to ensure that the protein powders dissolved thoroughly. A 100µl of sample solution was pipetted into each of three 1.5 µl centrifuge tubes.

A total of 50µl of BSA (Bovine Serum Albumin) (2mg/ml) was then mixed into 950µl of ddH_2O as 0.1mg/ml stock protein standard. From the stock standard solution, standards of different protein contents (0, 2, 4, 6, 8, 10 µg) were prepared in triplicate (Table 3.1).

One ml of Bradford reagent (100 mg, Coomassiebrilliant blue G-250; in 50 ml Ethanol 95%; 100 ml Phosphoric acid 85%; topped up to 1L withddH_2O) was added into all tubes containing samples and standards. The mixtures were vortexed and left to stand at 28 °C for 20 min.

The absorbance of the BSA protein standards and samples was recorded at 595 nm to measure the protein content in each sample solution. The protein content of the samples was determined by protein standard curve of the BSA protein standard (Fig 3.1) (Bradford, 1976).

Table 3.1: Compositionof BSA and water in the protein standards

Standard (µg)	BSA (µl)	ddH_2O (µl)
0	0	100
2	20	80
4	40	60
6	60	40
8	80	20
10	100	0

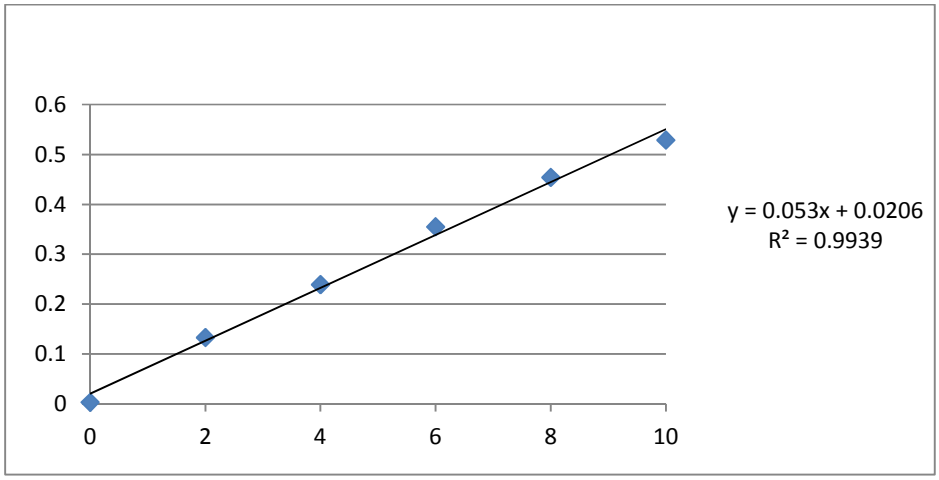

Figure 3.1: Protein standard curve of the BSA protein standard.

3.3 2D-ELECTROPHORESIS

3.3.1 Iso electric focusing (IEF)

Ten mg of DTT (GE healthcare, Sweden) and 2.5 µl of IPG buffer (GE healthcare, Sweden) was added to thawed rehydration buffer (12.0 g, Urea 8M; 0.5 g CHAPS 2%; trace amount, Bromophenol Blue; topped up to 25 ml, with ddH_2O;stored in 1 ml aliquots at -20 ^oC). Then, 125 µl of the mixed solution was spread evenly along a lane in the Immobiline™ Rehydration Tray using a micropipette. Immobiline™ Drystrip was taken out by holding its anodic end bare of any gel using a forcep. After removing the cover foil overlaying the gel surface, the drystrip was submerged into the rehydration buffer. Care was taken to remove any bubbles beneath the strip and the solution was distributed evenly along the strip by gentle lifting and lowering from one end as well as sliding it back and forth. Immobiline™ Drystrip Cover Fluid was used to overlay the strip and solution so that it will not dehydrate. The strip was left to rehydrate in the dark for 10-20 hours.

3.3.2 Cup loading of samples

To 50 mg samples of DTT and 2.5 µl of IPG buffer was added 1 ml of rehydration buffer. The solution was gently vortexed and left to incubate at room temperature for 30 min. The strip holder was carefully placed onto the bed of IPGphor 3 (GE Healthcare, Sweden). IPG strip (GE Healthcare PH 3-10, 7 cm, Sweden) was taken out from the rehydration tray and excessive cover fluid was blotted off with a piece of filter paper. The strip was gently placed with the gel side facing upwards onto the strip holder with its (+) end touching the anode side and its other end (-) touching

the cathode area. Paper wicks dampened with ddH_2O were placed at both ends of the strip with a portion of it covering the gel terminals. The electrodes are then secured with the conductor touching the paper wicks. The strip holder was filled with cover fluid. Loading cup was later placed near the anodic end. The cup was filled with cover fluid and adjusted for any leakage. Sample solution mixtures were centrifuged (Eppendrof, Germany) at 10,000 rpm for 3 min. Samples of 125 µl of supernatant was extracted and loaded into the cup before initiating the run. The duration of IEF runs ranged from 1 to 4 hr(Table 3.2). Paper wicks were change occasionally as they become saturated.

Table 3.2: Parameters used for IEF runs

Voltage mode	Voltage (V)	Duration (hr)
Step and Hold	200	1
Gradient	1,000	1
Gradient	5,000	4
Step and Hold	5,000	1

3.3.3 Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The gel caster was set up. Homogenous gel (31.7 ml, ddH_2O; 25.0 ml, 1.5M Tris-HCl, pH 8.8; 41.7 ml, Acrylamide Stock Solution (30%); 500µl, 10% APS (Ammonium Persulphate); 50µl, TEMED; 10% APS & TEMED added lastly) solution was prepared in a disposable bottle and later pipetted into the gel caster until it was almost full. Then, 0.1% SDS (1 ml,10% SDS Solution; topped up to 100 ml, with ddH_2O) overlay solution was sprayed onto the gel until the caster was full. The gel was left to harden for about 1-2 hours. Once the gel hardens, the setup was transferred into the electrophoresis instrument.

A 50 mg sample of DTT and 125 mg of Iodoacetamide (GE Healthcare, UK) were added separately into two 5 ml equilibration buffer (3.35 ml, Tris-HCl (1.5M, pH 8.8); 36.04, Urea 6M; 34.5 ml, 85% Glycerol; 2.0g SDS 2%; trace amount, Bromophenol Blue; topped up to 100 ml with ddH_2O, in 5 ml aliquots stored at -20 ^{o}C). Then, 2.5 ml of the equilibration buffer with added DTT was pipetted into an equilibration tray. The isoelectrically focused strip was then submerged into the buffer. They were left on a shaker for 10 min. Then, 2.5 ml of equilibration buffer with added IAA was pipetted into another lane of the equilibration tray. The strip was transferred into it and they were left on a shaker for another 10 min. The strip was rinsed with SDS electrophoresis buffer thereafter before placing it sideways onto the polyacrylamide gel. A small filter paper was soaked with 2.5 µl ladder (GE Healthcare, UK; Fig 3.2) solution before placing it on one side of the strip. Sealing solution (25 ml, SDS Electrophoresis Buffer; 125 mg, Agarose M; trace,

Bromophenol Blue; heat until agarose melted and stored at 4^{o}C) was heated in a microwave until it melts. Then, it was left to cool slightly before pipetting it to fill the void occupied by the strip. The top and bottom chambers of the electrophoresis instrument were then filled with SDS electrophoresis buffer (9.0 g, Tris 3 g/L; 43.2 g, Glycine 14.4 g/L; 3.0 g, SDS 1 g/L; topped up to 3L with ddH_2O; and stored at 4 ^{o}C). The instrument was connected to a power supply and electrophoresis was performed at 250V. The run was terminated when the bromophenol blue front nearly reaches the bottom edge of the gel. The gel was preceded to silver staining procedure (Table 3.3), andscanned using a computer scanner.

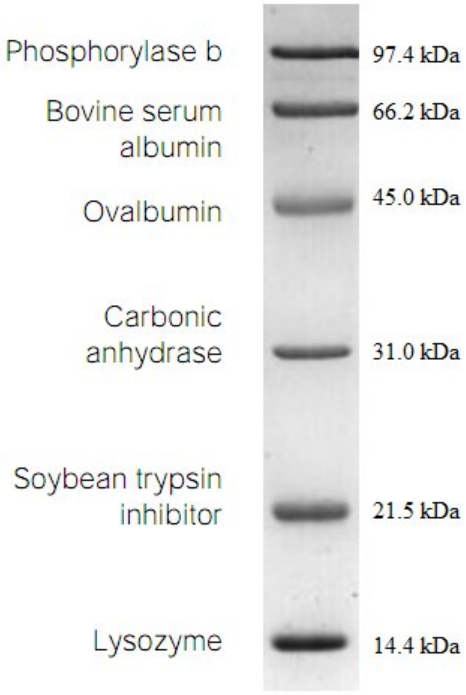

Figure 3.2: Constituents of the protein markers and their respective molecular weights

Table 3.3: Silver staining procedure (modified from Gromova and Celis, 2006)

Steps	Solutions	Duration of Shaking
Fixing	Fixing solution: 120ml glacial acetic acid; 500ml 96% ethanol; 500µl 35% formaldehyde; topped up to 1L with deionised water.	2hours or overnight
Washing	Washing solution 20% ethanol: 200ml 96% ethanol; topped up to 1L with dionised water.	3 x 20 minutes
Sensitizing	Sensitizing solution 0.02% (w/v): 200mg sodium thiosulphate anhydrate; topped up to 1L with deionised water.	2 minutes
Washing	Deionized water	2 x 1 minute
Silver staining	Staining solution 0.2% silver nitrate (w/v), 0.076% formalin: 2g silver nitrate, 760µl 35% formaldehyde; topped up to 1L with deionized water. Pre-cool solution in 4°C before use.	20 minutes
Washing	Deionized water	2 x 30 seconds
Developing	Developing solution: 60g sodium carbonate; 500µl formaldehyde; 4mg sodium thiosulphate; topped up to 1L with deionized water.	3-5 minutes

Table 3.3: Silver staining procedure (continued)

Stopping	Stopping solution, 12% (v/v) glacial acetic acid: 120 ml glacial acetic acid; topped up to 1L with deionized water.	10 minutes
Storing	1% (v/v) acetic acid; 10 ml acetic acid; topped up to 1L with deionized water	-

3.4 DATA ANALYSIS

Scanned gel pictures were saved in TIFF format and later analyzed using Bio Rad'sPDQuest 2D Analysis Software. The image was automatically rendered into grayscales by the software in order for it to perform further analysis on the picture. The background noise was filtered out using Gaussian filtering method. Once filtered, the software's 3D viewer was then used to analyze the area of interest within the gel picture.

3.5MASS SPECTROMETRY

The biomarker A of sample MON-A13 was identified on the 2DE profile of Tongkat Ali capsules and the gel plug of the spot was taken out, sent for nanoLC ESI MS/MS analysis by Australian Proteome Analysis Facility (APAF). Sample preparation consisted of tryptic digest done by APAF. Data from peptide mass fingerprinting combined with tandem mass spectrometry (PMF + MS/MS) was searched using mascot (Matrix Science London, UK) to identify the proteins. High scores in the database searches indicated a likely match. Significant sequences that were generated from LC-MS/MS data underwent de novo sequencing.

3.6 DATABASE PROCESSING:

Proteins were identified using the mono isotopic masses with the internet search program MASCOT(http://www.matrixscience.com).For MASCOT search, the search parameters were: MS/MS Ion Search, Enzyme: Trypsin, variable modification: oxidation (M) and propionamide (C), mass values: Monoisotopic, protein mass: unrestricted, peptide mass tolerance: ± 300 ppm, fragment mass tolerance: ±0.06 Da, max missed cleavage: 1, instrument type: ESI-QUAD-TOF, number of queries: 375.