# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1 TOTAL PROTEIN DETERMINED USING BRADFORD ANALYSIS

The data on concentrations of all the 29 samples (capsules) (Table 4.1 and 4.2) obtained from the spectrophotometer outputwere transferred using the standard formula to determine the final required amount of the capsules for 2DE protocol.

**Table 4.1:** Protein content in MOH approved samples.

| Code (non-MOH approved) | Protein content (µg/mg sample) ± SD | Amount of sample needed for 2D-Electrophoresis (25 mg) |
|---|---|---|
| CFPR-JV1 | 14.30 ± 0.09 | 3.50 |
| MON.JV1 | 16.70 ± 0.66 | 2.30 |
| MON-NC1 | 6.15 ± 0.09 | 8,12 |
| MON.A01 | 0.143 ± 0.02 | 32.98 |
| MON.A02 | 1.43 ± 0.02 | 18.22 |
| MON.A03 | 0.09 ± 0.008 | 15.64 |
| MON.A04 | 0.93 ± 0.87 | 6.16 |
| MON.A05 | 0.50 ± 0.25 | 23.34 |
| MON.A06 | 0.21 ± 0.04 | 8.86 |
| MON.A07 | 0.16 ± 0.02 | 9.46 |

**Table 4.1:** Protein content in MOH approved samples (continued)

| Code (non-MOH approved) | Protein content (µg/mg sample) ± SD | Amount of sample needed for 2D-Electrophoresis (25 mg) |
|---|---|---|
| MON.A08 | 11.28 ±0.20 | 4.43 |
| MON.A09 | 4.43 ±0.28 | 11.20 |
| MON.A10 | 4.38 ±0.22 | 11.40 |
| MON.A11 | 6.90 ±0.81 | 7.24 |
| MON.A12 | 7.02 ± 0.31 | 7.11 |
| MON.A13 | 2.27 ± 0.30 | 22.0 |
| MON.A14 | 5.05 ± 0.30 | 9.88 |
| MON.A15 | 1.55 ± 0.21 | 32.2 |
| MON.A16 | 2.47 ± 0.24 | 20.26 |
| MON.A17 | 8.11 ± 0.25 | 6.16 |
| MON.A18 | 6.24 ± 0.47 | 8.00 |
| MON.A19 | 4.50 ± 0.38 | 11.1 |
| MON.A20 | 7.18 ± 0.85 | 6.95 |
| MON.A21 | 6.89 ± 0.09 | 7.25 |
| MON.22 | 5.56 ± 0.14 | 8.98 |
| MON.A23 | 3.65 ± 0.14 | 13.67 |

**Table 4.2:** Protein content in non-MOH approved samples

| **Code (non-MOH approved)** | **Protein content (µg/mg sample) ± SD** | **Amount of sample needed for 2D-Electrophoresis (25 mg)** |
|---|---|---|
| MON.X01 | 2.59 ± 0.31 | 19.31 |
| MON.X02 | 2.73 ± 0.21 | 18.34 |
| MON.X03 | 2.12 ± 0.18 | 23.57 |
| MON.X04 | 0.37 ± 0.15 | 133.9 |
| MON.X05 | 0.80 ± 0.11 | 61.97 |
| MON.X06 | 2.55 ± 0.09 | 19.56 |

n=3

SD: Standard Deviation

Among all samples tested, the highest protein contentwas observed in MON-A08, while the lowest was in MON-X04. In samples with high to moderate protein content in the capsules, the needed amount for 2D-Electrophoresis was easily achieved. On the other hand, samples with low protein content required higher amounts of samples for 2DE and with it increasing the chances of possible contaminants.

## 4.2 VALIDATION OF BIOMARKERS

In this protocolwe were looking for 2 biomarkers (A and B) at 14.4 kDa molecular weights on polyacrylamide gel. What we could observe in CFPR-JV1 which were used as a positive control and provided from FRIM (a standardized Tongkat Ali extract), clear and reproducible spots of biomarker A and B (Fig 4.1). The second positive control was MON-JV1 which was extracted in our laboratory and it as well showed both biomarkers clearly to be present (Fig 4.2).While MON-NC was the negative control used and accordingly it did not display any spots for biomarkers A and B (Fig 4.3).

Clear and reproducible spots of Tongkat Ali biomarkers are presented in Figure 3.6. These spots came from a product with only Tongkat Ali (MON-A15) (not mixed with other herbs). Whereas,clear, A and B spots and many other spots were observed from another Tongkat Ali product stated to be mixed with other herbs (MON-A08) (Figure 4.5). Figure 3.8 showed product MON-A09 having no spots of interest to represent as an example of product having no Tongkat Ali active ingredient.

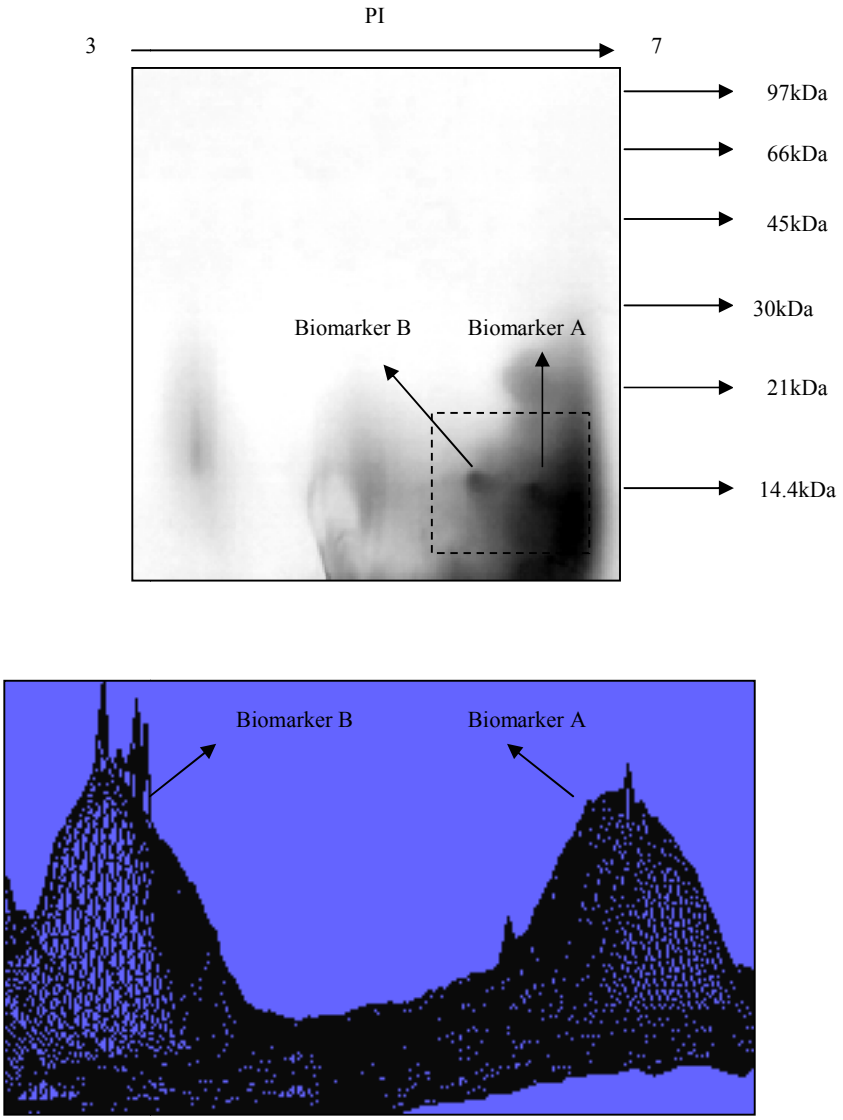


**Fig 4.1**: 2DE on CFPR-JV1 with boxed area showing biomarkers A and B. (Insert below showing 3D view of both biomarkers analyzed with Bio Rad'sPDQuest).

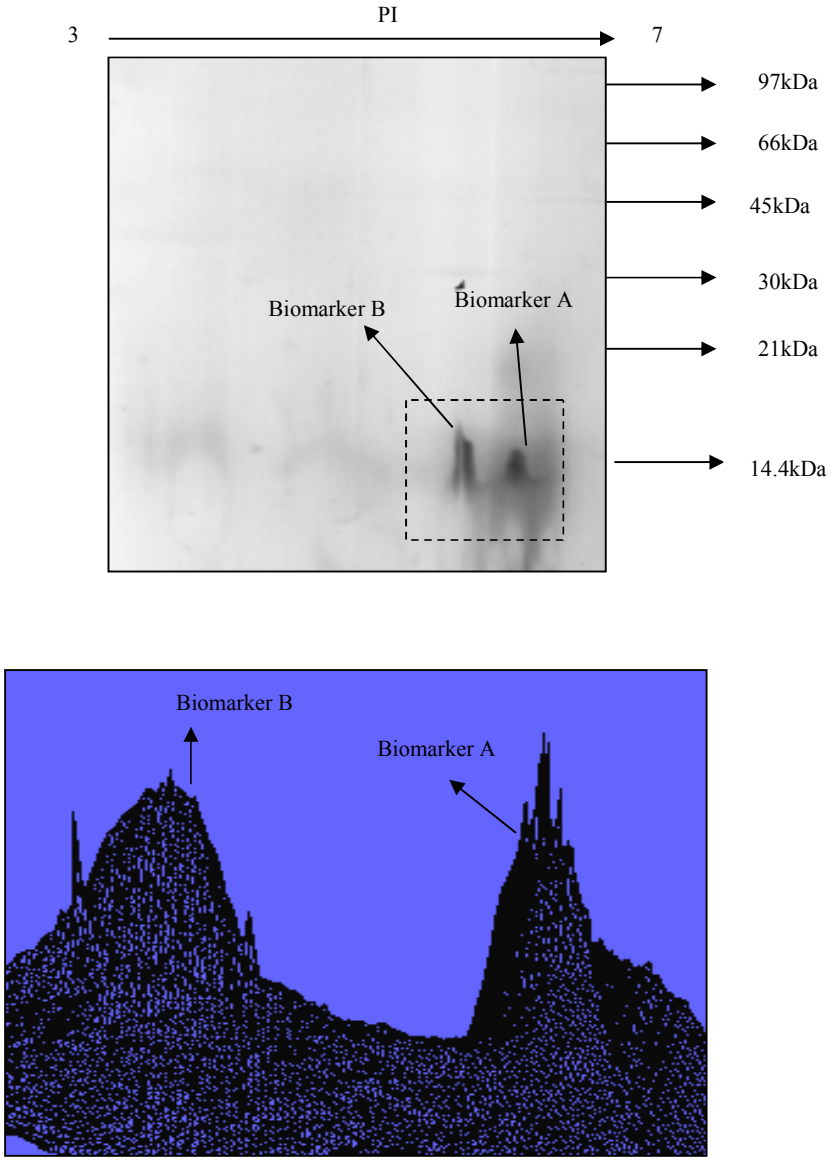


**Fig 4.2**: 2DE on MON-JV1 with boxed area showing biomarkers A and B. (Insert below showing 3D view of both biomarkers analyzed with Bio Rad'sPDQuest).

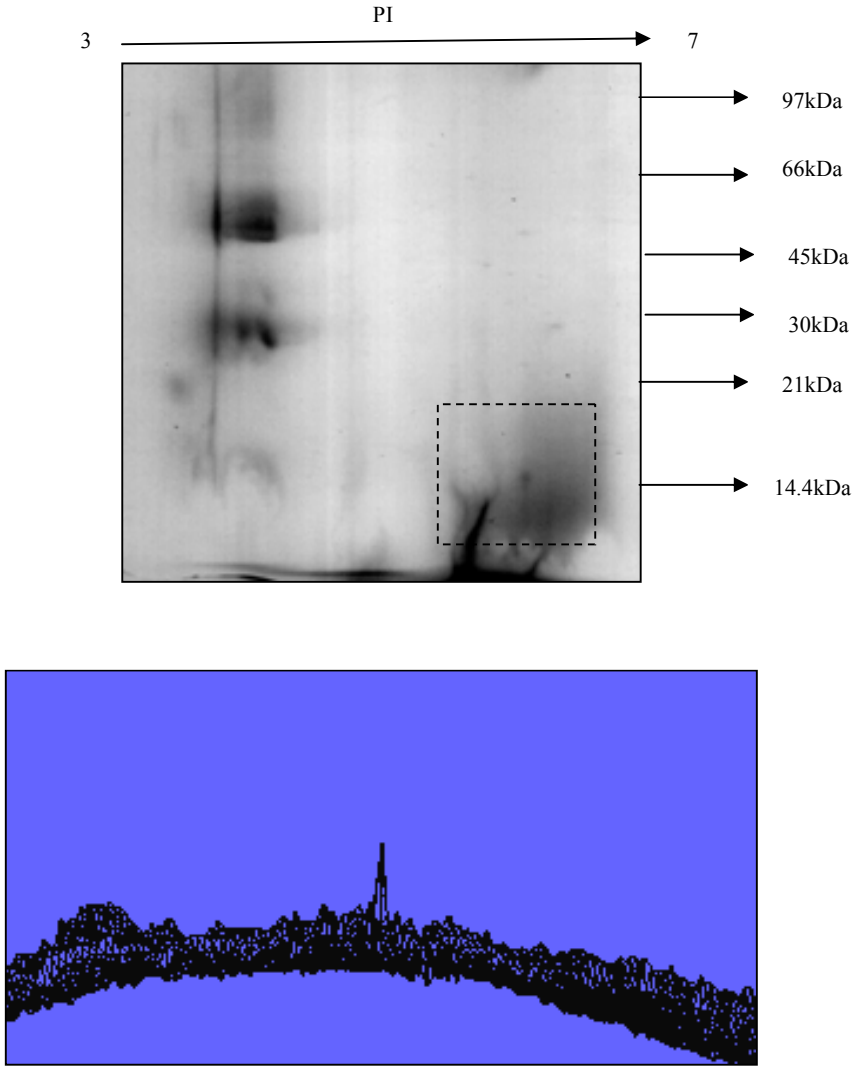


**Fig 4.3**: 2DE on MON-NC with boxed area showing where the biomarkers A and B should be. (Insert below showing 3D view with Bio Rad'sPDQuest).

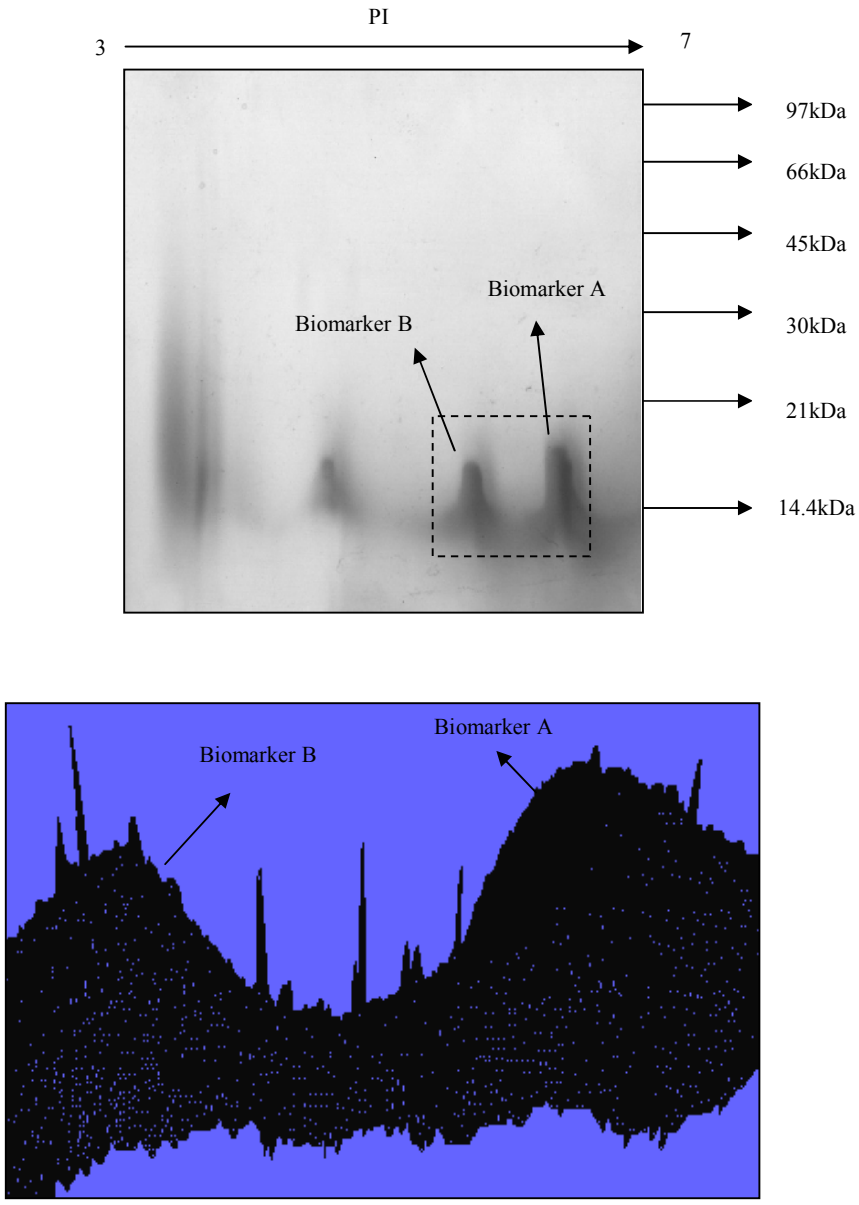


**Fig 4.4**: 2DE on MON-A15 with boxed area showing biomarkers A and B. (Insert below showing 3D view of both biomarkers analyzed with Bio Rad'sPDQuest).

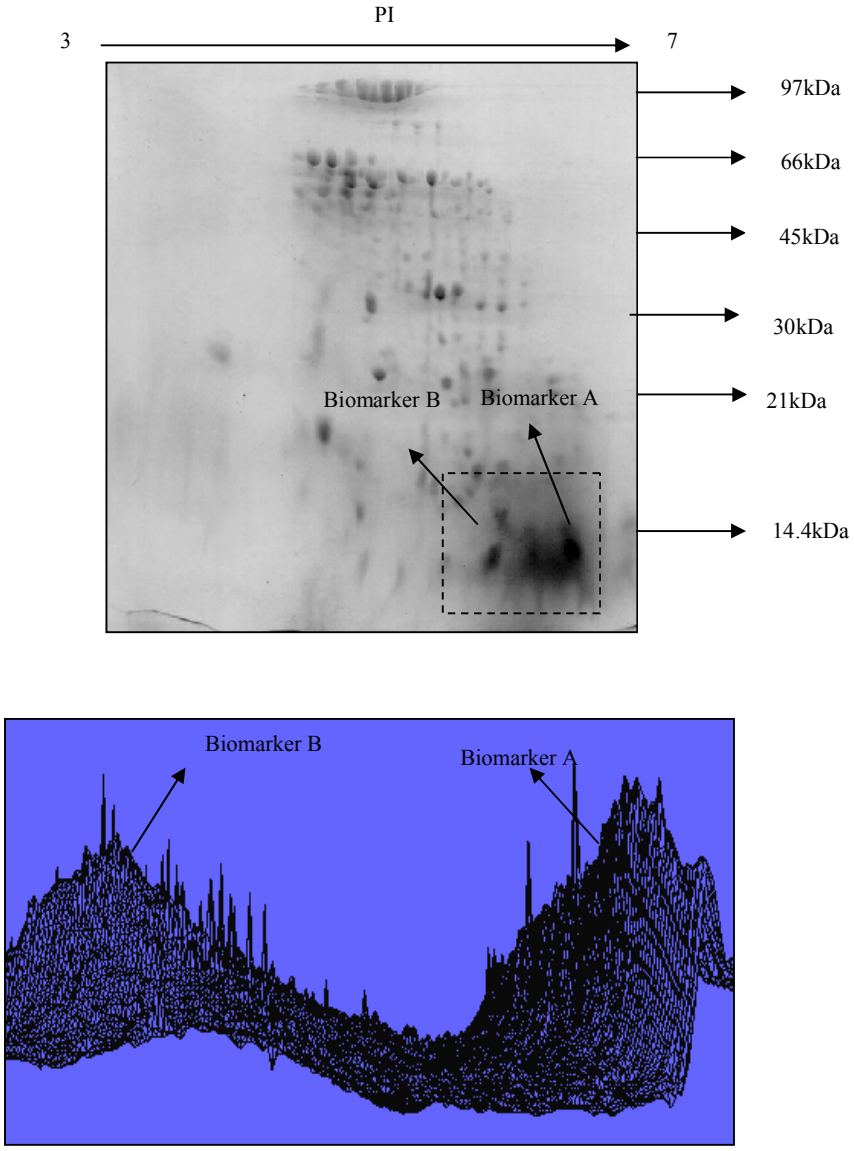


**Fig 4.5**: 2DE on MON-A08 with boxed area showing biomarkers A and B. (Insert below showing 3D view of both biomarkers analyzed with Bio Rad'sPDQuest).

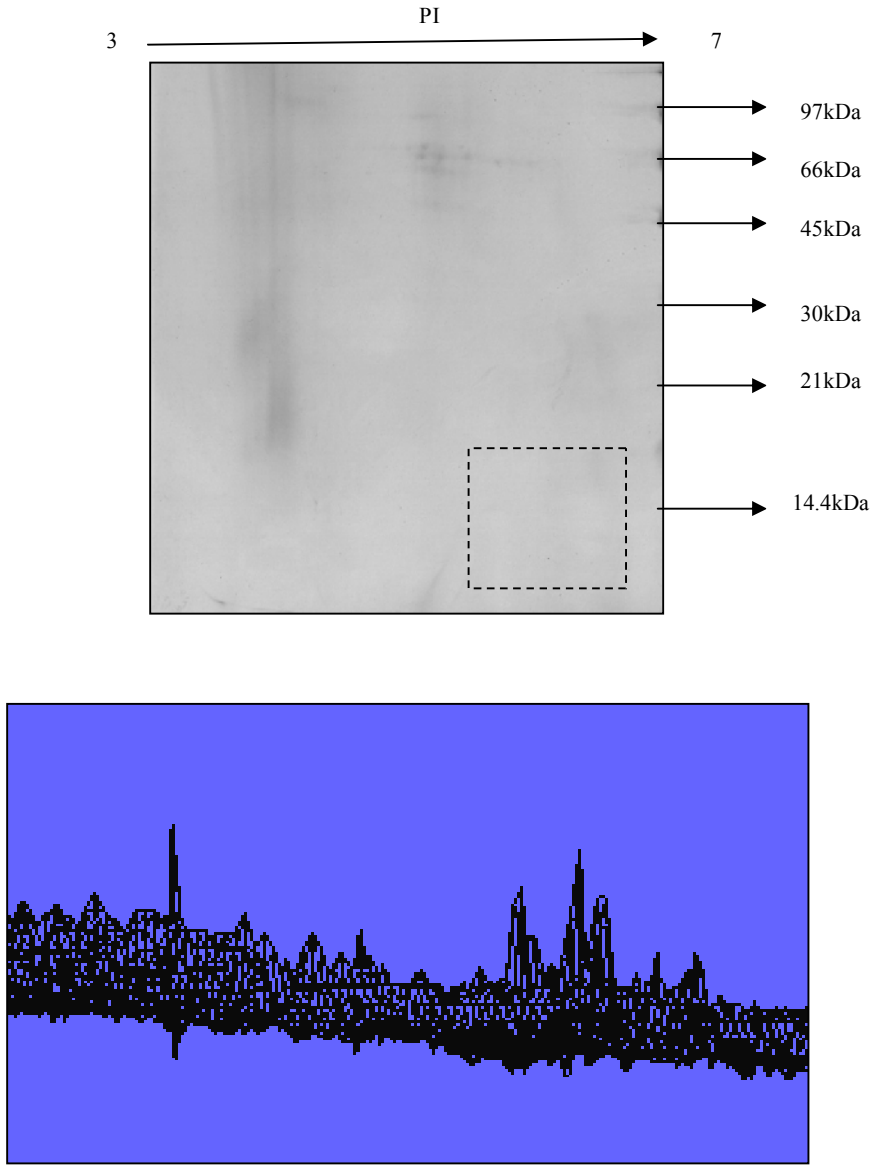


**Fig 4.6**: 2DE on MON-A09 with boxed area showing where the biomarkers A and B should be. (Insert below showing 3D view with Bio Rad'sPDQuest).

## 4.3 SUMMARY ON THE SCREENING FOR MARKERS

The investigation was focused on using proteomic for detecting the biomarkers which caused aphrodisiac activity in Tongkat Ali extracts. As noted previously, 29 products were evaluated in this study (Table 4.3). The 29 products, comprised of two groups, where 23 products were MOH approved (Table 4.1), while the other 6 products non-MOH approved (Table 4.2). Out of the 29 tested, eighteen productsshowed clear presence of Tongkat Ali biomarkers A and B (MON-A02, MON-A04, MON-A05,MON-A06,MON-A07, MON-A08, MON-A11.MON-A13,MON-A15, MON-A16, MON-A18, MON-A20, MON-A22,MON-A23, MON-X01, MON-X02,MON-X03, MON-X05) (group A) (Appendix B). While eleven products (MON-A01, MON-A03, MON-A09, MON-A10, MON-A12, MON-A14, MON-A17, MON-A19,MON-A21, MON-X04, MON-X06) (group B) (Appendix C) did not show any biomarker spots.

The group A products verified the usage of proteomics by detecting the protein biomarkers within them. In the case of group B, despite trials with various treatments, did not show any positive results. Samples MON-A10, MON-A12 and MON-A17 showed no positive result for the biomarkers, although they were repeated 3 times. In the last one the samples were left over night in IEF with 50V voltage then followed by the usual protocol (Table 3.2). This could be due to the fact all these three samples were a form of Maajun (a dark colored intoxicating confection, commonly sold in bazaars in India and Indonesia) (Fig 4.7)which could have been mixed with other plants. A mixture of plant extracts could interfere with the focusing in IEF to reachthe desired voltage (5000 V). It is important to reach

high voltage in IEF as it is relevant to move the protein to its respective pI. Salt contamination (ionic constituents) could be one of the reasons for this problem. The presence of salts could be due to contaminants from other plants. It was known in 2DE, DNA or certain macromolecules such as phenolic components could aggregate proteins thus disturbing the IEF. It was noticed however, that several of these products (MON-A03, MON-A09, MON-A14, MON-A19, MON-A21) had no problems in running 2DE (able to reach high voltage in the last phase) and they were mixed with other plants.

MON-A01 manufactured in Malaysia which did not show existence of the markers A and B, is the most famous product from Malaysia and Indonesia, and it was the only product with claims of US and EURO patent numbers. This product was pure (Tongkat Ali) and it was repeated 3 times with the same negative results In the third run the protocol was changed and the sample was left over night in IEF with low voltage (50V) for 10 hours followed by the usual protocol (Table 3.2). Nevertheless, neither of the treatments resulted in any biomarker spots. It is likely that the special extraction technique claimed to be used for this product may have an effect.

MON-X04 and MON-X05 contained very low protein content based on Bradford assay. In order to accommodate more than 25 mg load, the dry weight content of the capsule needed to be increased. As such this was an inevitable limitation that did not allow running of 2DE with higher protein load (eg 50 mg). The load of 25 mg gave negative results for MON-X04 while for MON-X05 faint spots for both biomarkers were observed.

The last sample MON-X06 did not show any spots. This product from Indonesia was like a small tough ball which needed to be crashed using pestle and mortar. It was not possible to run using 50 mg protein samples as the powder became semi solid upon adding the rehydration buffer, making it very difficult to transfer with a pipette. This is maybe attributed to the method of preparation of the product.

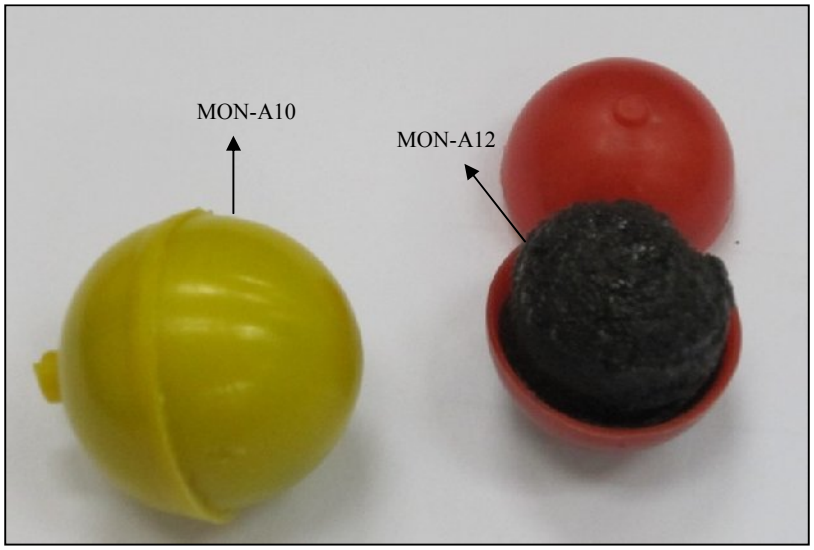


**Figure 4.7**: Maajun product (a dark colored intoxicating confection, commonly sold in bazaars in India and Indonesia).

**Table 4.3:** Tabulated results showing the presence or absence of marker A and B in each sample.

| Products Code | Marker A | Marker B | Preparation* | Content* |
|---|---|---|---|---|
| CFPR-JV1 | ✓ | ✓ | Standardized extract from FRIM | 100% radix EL (only Tongkat Ali) |
| MON-JV1 | ✓ | ✓ | Extracted of Tongkat Ali in laboratory (using hot distillation) | 100% radix EL (only Tongkat Ali) |
| MON-NC1 | ✗ | ✗ | Not stated | 100% "*UbiJaga"* (Smilax Myosotiflora) |
| MON-A01 | ✗ | ✗ | Advance extraction technique(freeze-dried water extraction) | 75 mg radix EL (only Tongkat Ali) |
| MON-A02 | ✓ | ✓ | Not stated | *Eurycoma longifolia* root extract: 160 mg,*Tinospora cordifolia*stem extract: 80 mg,*Glycyrrhizaglabra* root extract:80, *Centellaasiatica* herb extract:80. |
| MON-A03 | ✗ | ✗ | Modern technology in extraction | Radix *Eurycoma longifolia*:50 mg, Herba *Epimedium Brevicornum*:100 mg, *Actinolitum*:100mg, Herba*CuscutaChinensis*:100 mg, *Actinolitum,Herba Cynomorium Songaricum*:100 mg. |

**Table 4.3:** Tabulatedresults showing the presence or absence of marker A and B in each sample.(continued)

| Products Code | Marker A | Marker B | Preparation* | Content* |
|---|---|---|---|---|
| MON-A04 | ✓ | ✓ | Not stated | Radix *Eurycoma longifolia* Jack:354 mg , *Semen Pimpinella anisum*:10 mg, *Semen Cumimum cymimum*:22 mg, *Rhizome zingiber offcinale*:10 mg, *Sticepus variegahus*:10 mg, *Sticepus variegahus*:10 mg, *semen piper nigrum*:10 mg. |
| MON-A05 | ✓ | ✓ | Traditional preparations | Radix *Eurycoma longifolia*: 300 mg, Radix *Astragaliseu hedysari*:200 mg |
| MON-A06 | ✓ | ✓ | Traditional preparations | Radix *Eurycoma longifolia*:50 mg, *RhizomaSmilax Myosotiflora*: 70 mg, *Rhizoma Allomorphia Malacensis:*50 mg, *Semen Trigonellafoenum Graecum*:15 mg, *Rhizoma Zingiber Minus*:25 mg, *Caulis Leptosmermum Flavescens*: 25 mg, *Rhizoma AcorusCalamus* 15 mg, *Semen Nigella Sativa*:30 mg, *Semen Coriandrum Sativum*: 15 mg, *Fructus Piper Longum*:15 mg, *Semen Trachyspermum Ammi*: 10 mg. |
| MON-A07 | ✓ | ✓ | Traditional preparations | Radix *Eurycoma longifolia jack*: 425 mg, *Semen nigella Sativa*: 50 mg, *Folium Cassia Angustifolia*: 25 mg. |

**Table 4.3:**Tabulated results showing the presence or absence of marker A and B in each sample(continued).

| Products Code | Marker A | Marker B | Preparation* | Content* |
|---|---|---|---|---|
| MON-A08 | ✓ | ✓ | Not stated | *Eurycoma longifolia*: 120 mg, *Nigella sativa*: 100 mg, *Globia Pendula*: 50 mg, *Curcuma domestica*:10 mg, *Piper nigri fructus*:50 mg. |
| MON-A09 | ✗ | ✗ | Not stated | Root Tongkat Ali Powder Extract:60 mg, *Fructus Saw Palmetto* Power extract: 120 mg, *Pumpkin Seed* Powder Extract: 30 mg, *Fructus Tribulusterrestris* powder extract: 30 mg, Root *muriapuama* powder extract:60 mg. |
| MON-A10 | ✗ | ✗ | Traditional preparations | Not stated for Tongkat Ali,Herbs *coriondrum Sativum*: 1.67 gm, Radix *Smilax Myositiflora*: 0.34 gm, Herb *Pimpenella Anisum*:0.33 gm, Herbs *Cuminum Cyminum*:0.33 gm, Seed *Nigella Sativa*:0.33 gm, *Rizom Alpinia Galanga*:0.33 gm, *Rizom Curcuma Domestica*:0.25 gm, *Honey*:2.59 gm, *Sesame oil*:0.24 gm, *Rizom ZIngeber Officinal*:0.25 gm, *Flos Eugenia Aromatica*: 0.33 gm, *Helicteres Isora*: 0.17 gm, *Fructus Myristica Fragrans*:0.17 mg, *Seed Piper Nigrum*:0.17 gm, *Semen Tracchyspermum Ammi*:0.25 gm. |

**Table 4.3:** Tabulated results showing the presence or absence of marker A and B in each sample(continued).

| Products Code | Marker A | Marker B | Preparation* | Content* |
|---|---|---|---|---|
| MON-A11 | ✓ | ✓ | Not stated | Radix *Eurycoma longifolia*: 80 mg, *Angulliaangullia*: 150 mg, Radix *Striga Asiatica*:100 mg, *Zingiber Officinale Rhizome*:70 mg, Radix *Smilax Myositiflora Zingiber* :50 mg, *Bulbus Allium Sativum*:50 mg. |
| MON-A12 | ✗ | ✗ | Traditional preparations | Radix *Eurycoma longifolia*:0.125g , Honey:0.25g, *bean oil*: 0.175g, *Rhizomazingiber officinale*:0.025g, *Flos Eugenia Aromatica*:0.00375g, *Herba piper nigrum*:0.05g, *Herba croton Caudatum*:0.025g, *Herba Coriandrum Sativum*:0.0375, *Herba Nigella Sativa*:0.0125g, *Fructus Pimpinella Anisum*:0.0125g, *Stichopus Variegatus*:0.0125g, *Morinda Citrifolia*:0.125g. |
| MON-A13 | ✓ | ✓ | Traditional preparations | EL 500mg (only Tongkat Ali) |
| MON-A14 | ✗ | ✗ | Traditional preparations | Tongkat Ali water soluble extract: 119.85 mg, *Macaextarct/Rhizoma Lipidummeyenii*: 119.85 mg.*American Ginseng* extract: 119.85 mg.*Ginkgo Biloba* Powder: 109.51 mg. |

**Table 4.3:**Tabulated results showing the presence or absence of marker A and B in each sample(continued).

| Products Code | Marker A | Marker B | Preparation* | Content* |
|---|---|---|---|---|
| MON-A15 | ✓ | ✓ | Not stated | Tongkat Ali water soluble extract: 50 mg (only Tongkat Ali) |
| MON-A16 | ✓ | ✓ | Traditional preparations | Radix Tongkat Ali: 130.41 mg, Radix *achyranthesbindentata*: 18.63, mg,Cortex*eucommia ulmoides*:18.63 mg, radix *astrogalus membranaceus bge*:18.63 mg. |
| MON-A17 | ✗ | ✗ | Traditional preparations | Radix *Eurycoma longifolia jack*:2.56g , *Euginia Caryaphylata Fructus*:0.105g, Radix *Curcumae Zadoarria*:0.215g, *Zingiber Officinale roscope*:0.215g, *Rhizome Cuminum Cyminum*:0.125g Semen *Piper Nigrum*:0.105g, *Rhizome Alpina galanga*:0.105g, Semen *Pimpinella Anisum*:0.215g , Semen*cariandrum sativum*:065g, Semen *nigella sativa*:0.6g, Cortex*cinnamomum zeylanicum*:0.6g, Fructus*myristica fragrans:*0.09g. |

**Table 4.3:**Tabulated results showing the presence or absence of marker A and B in each sample(continued).

| Products Code | Marker A | Marker B | Preparation* | Content* |
|---|---|---|---|---|
| MON-A18 | ✓ | ✓ | Traditional preparations | *Eurycoma longifolia* Root Extract:5mg,*Piper Longum* Fructus: 10mg, *Helicteres Isora*Stem: 5 mg, *Coriandrum Sativum*Seed: 480mg. |
| MON-A19 | ✗ | ✗ | Traditional preparations | . Radix *Eurycoma longifolia*:27 mg, Stem *Ardisia crispa*:81mg, Stem *Cinnamoum iners*:54mg, Radix *Moringa elliptica*:54mg, *Rhizome Smilaxmyositiflora:*13.5mg, Stem *Alyxia reinwardtii*:13.5mg, Stem *Alyxia indica*:13.5 mg. |
| MON-A20 | ✓ | ✓ | Traditional preparations | Radix *Eurycoma longifolia*:45mg , *Herba Cynomorium songaricum*:70 mg, Semen *Cuscuta chinensis*:65 mg, Cortex *Eucommia ulmoides*:50mg, Radix *Panax ginseng*:50 mg, *Fructus Tribulus terrestris*:45mg, *Rhizoma Dioscorea opposita*:40 mg. |
| MON-A21 | ✗ | ✗ | Traditional preparations | Tongkat Ali exract: 50 mg, Tongkat Ali powder: 100mg, *Eleuthercoccus Senticosus*Root Extract: 100 mg, *Fructus Tribulus Terrestris* Extract: 100 mg. |

**Table 4.3:**Tabulated results showing the presence or absence of marker A and B in each sample(continued).

| Products Code | Marker A | Marker B | Preparation* | Content* |
|---|---|---|---|---|
| MON-A22 | ✓ | ✓ | Traditional preparations | Radix *Eurycoma longifolia jack*: 325mg (only Tongkat Ali) |
| MON-A23 | ✓ | ✓ | Traditional preparations | . *Eurycoma longifolia jack* Radix-100%:350mg (only Tongkat Ali) |
| MON-X01 | ✓ | ✓ | spray-dried water soluble extract | Radix *Eurycoma longifolia* Extract: 60 mg (only Tongkat Ali) |
| MON-X02 | ✓ | ✓ | Not stated | Tongkat Ali: 250 mg, *Ginseng*: 250 mg. |
| MON-X03 | ✓ | ✓ | Not stated | 100% radix EL (only Tongkat Ali) |
| MON-X04 | ✗ | ✗ | Not stated | Extract *Eurycoma longifolia* Radix:15% , Extract *Miristica Fragrans*: 5%, Extract *Yohimbin*: 10%, *Zingiber Rhizoma*:20%, *Curcumae Rhizoma*:30% Other ingredients in the capsule to:100%. |

**Table 4.3:**Tabulated results showing the presence or absence of marker A and B in each sample(continued).

| Products Code | Marker A | Marker B | Preparation* | Content* |
|---|---|---|---|---|
| MON-X05 | ✓ | ✓ | Not stated | Radix EL: 40%, *Pantrocinum*:10%, *Syngnathoides biaculeatus*:10%, *Panax Ginseng* Radix:10%, *Yohimbehae*:10%, *Ganaderma*:10%, *Hypocampus*:10% in the form ofextract. |
| MON-X06 | ✗ | ✗ | Not stated | Tongkat Ali Akbar,Some kinds of flower seeds and herb. |

✓： Presence of marker

✗： Absence of marker

*note: as stated within product description on the bottle.

## 4.4 ESI MS/MS ANALYSIS

LC MS/MS was performed on the reduced gel band (biomarker A, approximate MW: 10 – 14kDa, pI: 7 -10 with silver staining). Using Mascot database search, peptide mass finger printing results obtained concluded no match. This was expected as the available database for this species (*Eurycoma longifolia*) was only four proteins, which were related to photosynthetic mechanism and none of these proteins were linked to aphrodisiac activity. Due to insufficient library information, we found no match to the proteins at these spots. As such we concluded the protein is a novel one yet to be identified. The service provider suggested de novo sequencing for selected ions. Sequence coverage of nano LC ESI MS/MS for a gel spot depends on the amount of protein in the spot, tryptic peptide size, and the peptide ionization efficiency. It was unlikely toobtain the full amino acid sequence coverage of the whole protein. The full sequence of certain ions upon de novo sequencing could be helpful for designing primers.

In general, the ESI-MS/MS run led to fragmentation data on peptides of sufficient quality to undergo de novo sequence. It was found that a total of ten peptides had significant fragmentation and could be sequenced well enough (Fig 4.8 to 4.17). APAF performed the Mascot search for the MS/MS data on all ten peptides and they suggested these peptides appeared to proteins shown in Table 3.7 and with higher chances to match to rubber elongation protein. This was confirmed by MS/MS of 1157.6404 peak which had a sequence of DASIQVVSAIR. The ions score was 71 with matched of 26/106 fragment ions using 38 most intense peaks.

Nevertheless it was unlikely this protein could be a match to marker A of *Eurycoma longifolia* even though it is from a plant species commonly found in Malaysia that is *Hevea brasiliensis* (Rubber tree). Nevertheless the sequence of its peptide as well as others will be useful for future application. De novo sequenced data and the peptides found in the unassigned peptides list are included in the appendix C.

### 4.4.1 Mascot search on peak list

**Table 4.4:** shows the match of 10 proteins upon conducting peptide mass fingerprinting on the peak list.

| | | |
|---|---|---|
| 1 | gi\|21050 | Ribulosebisphosphate carboxylase (*Phaseolus vulgaris*) |
| 2 | gi\|13241107 | Ribulose-1,5-bisphosphate carboxylase small subunit P1A (*Flaveria palmeri*) |
| 3 | gi\|132270 | RecName: Full=Rubber elongation factor protein; Short=REF; AltName: Allergen=Hev b 1 |
| 4 | gi\|109892850 | RecName: Full=Putative cytochrome c oxidase subunit II PS17 |
| 5 | gi\|147838966 | hypothetical protein (*Vitisvinifera*) |
| 6 | gi\|147838966 | unnamed protein product (*Vitisvinifera*) |
| 7 | gi\|118482129 | unknown (*Populustrichocarpa*) |
| 8 | gi\|172046673 | RecName: Full=Unknown protein 1 |
| 9 | gi\|157343282 | unnamed protein product (*Vitisvinifera*) |
| 10 | gi\|56744298 | Putative gag-pol polyprotein, identical (*Solanumdemissum*) |

The MS/MS spectra (Fig 4.8 to 4.17) for each of the sequenced peptides have been included with a number of possible sequences determined by the software (including an assigned score).

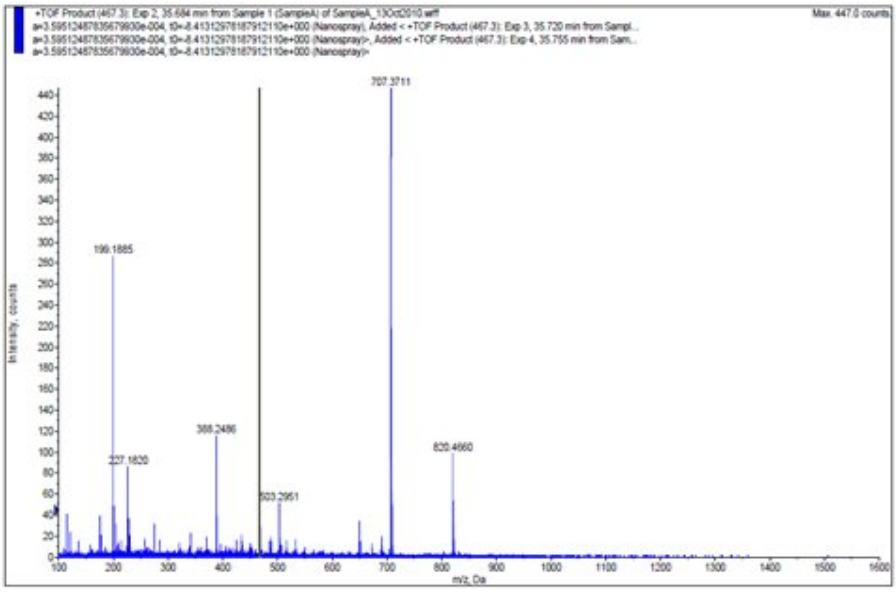


**Figure 4.8**: MS/MS spectra of peak 467.3 with possible sequence of LLGMDGGVR with 98.57 score.

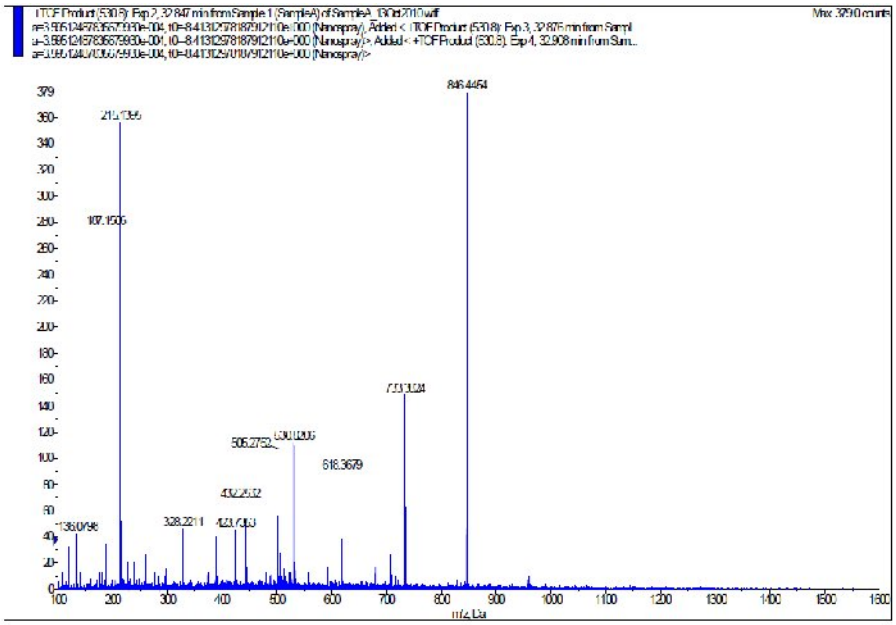

**Figure 4.9**: MS/MS spectra of peak 530.8 with possible sequence of TLLDDAGLDK with 98.15 score.

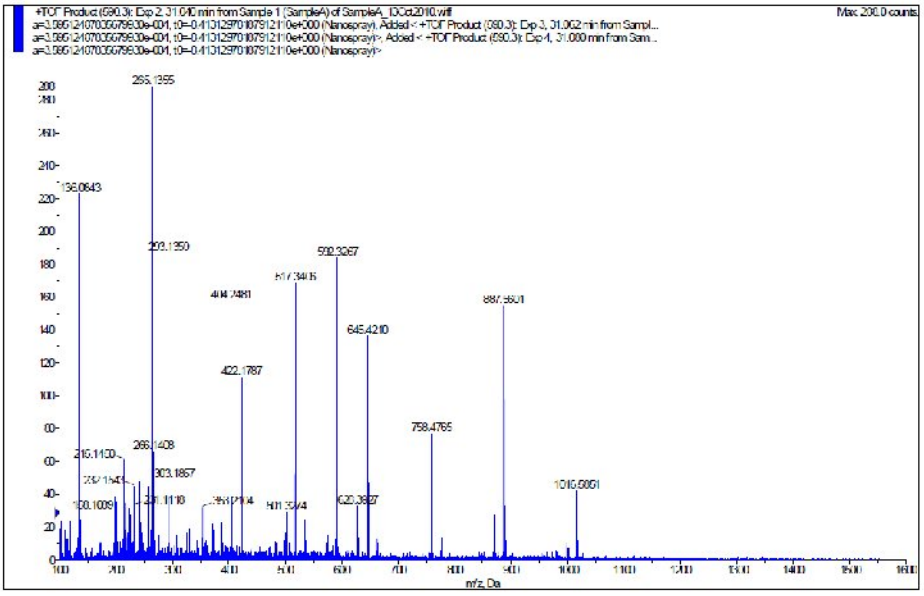

**Figure 4.10**: MS/MS spectra of peak 590.3 with possible sequence of YEELGALTAGR with 99.44 score.

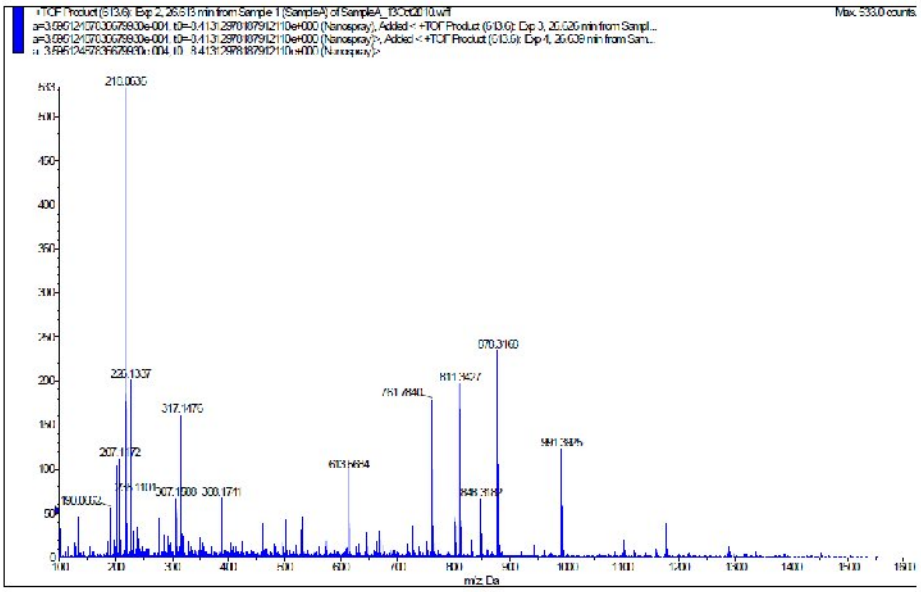

**Figure 4.11**: MS/MS spectra of peak 613.6 with possible sequence of YDCPNGGALASGFGAAVAK with 100.00 score.

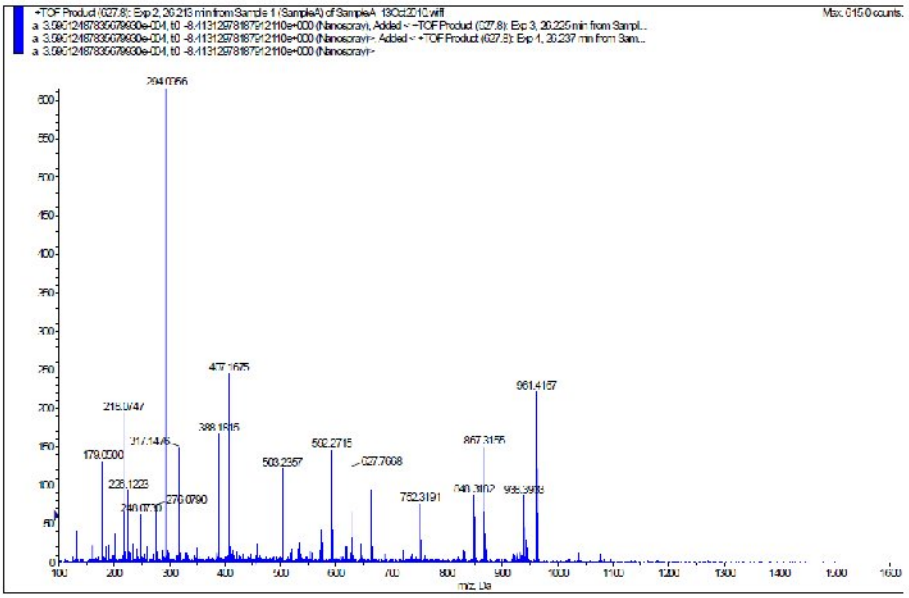

**Figure 4.12**: MS/MS spectra of peak 627.8 with possible sequence of DMSGAGGAGMAVAK with 91.69 score.

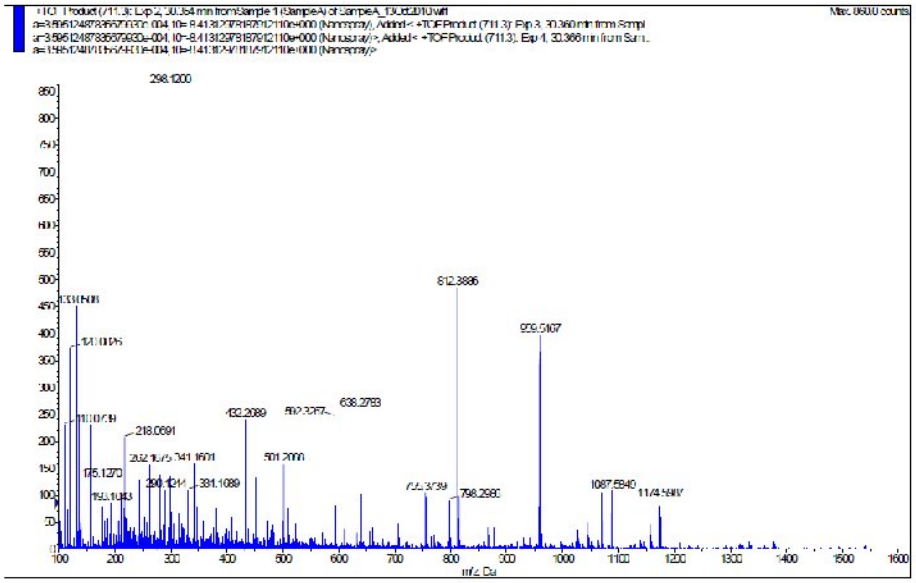


**Figure 4.13**: MS/MS spectra of peak 711.3 with possible sequence of TQGASMYGMTLMGYGPGYAK with 97.61 score.

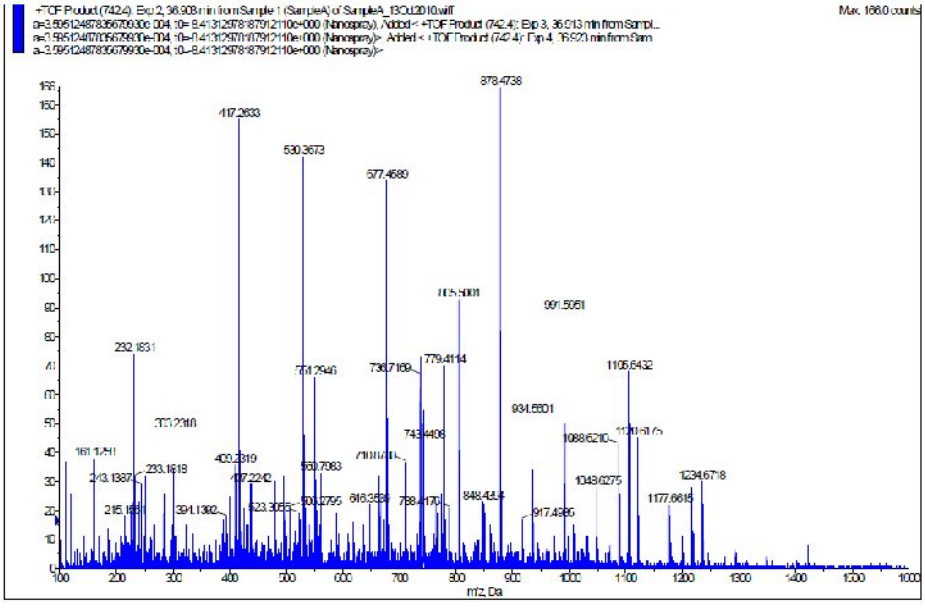


**Figure 4.14**:MS/MS spectra of peak 742.4 with possible sequence of TSAAHHVTEGEGGGEGAMLGGAGR with 100.00 score.

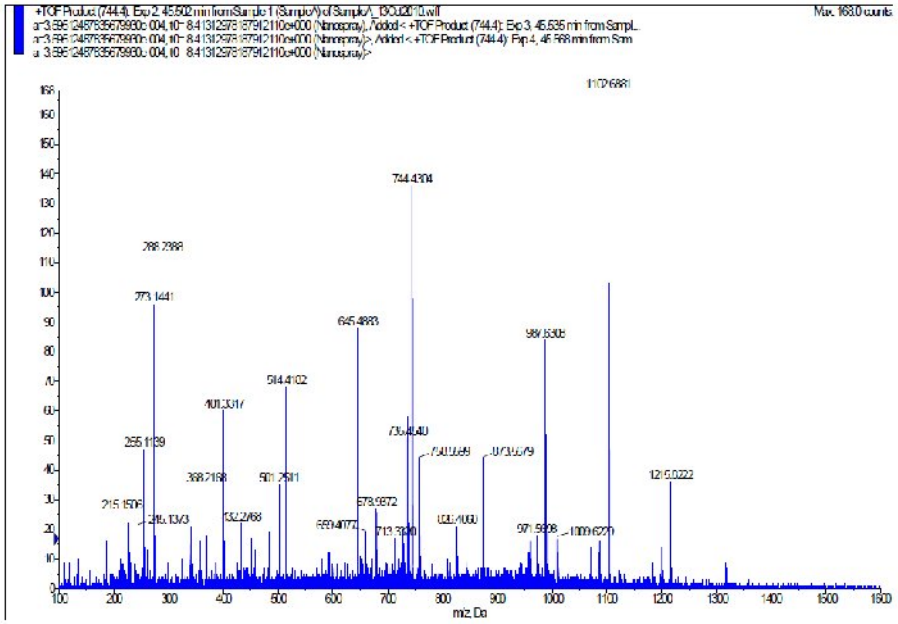


**Figure 4.15**: MS/MS spectra of peak 744.4 with possible sequence of NGTLGATEVGSTGAPR with 96.17 score.

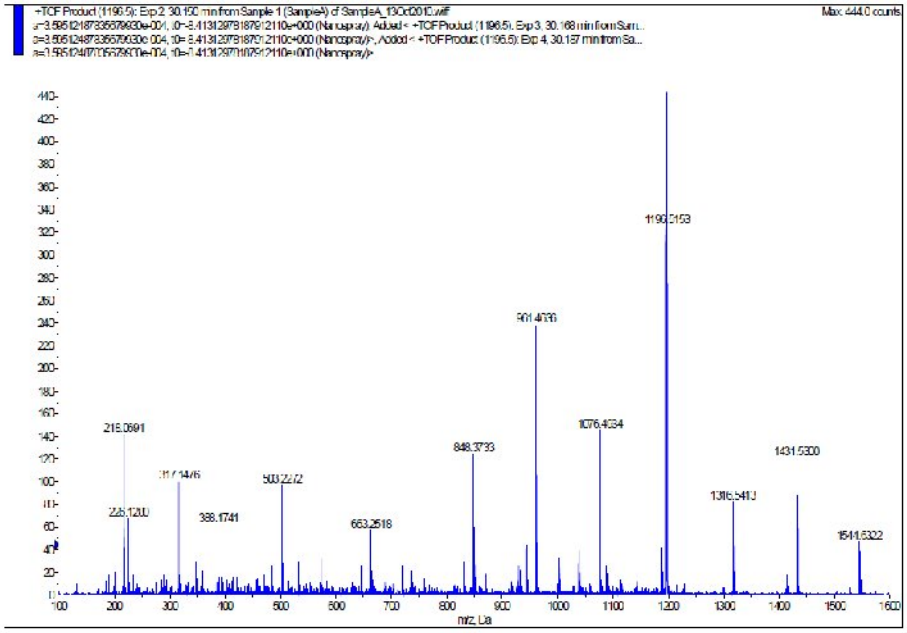


**Figure 4.16**:MS/MS spectra of peak 1196.5 with possible sequence of QPAYVSSDLDSNGPLAGGMGAAVAK with 98.33 score.

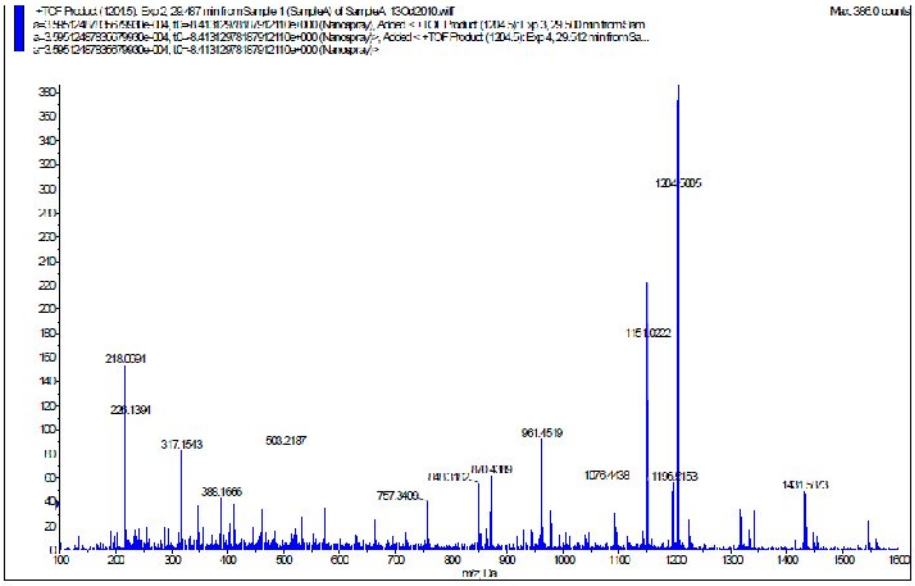


**Figure 4.17**:MS/MS spectra of peak 1204.5 with possible sequence of QGPGGSSGGYEVCLPTAGGDSGSVAK with 96.13 score.

## 4.5 DISCUSSION AND CONCLUSSION

There were very significant difference in protein content among the samples ranging from the highest 11.2840μg protein per mg sample (MON-A08) to the lowest 0.3734 μg protein per mg sample (MON-X04) (Table 4.1). These differences could be one of the reasons why some samples produce markers while others do not. Some samples, MON-X04 for instance was found to have a low protein content and hence it is not surprising that the markers were not detected at all in 2D-GE, though it is equally possible that the markers are not present. Nevertheless, for the sake of product evaluation, these products can be safely 'rejected' because it may not have pharmaceutical affects whatsoever since the active constituents are present only in negligible amounts. Levels of each product in *E. longifolia* extract varied significantly (Table 4.3). Hence for some product, there is a possibility that the markers are present in minute amounts even though the protein content is high. For all products that did not show presence of any marker when 25 μg of protein was used, further investigation was performed with 50 μg of protein. Again however, it remains questionable whether such a low quantity of biomarkers can really elicit any aphrodisiac effect on the capsules taken. Further evaluation was required in the event the biomarker is detected at 25 μg protein load, whether the product will show efficacy on humans.

Among the 23 MOH approved products, 14 showed presence of marker A and B. The same markers appeared in 4 out of the 6 MOH unapproved products tested. This means, 60.9% of the approved products and 66.7% of the unapproved products contained the markers. Overall 62.1% of all tested samples (excluding

positive and negative controls) had the markers. In terms of irregularities, MON-A10 was a product labelled as *E. longifolia* herbal supplement, but did not have *E. longifolia* in its ingredient information. This questions the role of MOH in the control of *E. longifolia* products in the market. A surprising 37.9% of the approved and marketed products did not have the markers, and possibly there are many more products which do not contain *E. longifolia* thatmay have been given the green light by MOH and sold in pharmacies all around the country as well as exported to other countries in the world. There are currently 224 *E. longifolia* MOH approved products that are registered under Malaysian National Pharmaceutical Control Bureau (NPCB), which is a body under MOH that is responsible in the regulation of pharmaceutical products in the country (NPCB, 2011). It is likely that NPCB has no real means of validating the presence of *E. longifolia* in the products other than assessing the application forms filled by the manufactures and investigating the lead load and microbial content in the products. Therefore, the markers will be very useful in this case, to help in the screening of products for the presence or absence of *E. longifolia* extract. With these biomarkers, NPCB can quickly reject any new product alleged to contain *E. longifolia* extract, but do not show the presence of markers A and B in 2DE.

The markers are only meant for *E. longifolia*. It is nevertheless possible though for products lacking in the biomarkers to still show aphrodisiac activity due to incorporation of other ingredients. Plants with aphrodisiac activity such as *S. myosotiflora*, *E.brevicornum, C.songaricum,* Yohimbine, etc have been known to be included in some of the products, possibly to enhance the power and potency of the concoction (Burkill, 1966; Yonezawa*et al*, 2005; Chen and Chiu, 2006; Damayanthi

*et al*, 2011andHuiping*et al*, 2011). Besides that, there is a possibility that manufacturers may have used immature *E. longifolia* root and even bark in their products (Cash, 2010). Doubts on the authenticity of the materials used may arise because *E. longifolia*are not cultured and harvested as an agricultural crop. Their quantities are not obtained in bulk and their supply is not consistant. They are generally found in the jungle as rare plants (especially mature ones). It is also possible for certain unscrupulous manufacturers to incorporate acetildenafil into their *E. longifolia* products (Becoat, 2006). Acetildenafil is a sildenafil analogue which has not undergone human testing yet.

It must be noted that the sample size of the unapproved products was small compared to the approved ones because of the difficulty to obtain them. The unapproved products were not available in any registered pharmacy. However, we still managed to get samples from small roadside and market stalls. Though the sampling size was relatively small, however quite a number of the unapproved products contained the markers as well. Despite the lack of quality control and regulation by pharmaceutical bodies, most of the MOH unapproved products still retained the biomarkers. The efficacy of these unapproved products might match or be even better than the approved ones, although there are still risks of other contaminants (fungi and bacteria and heavy metals) which are supposed to be tightly regulated by NPCB. The reason why most MOH unapproved products contains the biomarkers can be due to the fact that *E. longifolia* extract can be obtained easily with little processing. This was demonstrated by the positive results with MON-JV1 which was extracted in our laboratory.

Markers A and B always appear together, but in most cases, the former is more prominent and conspicuous (especially when the protein spots are faint) compared to the latter (Fig 4.1- Fig 4.5). Hence, it appears that marker A is the more suitable biomarker that can be used for *E. longifolia* validation purposes by NPCB. For this reason, marker A was chosen for further characterization and analysis. Marker A was isolated as a gel plug and was sent to APAF for 1D nanoLC ESI MS/MS run. The results were somehow as predicted. Owning to the fact that there are only four protein entries from the plant *E. longifolia* in the NCBI database (Tee and Azimahtol**,** 2005),it is therefore not surprising that the peptide fingerprint did not match any of the four proteins (all of which are related to the chloroplast and nuclear phylogenetic marker including AtpB, Ribulose-1,5-bisphosphate carboxylase/oxygenase subunit, PhyC and Maturase K). Hence, there is a high probability that this protein is novel, at the very least, for this plant. However, it was matched to an unrelated protein (rubber elongation factor protein) from the plant *Heveabrasiliensis*and several other plant proteins such as the ribulosebisphosphate carboxylase and Putative cytochrome c oxidase subunit II PS17(Table 4.4). Hence, there is a high probability that marker A is a novel and unreported protein. It also has the potential to be one of the bioactive proteins that is responsible for *E. longifolia* aphrodisiac activity. Whether the protein within marker A has similarities to the Eurypeptide reported in the media is difficult to ascertain as no records on the sequence can be obtained for comparison (Sambandan*et al*., 2004). It is difficult to obtain a full protein sequence based on gel plugs.

2DE is the ideal method for the study of *E. longifolia* proteome. Firstly, due to reporteds suggesting that the active constituent for aphrodisiac activity of *E.*

*longifolia* being protein related, and hence the choice for the use of 2DE (Sambandan *et al*., 2004).Secondly, 2DE has a high separation resolution. Being able to separate proteins based on their charge and size, that is, it helps in the separation of marker A, B and other proteins in herbal products. Otherwise, they will remain together if they were to be separated by SDS-PAGE alone. Besides that, the chosen method was relatively quick and cheap.

Future research work should include the isolation of marker A and B by utilizing conventional chromatography technique generally used to purify proteins (size-exclusion, ion-exchange, or hydrophobic interaction chromatography) and establishing their full sequence. Both of these will be useful in elucidating their function. The isolated proteins can then be tested on mice. The information will be useful in validating more products, especially the MOH unapproved ones to further support the findings of this study [i.e. that most MOH approved and unapproved products do contain the markers and the extraction of *E. longifolia* root extract is indeed simple and its proteins are not easily denatured through high temperature processing (> 80 °C)]. Somatic embryogenesis and direct plant regeneration techniques need to be further exploited to culture and produce this plant in large quantities. This would help in reducing the price of *E. longifolia* so that its pharmacological goodness can be enjoyed by all, while preserving natural forest resources.