CHARACTERIZING AND ISOLATING PROTEIN MARKER IN AUTHENTICATING *EURYCOMA LONGIFOLIA* HERBAL PRODUCTS

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ABSTRACT

Eurycoma longifolia or commonly known as Tongkat Ali has been identified as a valuable product in phytochemical industry due to its reputation in enhancing sexual properties. The proliferation of *E. longifolia* based herbal products renders quality control measure to be an important task. Standardization should be carried out, and currently the products are standardised to eurycomanone, the primary compound in the plant. Current research is on preliminary work in developing and isolating protein as marker compound to authenticate *E. longifolia* herbal products. From the market, 16 Malaysian Registered Products, 14 Malaysian Unregistered Products, 12 International Products and 8 Beverages were sampled. Eurycomanone analysis revealed that 24 or 48% of the total products contained eurycomanone while 26 or 52% did not. Protein marker analysis was commenced with Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE). The results indicated that a distinctive single protein band appeared in the SDS PAGE gel of product containing *E. longifolia*. Further inspection by 2 dimensional gel electrophoresis (2DE) revealed that *E. longifolia* consisted of four proteins, with similar molecular weight, but differed by isoelectric point. The four proteins were denoted as Marker A, B, C and D. Marker A was chosen as the ultimate marker as it was consistently presented in products containing *E. longifolia*. The presence and quantity of eurycomanone and Marker A in products containing *E. longifolia* was comparable with minor exception for four products (C1, C4, C7, and C21). Marker A was isolated using subsequent size exclusion chromatography and anion exchange chromatography. The purity of Marker A was proven by the appearance of single spot in 2DE gel, with the same electrophoretic profile of Marker A in *E. longifolia* extracts. Marker A then characterized by MALDI TOF MS and partially sequenced using *de novo* sequencing method. Marker A consisted of 22 amino acids. This study has led to the isolation of homogenous protein that can be utilized as novel and comparable marker to the chemical marker; eurycomanone, to authenticate *E. longifolia* products. Being a protein, subsequently an antibody can be developed and incorporated into biosensor device.

ABSTRAK

Eurycoma longifolia atau lebih dikenali sebagai Tongkat Ali adalah satu produk yang bernilai di dalam industri fitokimia disebabkan reputasinya untuk meningkatkan fungsi seksual. Peningkatan produk *E. longifolia* di pasaran menjadikan kawalan kualiti sebagai sesuatu yang penting, tambahan pula timbul isu produk ini dipalsukan. Piawaian mesti dijalankan dan pada masa ini, ia dipiawaikan kepada eurycomanone, bahan utama di dalam *E. longifolia*. Kajian ini adalah berkenaan menjadikan *protein* sebagai penanda kepada produk *E. longifolia*. 16 Produk Malaysia Berdaftar, 14 Produk Malaysia Tidak Berdaftar, 12 Produk Antarabangsa, 8 Produk Minuman telah disampel dari pasaran. Analisa *eurycomanone* menunjukkan 24 atau 48% dari keseluruhan produk mengandungi *eurycomanone* manakala 26 atau 52% tidak mengandungi *eurycomanone*. Analisa *protein* penanda dimulakan dengan *Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis* (SDS PAGE). Hasil analisa mendedahkan satu jalur *protein* di dalam gel produk yang mengandungi *E. longifolia*. Analisa lanjutan untuk *protein* penanda dijalankan dengan gel electrophoresis dua dimensi (2DE) mendedahkan *E. longifolia* terdiri dari 4 *protein*, dengan berat molekul yang sama tetapi berbeza takat isoelectrik. 4 *protein* tersebut dinamakan Penanda A, B, C dan D. Penanda A di pilih sebagai penanda terbaik kerana sentiasa hadir di dalam produk yang mengandungi *E. longifolia*. Kehadiran dan kuantiti *eurycomanone* dan Penanda A di dalam produk yang mengandungi *E. longifolia* didapati setara kecuali untuk 4 produk (C1, C4, C7, C21. Penanda A berjaya dipencil menggunakan kromatografi berasas saiz dan kromatografi penukaran ion. Ketulenan Penanda A dibuktikan dengan penampakan satu titik *protein* di dalam gel 2DE dengan sifat elektroforetik yang sama dengan Penanda A. Penanda A dikenal pasti menggunakan MALDI TOF MS dan penjujukan dilakukan menggunakan kaedah penjujukan *de novo*. Hasil penjujukan menunjukkan Penanda A terdiri daripada 22 asid amino, tetapi penjujukan ini adalah separa. Kesimpulannya, kajian ini telah berhasil untuk memencilkan *protein* yang berpotensi untuk menjadi penanda kepada produk *E. longifolia*. Penanda *protein* mempunyai potensi untuk menghasilkan antibodi dan diaplikasi di dalam alat biosensor.

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LIST OF SYMBOLS

m	meter
cm	centimetre
µM	micro molar
LC_{50}	lethal concentration
%	percentage
>	more than
<	less than
µg	micro gram
mL	mili litre
mg	mili gram
ppm	part per million
°C	degree Celsius
nm	nano meter
pH	hydrogen concentration
m/z	mass to charge ratio
kDa	kilo Dalton
µm	micro meter
µL	micro litre
L	litre
N	normality
V	voltage
hr	hour
mBar	mini bar
µJ	micro joule
mM	mili molar
mm	mili meter
v/v	volume/volume

LIST OF SYMBOLS

ng/µL	nano gram/micro liter
kV	kilo volt
T	total concentration of acrylamide and bis-acrylamide monomer

LIST OF ABBREVIATIONS

2DE	Two Dimensional Electrophoresis
APCI	Atmospheric Pressure Chemical Ionization
APS	Ammonium Persulphate
B-ME	2-Mercaptoethanol
BSA	Bovine Serum Albumin
CAPD	Computer Aided Process Design
CC_{50}	Critical Concentration
cGMP	Cyclic Guanoside Monophosphate
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
DAD	Diode Array Detector
DCA	Drug Control Authority
DHEA	Dihydroepiandosterone
DTT	Dithiothreitol
ED	Erectile Dysfunction
EL	*Eurycoma longifolia*
EMEA	European Medicines Agency
EPP1	Entry Point Project 1
ESI	Electro Spray Ionization
EU	European Union
EUR	European Dollar
FRIM	Forest Research Institute of Malaysia
GCMS	Gas Chromatography Mass Spectrometry
GI_{50}	Growth Inhibition
GMP	Good Manufacturing Process
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography

LIST OF ABBREVIATIONS

IAC	Immuno Affinity Column
IEX	Ion Exchange Chromatography
IMP7	Image Master 2D Platinum 7
IP	International Products
IPG	Immobilized pH Gradient
LC	Liquid Chromatography
LCMS	Liquid Chromatography Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
MALDI	Matrix Assisted Laser Desorption Ionization
MIT	Massachusetts Institute of Technology
MOH	Ministry of Health
MRP	Malaysian Registered Products
MUP	Malaysian Unregistered Products
NIR	Near Infra Red
NPCB	National Pharmaceutical Control Bureau
OHCT	Oxidized Hydroxyl Cis Terpenone
PDE6	Phosphodiesterase Type 6
POC	Point of Care
QToF	Quadrupole Time of Flight
Rf	Retention Factor
SD	Standard Deviation
SDS PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
SELDI-MS	Surface Enhance Laser Desorption Ionization Mass Spectrometer
SNPs	Single Nucleotide Polymorphisms
TA	Tongkat Ali

LIST OF ABBREVIATIONS

TCM	Traditional and Complimentary Medicine
TCM	Traditional Chinese Medicines
TEMED	Tetramethylethylenediamine
TLC	Thin Layer Chromatography
TOF	Time of Flight
UFLC	Ultra Fast Liquid Chromatography
US	United States
UV	Ultra Violet
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Research Background

Being considered as national treasure while other countries refer it as Malaysian Ginseng, Malaysian government is investing effort to license, develop, and sustain research into the potential health benefits of Tongkat Ali or *Eurycoma longifolia* (Talbott et al., 2013). Due to the benefits of herbal medicine, World Health Organization (WHO) encourages countries to promote the application of herbal medicines effectively (Charoonratana et al. 2014). Global herbal industry is expected to worth RM16 trillion by 2050 and Malaysia has made *E. longifolia* as one of five herbs that will be given priority in commercialization and standardization under Entry Point Project 1 (EPP1) of Agriculture (Utusan Malaysia, 2014). For instance, raw *E. longifolia* that is priced at RM2/kg jumped to RM2800 per kg after extraction and standardization (The Star, 2015).

Based on the wisdoms of traditional herbal practitioners who formulated the preparation of *E. longifolia*, the consumption benefits of this plant have been passed from generation to generations. Usually, it is prepared in the form of water decoction. The decoction is bitter, and it is assumed that the more the bitterness, the better the efficacy (Bhat and Karim, 2010).

Famous for its aphrodisiac property, the traditional uses range from tonic after childbirth to treating malaria, while published researches reveal that this plant has potential to treat various diseases such as malaria and cancer. Phytochemical studies revealed the presence of bioactive compounds in *E. longifolia* such as quassinoids, alkaloids, squalene derivatives, tirucallane-type triterpenes and biphenylneolignans (Bhat and Karim, 2010). Laboratory research, animal study, clinical trials have confirmed the pharmaceutical advantages of this plant, making *E. longifolia* as the most commonly used and extensively researched herbs in this region (Pan et al., 2014).

Despite the wide range of medicinal uses, *E. longifolia* is most famous through its aphrodisiac property. Its reputation in the western world also is probably gained by its potential in enhancing sexual performance. Aphrodisiac property of *E. longifolia* is contributed by its testosterone enhancing effect. Instead of containing testosterone itself, *E. longifolia* works by promoting the testes to increase the production of testosterone naturally (Bhat and Karim, 2010). Current androgen replacement therapy applies supplementation of testosterone gel, injection or pellet. The continuous supplementation can result in the stopping of body natural production system, thus, the natural testosterone promoting effects of *E. longifolia* can overcome the adverse reaction of the treatment. Due to its energy enhancing property and aphrodisiac potential, lots of products with *E. longifolia* formulations have been sold in the market and it has been identified as a potentially lucrative commercial product (Kumaresan and Sarmidi, 2003). Usually from the raw crude powder or after the freeze-drying process of the water extracts, the products come in the form of capsule, pills, liquid formulation, premixed coffee and canned processed drinks (Mohd Effendy, 2012). The standardized water soluble extract has been patented in 2006 (US 7.132.117.B2) after having gone extensive animal and human clinical evaluation (Tambi, 2009).

Usually in the market, the concentration of *E. longifolia* in a product is declared as x:y which means x gram of *E. longifolia* is used to produce y gram of extract. However, this claim cannot be verified. Some manufacturers deceive consumers by putting very little amount of *E. longifolia* where very low dose will not give the beneficial therapeutic value. Sometimes substances that are not declared at the packaging such as controlled drugs are also added. Consumers are also at risk of fake products. For example, last year, 40000 sachets of premixed coffee, worth RM60000 which imitates famous products bearing Tongkat Ali name were seized in a raid in Sabah (The Star, 2014).

Numerous *E. longifolia* products are being sold worldwide, but currently there is no standard method in controlling the quality of the products. Most of the products are processed traditionally where the quality are not well regulated. Most common method in determining the quality of the products is by analyzing the eurycomanone content, which is the major compound in the plant. Besides eurycomanone, which is a secondary metabolite, there are few researches who studied protein as the marker in *E. longifolia*. Asiah et al. (2007) utilised surface-enhanced laser desorption/ionization mass spectrometer (SELDI-MS) in identifying the potential protein marker in several plant famous for sexual enhancing effects including *E. longifolia* while Vejayan et al. (2013) detected two distinctive protein markers using two dimensional electrophoresis (2DE) in standardized extracts and in several *E. longifolia* products sold in Malaysia. The marker was not detected in selected herbal products devoid of *E. longifolia* extracts.

It should be noted here that eurycomanone is not well proven to be responsible for male virility and enhancing sexual performance except a study conducted by Low et al. (2013b) which indicated eurycomanone could increase spermatogenesis. Meanwhile, Sambandan et al. (2006) indicated that the compound responsible for aphrodisiac property of the plant could be a peptide. They identified a bioactive fraction containing peptide of 4.3 kDa, labelled as eurypeptide, which enhance synthesis of various androgens. The bioactive peptide (4.3 kDa) in the fraction of the water extract (patented: PI 20003988, MAL; 10/362697, USA; 01920972.5, EUROPE and 2002-522919, JAPAN) is a potential phytoandrogen, which has been reported to increase the testosterone level in rat Leydig cells.

Therefore, it is a scientific and commercial interest to identify this protein as a possible marker to determine the authenticity of *E. longifolia* products and to verify which compound in *E. longifolia* that contributes to aphrodisiac property. However, it is perplexing that the peptide found in the patented bioactive fraction has not been isolated and identified till today. It is most probably because of the problems encountered in isolating or identifying the protein due to the fragility, lability and sensitivity of the protein. Because of the challenges in studying this protein, current study deviced the method to isolate the protein with precautinary steps such limiting the number of steps in purification procedure and avoiding the use of organic solvent to minimize protein loss due to degradation.

This study would be performing proteomic evaluation on several *E. longifolia* products sold in Malaysia and international market. This marker would be generated using 2DE analysis, which is much cheaper to be procured, less maintenance and lower running cost. The potential protein marker would be identified, isolated and the amino acid sequence of the protein would be determined. This protein marker analysis would be compared with the existing chemical marker, eurycomanone and was expected that both of these markers may serve to be useful in authenticating genuine or unadulterated *E. longifolia* herbal aphrodisiac products. This finding is useful in setting the baseline for new biomarker development for *E. longifolia* commercial products. A marker of protein origin can be useful in generating antibody that can be developed into biosensors.

1.2 Problem Statement

E. longifolia commercial products are in high demands. However, questions have been raised about the quality of these products. Very few know the exact contents of the products that they have purchased or consumed. Consumers are at risks of products mislabelling and too little amount of active ingredient that they intent to get in the products. Mislabelling can delay adequate treatment in case of medical emergency, very low dose will result in no therapeutic value. In addition, manufacturers often make unrealistic claims such as fast efficacy period and high doses of active ingredients. *E. longifolia* products concentration is always advertised and promoted based on extraction ratio such as 200:1, 2000:1. However, this claim cannot be verified.

In Malaysia, pharmaceutical and traditional products for medical purposes need to be registered with Drug Control Authority (DCA). The registration criteria are limit of heavy metal, absence of steroid and synthetic adulterant and microbial load. It can be concluded here that products registration only confer guarantee on the product safety, but not the quality. Since phytochemical industry is developing and becoming a lucrative industry, critical evaluation on the product quality is also needed. This is to ensure that customers get the best of what they have purchased and to prevent unscrupulous manufacturer from making misleading claim. Moreover, Malaysia, as the biggest producer, should produce high quality and better standardized products

Currently there is no other method to authenticate the products except by determining the eurycomanone content, a major compound in the plant. However, eurycomanone is not verified to be the compound responsible for aphrodisiac property of this plant. A patent (US 7,132, 117 B2), found a peptide in a bioactive fraction of *E. longifolia* extracts. It could be proposed that the protein can be the marker to authenticate efficacy of a product. However, the protein is not isolated till today. Successful isolation and characterization of the protein will pave the way in producing protein marker that can be incorporated into cheaper and user friendly device such as biosensor. This is because, the method to determine eurycomanone involves the use of HPLC and costly eurycomanone standard needs to be purchased regularly.

1.3 Objectives of Study

- To determine the presence of eurycomanone in *E. longifolia* extracts and commercial products
- To develop and determine protein marker in *E. longifolia* extracts and commercial products
- To compare the usefulness of protein marker (new marker) with the current existing marker, eurycomanone
- To isolate and characterize the protein marker

1.4 Scopes of Study

The general scopes of the study are summarized as following;

- Sampling of *E. longifolia* commercial products from the markets
- Determine the presence of eurycomanone in *E. longifolia* extracts and commercial products using HPLC
- Determine total protein content in *E. longifolia* extracts and commercial products

- Analyze electrophoretic profile of *E. longifolia* extracts and commercial products using SDS PAGE analysis
- Analyze protein profiles of *E. longifolia* extracts and commercial products using 2DE analysis
- Study eurycomanone and protein profile in plant species with same local name with *E. longifolia*; Tongkat Ali Hitam, Tongkat Ali Merah
- Analysis of 2DE gels using 2DE software, Image Master Platinum 7
- Isolation of the protein marker
- Purity test of the isolated marker
- Peptide mass finger printing of protein marker
- *De novo* sequencing of the protein marker

CHAPTER 2

LITERATURE REVIEW

2.1 *Eurycoma longifolia* Nomenclatures and Taxonomy

Tongkat Ali is from Simaroubaceae family and *Eurycoma longifolia* is the Latin name. In Malaysia, it is also known as Payung Ali, Penawar Pahit, Setunjang Bumi, Bedara Pahit, Tongkat Baginda, Pokok Syurga, Tongkat Ali Hitam, Pasak Bumi, Pokok Jelas and Jelaih (Athimulam et al., 2006). This plant is also popularly called as Long Jack, Malaysian Ginseng, Local Ginseng, Natural Viagra (Bhat and Karim, 2010). In Singapore, it is also known as Tongkat Ali while in Indonesia as Pasak Bumi, Tung Saw in Thailand and 'Cay ba binh' in Vietnam (Meng et al., 2014). 'Cay ba binh' is translated as 'a tree which cures hundreds of diseases' (Tee and Lope Pihie, 2005). Among these common names, Tongkat Ali is the most famous of all in Malaysia as well as internationally. The latter is indicative based on the many products sold in western world stating "Tongkat Ali" at their labels or packaging. Tongkat Ali literally means Ali's walking stick, where "Ali" might refer to the claimed sexual enhancing property for men while "walking stick" might refer to the morphology of the long straight root (Mohd Effendy et al., 2012; Bhat and Karim, 2010).

Geographically, *E. longifolia* is endogenous to Southeast Asia, including Malaysia, Indonesia, Thailand, Laos, Cambodia and Vietnam. It is an evergreen, slender shrub, single stemmed, slow growing and grows along hilly slopes of rainforests, as an understorey in the lowland forests at up to 500 m above sea level (Ang et al., 2002; Tee

and Lope Pihie, 2005; Razak and Aidoo, 2011). It can be found in primary and secondary, evergreen and mixed deciduous forest (Kavitha et al., 2010), on well drained sandy soil (Chen et al., 2012), with partial shade and adequate quantity of water (Bhat and Karim, 2010). The maximum height of this plant is 15–18 m, bears fruit about two and half years after cultivated, while the root is usually harvested for traditional and commercial uses after four years (Athimulam et al., 2003). However, it is generally believed that this plant actually reaches its complete maturity after 25 years (Bhat and Karim, 2010). The production of the roots is time consuming and fluctuates depending on seasons (Lulu et al., 2014). Root harvesting is carried out manually and to uproot a single plant might take between 8 to 10 hr (Bhat and Karim, 2010).

An example of *E. longifolia* root and tree is in Figure 2.1. This plant is a dioecious, where male and female flowers are produced on different trees. The leaves are pinnate, 20-40 cm long, with ovate–lanceolate leaflets, spirally arranged (Zanoli et al., 2009). The fruit is ovoid, 1–2 cm long and 0.5–1 cm broad; its color moves from green to blackish-red when it ripens (Mohd Effendy et al., 2012; Zanoli et al., 2009). Seedlings are the main mode of planting for this plant (Hassan et al., 2012).

Besides *E. longifolia*, there are two other plants that are referred by the locals as Tongkat Ali; *Polyalthia bullata* and *Goniothalamus* sp (Mohd Effendy et al., 2012; Athimulam et al., 2006; Bhat and Karim, 2010; Chua et al., 2011; Hassan et al., 2012). The local names for *Polyalthia bullata* and *Goniothalamus* sp are Tongkat Ali Hitam and Tongkat Ali Merah respectively. *E. longifolia* and these two plants share the same local name due to the similar morphology, which is a long, straight root. *Hitam* and *Merah*, which means black and red, signify the color of the root. Figure 2.2 shows the roots of both plants. Traditionally, Tongkat Ali Hitam is used for aphrodisiac purpose (Connolly et al. 1996), as tonics (treat kidney infections, diabetes and high blood pressure (Ong and Nordiana, 1999). In terms of medicinal uses, *E. longifolia* is more routinely used (Bhat and Karim, 2010) and scientific research is also more numerous on *E. longifolia* (Hassan et al., 2012).

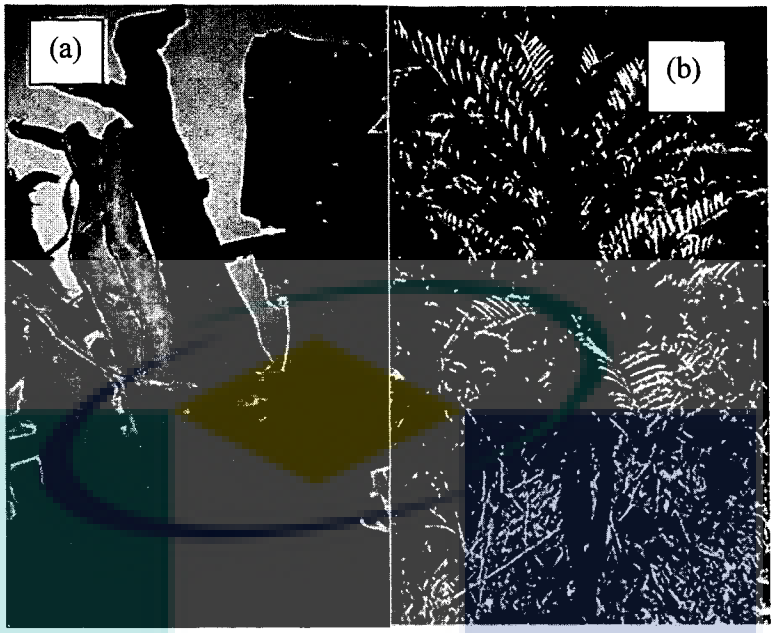

Figure 2.1: *E. longifolia* roots and tree (a) *E. longifolia* roots, (b) *E. longifolia* tree. The long, straight root is the characteristic of *E. longifolia*.

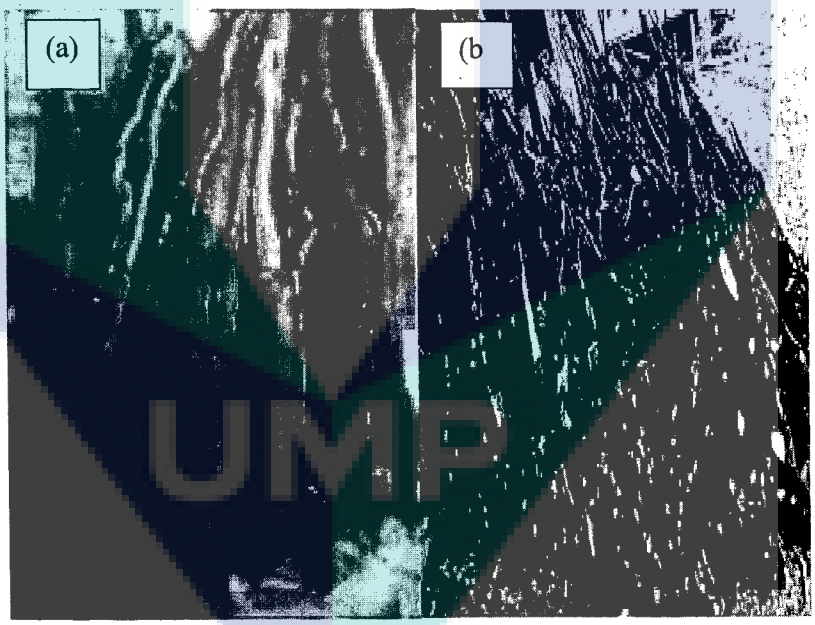

Figure 2.2: Roots of TA Merah and TA Hitam (a) TA Merah (b) TA Hitam. The local names signify root color of both plants.

2.2 *Eurycoma longifolia* Traditional and Medicinal Uses

E. longifolia possess lots of medicinal properties and has been sought by Southeast Asian community to improve general health. This plant is called "Malaysian Ginseng" because a tea is prepared from this plant, by boiling the root that has been cut into smaller portions (Zanoli et al., 2009). Almost all parts of *E. longifolia* have therapeutic uses, but the root is considered as the most valuable (Bhat and Karim, 2010). The root of this plant has been used as single or essential component to increase libido, treat malaria, bleeding, ulcers, hypertensions, dysentery, glandular swelling, antipyretic, complications after childbirth (Ang and Cheang, 1999; Tee and Lope Pihie, 2005; Razak and Aidoo, 2011). It has also been prescribed as remedy for age related problems such as reduced energy, mood, and libido (Talbott et al., 2013). The leaves are used to cure malaria, ulcers, syphilis, gonorrhoea, gum diseases prevention and relieve insect bites (Athimulam et al., 2003). Since the root decoction is very bitter, adding honey, or dates, or sugar is suggested to remove the bitterness. However, a few experienced traditional healers claimed that the efficacy lies with the bitterness (Bhat and Karim, 2010).

The traditional uses of this plant are well documented in literatures. Hout et al. (2006) surveyed the ethnobotanical application of *E. longifolia* among traditional healers in Cambodian regions. The survey demonstrated that *E. longifolia* has been used as tonic to treat fever, dysentery, rheumatism and as diuretic. Adnan and Othman (2012) interviewed urban folks in Kampung Bharu, Kuala Lumpur and discovered that *E. longifolia* is a common plant species to them and it is often used for healing and general consumption. *E. longifolia* has also been used to treat malaria. A survey was conducted by Al-Adhroey et al. (2010) in among 233 aboriginals as well as rural households and traditional healers in malaria endemic areas in Malaysia. The study revealed that *E. longifolia* was mentioned by the entire respondent group as a plant to treat malaria.

Despite the wide range of traditional uses known, *E. longifolia* is most famous solely for its aphrodisiac property. It is notorious among Malaysian men because it increases male virility and sexual prowess during sexual activities (Ang and Cheang, 2001). Besides the Malay community, *E. longifolia* also has been used by the Chinese and Indian ethnics in Malaysia (Low and Tan, 2007). Ethnobotanical survey by Ong and Nordiana, (1999) in the states of Kelantan and by Samuel et al. (2010) in Perak, Malaysia

discovered that the decoction of the roots was taken as aphrodisiac by most men. Samuel et al. (2010) carried out a survey among a local Orang Asli tribe to gather information on the use of medicinal plants in the region of Kampung Bawong, Perak and *E. longifolia* was disclosed as the plant that they used most to treat sexual problems. Ab Rahman et al. (2011) found out, among 1331 men aged 40 years and above who were generally seeking treatments for their sexual problems, used *E. longifolia* besides Viagra, traditional massages and ginseng. *E. longifolia* is part of traditional and complementary medicines (TCM) and it is the favored treatment among Malaysian for aphrodisiac purpose. This situation might be due to this plant is easily available in Malaysia and the perception that TCM is safer than conventional medicines (Hassali et al., 2012).

2.3 Scientific Findings on *Eurycoma longifolia*

Besides traditional uses, *E. longifolia* pharmacological effects have been proven by numerous studies. Table 2.1 lists scientific studies that have been conducted on *E. longifolia.*

Table 2.1:
Scientific studies carried out on *E. longifolia*

Activity	Source
Antimalarial	Ang et al. (1995)
	Tran et al. (2003)
	(Chan et al. 2004)
	Kuo et al. (2004)
	Chan et al. (2005)
	Hout et al. (2006)
	Mohd Ridzuan et al. (2007)
	Nguyen-Pouplin et al. (2007)
	Sholikhah et al. (2008)

Table 2.1(continued):
Scientific studies carried out on *E. longifolia*

Activity	Source
Antimalarial	Wernsdorfer et al. (2009) Astelbauer et al. (2012)
Administration routes	Mohd Ridzuan et al. (2007)
Anti parasite	Jiwajinda et al. (2002) Kavitha et al. (2010) Kavitha et al. (2012)
Toxicity	Hout et al. (2006) Mayer et al. (2009) Kavitha et al. (2012)
Antibacterial	Farouk and Benafri (2007) Tzar et al. (2011) Khanam et al. (2015)
Anti tumor related activity	Okano et al. (1995) Chan and Choo (2002) Kuo et al. (2004) Chuen and Lope Pihie, (2004) Tee and Lope Pihie (2005) Nurhanan et al. (2005) Tee et al. (2007) Mahfudh and Pihie (2008) Purwantiningsih et al. (2011) Wong et al. (2012) Meng et al. (2014) Park et al. (2014)
Modulatory and metabolism activity	Low et al. (2011) Pan et al. (2014)
Anti-inflammatory effect	Tada et al. (1991) Ang and Cheang (1999)
Ergogenic effects	Muhamad et al. (2010) Chen et al., 2012) Shuid et al. (2012)

2.3.1 Antimalarial Effects

Tran et al. (2003) and Nguyen-Pouplin et al. (2007) tested the antimalarial activity of plants that are used in Vietnam traditional medicines and compared the results with current drugs to treat malaria; chloroquine, artemisinin and clindamycin. Nguyen-Pouplin et al. (2007) discovered that *E. longifolia* extracts showed high antimalarial activity while Tran et al. (2003) found *E. longifolia* could inhibit the growth of chloroquine resistant *Plasmodium falcifarum*. Butanol extracts were more potent than diethyl ether extracts, but compared to chloroquine, both extracts were more potent against chloroquine-resistant Gombak A *Plasmodium falcifarum* isolate (Chan et al., 2004). Hout et al. (2006) observed a very high anti-plasmodial activity of the plant aqueous extracts, which is the preferred extraction method in traditional medicines and it was as active as dichloromethane extracts. As combination of treatment is said to result in increased efficacy, Mohd Ridzuan et al. (2007) compared anti-malarial activity of standardized extracts which contained three major quassinoids (eurycomanone, 13,21-dihydroeurycomanone, and 13α(21) epoxyeurycomanone) and as well as combination of standardized extracts with artemisinin. It was discovered that combination of the extracts and the drug showed promising activity than standardized extracts.

Wernsdorfer et al. (2009) compared the anti-plasmodial activity of the standardized extracts with artemisinin activity. They reported that artemisinin showed a higher activity as compared to the standardized extracts, but the activity of the latter was relatively high to be ignored for further studies. Activity of the standardized extracts was higher than isolated quassinoids, consequently they claimed synergism between the combined three quassinoids. Astelbauer et al. (2012) compared the activity of plant derived compounds such as aglafolin, rocaglamid, against standardized quassinoid extracts from *E. longifolia* for anti-plasmodial activity. The result demonstrated that the standardized quassinoid extracts showed higher activity than the two compounds. In determining which stage of *P. falcifarum* is susceptible towards *E. longifolia* treatment, Sholikhah et al. (2008) discovered that trophozoites stage of *P. falcifarum* was the weakest when treated with *E. longifolia*.

The antimalarial activity of individual compound from *E. longifolia* especially the various quassinoids has also been tested by Ang et al. (1995). They assayed three quassinoids (eurycomanol, eurycomanol 2-O-β-D-glucopyranoside, and 13β,18-

dihydroeurycomanol) against chloroquine resistant *Plasmodium falcifarum*, but chloroquine posed higher activity against the *P. falcifarum* where the LC_{50} for the compounds were 1.231-4.899 µM., 0.389-3.498 µM, and 0.504-2.343 µM, respectively, compared with 0.323-0.774 µM for chloroquine. They discovered that LC_{50} of longilactone were from 5.5 to 13.7 µM while 11-dehydroklaineanone ,15β-hydroxyklainone, 14,15 β-dihydroxyklaineanone, 15β-O-acetyl-14-hydroxyklaineanone showed LC_{50} of 5.5, 5.3, 5.0, 23.8 µM. Kuo et al. (2004) isolated eurycomanone and pasakbumin B. The compounds displayed potent antimalarial activity against the resistant *Plasmodium falciparum*. Chan et al. (2004) isolated four quassinoids, (eurycomanone, 13,21-dihydroeurycomanone, 13α(21)-epoxyeurycomanone, eurycomalactone) and an alkaloid (9-methoxycanthin-6-one). Using lactate dehydrogenase assay, the four quassinoids showed high activity against chloroquine resistant Gombak A isolate, but less active on chloroquine sensitive compared to chloroquine. Ultimately, all the quassinoids and chloroquine tested were found less potent than artemisinin.

As eurycomanone is considered to be the most potent anti-malarial compound in *E. longifolia*, Chan et al. (2005), studied the anti-malarial activity against chloroquine-resistant isolate using semi synthetic forms of eurycomanone; referred as diacylated 1,15-di-O-isovaleryleurycomanone, 1,15-di-O-(3,3-dimethylacryloyl) eurycomanone, 1,15-di-O-benzoyleurycomanone and the monoacylated 15-O-isovaleryleurycomanone. The result showed that the monoacylated showed high activity and lower toxicity, while the diacylated showed lower activity, thus acylation only at the C-15 hydroxyl group may be worthy of further antimalarial investigation. Mayer et al. (2009) studied the activity of oxidized hydroxyl of cis terpenone (OHCT) against chloroquine and artemisinin resistant. The result showed that OHCT was more active than chloroquine on chloroquine resistant, but less active against chloroquine sensitive while OHCT and chloroquine exhibited similar potency against chloroquine resistant clone. OHCT found even at low nano molar concentration, able to inhibit survival of artemisinin resistant isolates. It was therefore concluded that OHCT has the potential capabilities to be developed as anti-malarial lead compound especially as it is easily synthesized (Mayer et al., 2009).

2.3.2 Administration Routes

In ensuring effective treatment, how the drug is administered in the body also plays its role. Mohd Ridzuan et al. (2007) discovered subcutaneous route of *E. longifolia* extracts was more effective than oral administration. They claimed that was because with subcutaneous route, drugs are directly exposed and absorbed to the bloodstream while with oral administration, the drug is absorbed through the digestive tract and passed through the liver before it is transported via the bloodstream causing inactivation and incomplete absorption.

2.3.3 Anti Parasitic Effects

Jiwajinda et al. (2002) studied the inhibitory effects of three quassinoids (longilactone, 11-dehydroklaineanone and 14,15 β-dihydroxyklaineanone) on a parasite, an adult schistosomes movement and egg-laying of *S. japonicum*. All the three quassinoids showed significant inhibitory effects on *S. japonicum* but the activities were found to be weaker than the current drug for schistosoma treatment, the praziquantel.

Kavitha et al. (2010 and 2012) investigated the anti-parasitic activities of *E. longifolia* on toxoplasmosis caused by *Toxoplasma gondii,* one of the most widespread protozoan parasites, chronically infecting approximately 30% of the global human population. Before studying the inhibitory effects, they first identified an appropriate host to support the growth of the parasite to ensure that the consumed *E. longifolia* extracts is not toxic to the host and affect host performance. Kavitha et al. (2010) tested the cytotoxicity of *E. longifolia* extracts against two mammalian cell lines (Vero and HS27). One of the *E. longifolia* fraction did not show toxicity ($CC50 > 20$ µg/mL) towards Vero cells, thus the Vero cells was chosen as the host for anti-parasitic studies. The inhibitory study showed *E. longifolia* fraction significantly inhibited *T. gondii* growth even at concentration as low as 0.369 µg/mL (Kavitha et al., 2012). Further advantage is that the *E. longifolia* fraction was less toxic to host cells than clindamycin, the current drug for toxoplasmosis treatment.

2.3.4 Toxicity Tests

To avoid the site effects of treatment, anti-parasite medicine that is given should only be toxic towards the parasite but not on the healthy host cells. There are several studies reporting the selectivity of *E. longifolia* towards parasite and normal cells. Chan et al. (2005) revealed that monoacylated eurycomanone displayed anti-plasmodial potency but toxicity was low in brine shrimp assay test. Mayer et al. (2009) reported that hydroxy cis terpenone from *E. longifolia* displayed strong growth inhibitory against all stages of *P. falcifarum* but not toxic to cultured human liver cells. There was a discouraging report by Hout et al. (2006) where they revealed *E. longifolia* extracts showed low selectivity between human and plasmodium cells. Kavitha et al. (2012) in their studies on the treatment of toxoplasmosis, compared the toxicity of several *E. longifolia* extracts and standard drug clindamycin. Results showed that one extract did not show any toxicity towards Vero cells while clindamycin showed toxicity at CC50 < 20 µg/mL.

2.3.5 Anti-Bacterial Effects

Farouk and Benafri (2007), investigated the anti-bacterial properties of methanolic, ethanolic, acetone, aqueous extracts from different parts of *E. longifolia* against several bacteria. The alcoholic and acetone extracts of the leaves and stem were active against *Escherichia coli* and *Salmonella typhi* microbes while the aqueous leaves extracts were found to be active against *Staphylococcus aureus* and *Serratia marscesens*. However, the root extracts, which has been the preference in traditional medicines and modern researches, failed to show any antibacterial property. The bacterial strains used in this study has no significant clinical importance, however in another study done by Tzar et al. (2011), *E. longifolia* aqueous extracts were studied on bacterial strains that cause diseases which are difficult to be treated. The pathogenic bacterial strains were methicillin-resistant *Staphylococcus aureus*, *Enterococcus faecium*, extended-spectrum beta lactamase-producing *Klebsiella pneumoniae*, group-1 beta lactamase-producing *Pseudomonas aeruginosa*, multidrug-resistant *Acinetobacter baumanii* and *Salmonella typhi*. The result was in agreement with Farouk and Benafri (2007), where anti-bacterial effects were only observed from the leaves and stems, but not from the root extracts. They also studied the anti-fungal effects as fungal infection is fast becoming an emerging

infection especially among immune compromised patients. Three species of *Candida* (*C. albicans, C. glabrata* and *C. krusei)* were used. However, the *E. longifolia* extracts did not show any antifungal activities at the concentrations tested in this study (10, 5, 2.5, 1.25 and 0.625 mg/mL). Khanam et al. (2015) screened the antimicrobial activity of methanol, acetone, ethyl acetate, chloroform and petroleum ether extracts of the stem and root of *E. longifolia*. All of the extracts exhibited highest antibacterial activity against gram positive bacteria, however, stem extracts showed higher activity. They claimed this is probably because stem has richer source of phytochemicals than the root. Among all the tested extracts, stem, methanol and ethyl acetate extracts were the most effective in inhibiting microbes.

2.3.6 Anti Tumor Related Activity

Okano et al. (1995) assayed the anti-tumor promoting activity of quassinoids. Some of the quassinoids showed potent activity, with more than 50% inhibition. They also to a certain extend studied the mode of action of the quassinoid whereby it was discovered a methyleneoxy bridge and a side chain found to enhance the activity while a sugar moiety reduces the activity instead. In 2002, Jiwajinda et al. (2002) discovered 14,15β-dihydroxyklaineanone (LC_{50}= 5μM) has the highest anti-tumor promoting activity and the inhibitory potential was much higher than that of quercetin (LC_{50}= 23μM) and β-carotene (LC_{50}= 30μM) i.e. two common anti-tumor promoting natural agent. Dehydrolongilactone, longilactone, 11-dehydroklaineanone and 15β hydroklaineanone were comparable to querecetin or β-carotene, while 15b-O-acetyl-14-hydroxyklaineanone was classified altogether as a less active compound.

Kuo et al. (2004) isolated 65 compounds from *E. longifolia*. Some of the compounds; eurycomalactone, 6-dehydroxylongilactone, 9-methoxycanthin-6-one, canthin-6-one, longilactone, 14,15b-dihydroxyklaineanone, pasakbumin C, and canthin-6-one-9-O-b-glucopyranoside demonstrated strong cytotoxicity toward human lung cancer (A-549) cell lines while eurycomalactone, 6-dehydroxylongilactone, 9-methoxycanthin-6-one, 14,15b-dihydroxyklaineanone, eurycomanone, pasakbumin B, and pasakbumin C exhibited strong cytotoxicity toward human breast cancer (MCF-7) cell lines. This research proved that eurycomanone poses cytotoxicity towards breast cancer cells while Chuen and Lope Pihie, (2004) substantiated this finding by showing

that their semi purified eurycomanone also conferred toxicity towards breast cancer cell. Moreover, it was discovered this compound was less toxic on non-cancerous breast cell (MCF10A) with EC value of 30.90 ± 0.99 µg/mL, compared to Tamoxifen, the drug for breast cancer treatment with EC value of 2.59 ± 0.11 µg/mL. Chuen and Lope Pihie, (2004) also elucidated the mode of action of the semi-purified eurycomanone where they discovered the treatment resulted in apoptotic cell death of MCF-7 breast cancer cells involving down-regulation of an apoptosis regulator protein (BCL-2). Meng et al. (2014) discovered eurycomanone and a newly found quassinoid, 5-iso-eurycomadilactone exhibited potent cytotoxicity towards MCF-7 and MGC-803 cancer cell lines. Park et al. (2014) isolated several quassinoids and those compounds showed significant cytotoxicity towards A549 and MCF-7 cancer cells.

Previous studies tested the anti-proliferative property of isolated compounds (Okano et al., 1995; Jiwajinda et al., 2002; Kuo et al., 2004) and synthetic compounds (Chuen and Pihie, 2004). Nurhanan et al. (2005) evaluated the cytotoxic effects of *E. longifolia* root extracts (methanol, n-butanol, chloroform and water) against human papillomavirus (KB), human prostate cancer (DU-145), human rhabdomyosarcoma (RD), breast cancer (MCF-7), ovarian cancer (CaOV-3), normal kidney (MDBK) cell lines. The study showed the entire root extracts except the water extracts gave significant cytotoxic effects on all the cell lines except MDBK (kidney) normal cell lines. Tee and Lope Pihie (2005) tested the anti-tumor effects of *E. longifolia* chromatographic fractions on breast cancer cell lines (MCF7) as they claimed multi composition of herbal medicines were more effective than single compound, probably due to the synergism of the contents. They discovered methanolic extracts showed higher anti-proliferative activity than the aqueous extracts towards MCF7 cells. Three fractions showed high toxicity towards MCF7 and significantly increased apoptosis in MCF 7 cells, but only one fraction was chosen for further purification as the two fractions contained eurycomanone, which have been well studied. Mode of action was elucidated and it was discovered that the anti- apoptotic protein, which is the Bcl-2, was down regulated in the fraction treated MCF7 cells.

Following this result, Tee et al. (2007) elucidated further the mode of action of the fraction. They discovered Bcl-2 protein was reduced followed by the cleavage and activation of caspase-7, which is an executioner protein of apoptosis. Mahfudh and Pihie (2008) also discovered the same mode of action of eurycomanone against cervical cancer HeLa cells. The apoptosis triggered by eurycomanone involved the up-regulation of p53

tumor suppressor protein and the increase of pro - apoptotic Bax protein. Besides that, in the study, eurycomanone also reduced the viability and proliferation against ovarian cancer *cells* (CaOv-3), immortal cell lines (HeLa), liver hepatocellular cells (HepG2), human melanoma cells (HM3KO), breast cancer cells (MCF-7) and less toxic on normal cells (MDBK, Vero). Zakaria et al. (2009) also discovered that apoptosis through the up regulation of p53 and Bax protein, with the down regulation of Bcl-2 protein was the mode of cell death in liver cancer cells (HepG2) treated with eurycomanone. Within four cancer cell lines (HepG2, Hela cells, CaOV3, HM3KO) tested by Zakaria et al. (2009), crude extracts of *E. longifolia* was the most potent towards cancerous liver cell (HepG2) and showed very little toxicity towards normal cells (Chang's liver and WLR-68). Tamoxifen, a standard drug showed great toxicity towards all the cancer cell lines including the normal cells.

Most studies on *E. longifolia* were conducted on the root extracts. However, Miyake et al. (2010) studied the phytochemical property of the stem extracts. Isolates from the stem extracts were evaluated for their anti-cancer activity against human fibrosarcoma (HT-1080) cell lines. Among the isolates, 9, 10-dimethoxycanthin-6-one, 10-hydroxy-9-methoxycanthin-6-one, and dihydroniloticin, showed strong toxicity and an isolate, 14-deacetyleurylene displayed stronger activity than standard drug, the fluorouracil. Wong et al. (2012) studied the effects of eurycomanone on lung cancer cells (A549) proliferation, clonogenic cell growth, expression of lung cancer markers and cancer related genes. The result showed that eurycomanone inhibited lung cancer cells proliferation in a dose dependent manner in a concentration ranging from 5 to 20 µg/mL and the concentration that inhibited 50% cell growth was 5.1 µg/mL. Cisplatin, the standard drug, displayed GI_{50} at 0.58 µg/mL, proving that cisplatin was at least 10 folds more potent than eurycomanone. However, cisplatin comes with adverse effects, thus, eurycomanone at the viable therapeutic concentrations (5-20 µg/mL) could inhibit lung cancer cells proliferation. After the eurycomanone removal, the proliferation activity of the A549 was not fully restored. The irreversible inhibition is good for anti-tumor activity especially if the half-life of the anti-tumor compound in plasma is short. The treatment with eurycomanone also resulted in down regulation of cancer cell growth associated genes, which were the prohibitin and endoplasmic reticulum luminal protein (ERp28). Inhibition of angiogenesis is one of the focuses in cancer research. Al -Salahi et al. (2013) evaluated the potential of partially purified quassinoids (TAF273) in inhibiting

angiogenesis, using *ex vivo* and *in vivo* angiogenesis models. Results demonstrated that the inhibition is probably due to the disturbance of endothelial cells proliferation, differentiation and migration.

The anti-tumor activity shown by *E. longifolia* probably is partly contributed by the free radicals scavenging property of the plant. This is because one of the fundamental step of cancer is the presence of free radicals that are damaging to DNA, leading to cancer. Free radical scavengers can get rid of free radicals, help in preventing and fighting cancer. Purwantiningsih et al. (2011) investigated the free radicals scavenging activity of the standardized ethanolic extracts of *E. longifolia* (TAF-273) related to its total phenolic and flavonoid contents. They discovered free radical scavenging activity increased in correlation with total flavonoid and phenolic contents. However, the activity was lower than gallic acid, which is the standard anti-oxidant or free radical scavenger.

2.3.7 Cytoselectivity

One of the adverse effects for current cancer treatment is that cancer drug is also toxic towards normal cells. To reduce side effects during treatment, anti-tumor medicines should have cytoselective property, which is highly selective towards cancer cells and ineffective towards normal cells. There are several studies reporting the toxicity of *E. longifolia* on normal and cancer cells. Chan and Choo (2002) studied the toxicity of diethyl ether, n- butanol and water fractions of *E. longifolia* that were fed on mice and brine shrimps. The results showed that butanol fraction was the most toxic and eurycomanone was identified as the most toxic compound in the butanol fraction. Structure activity relationship study showed that a C20 type quassinoid, an alpha, beta-unsaturated ketone in ring A, an exomethylene function at C-13 and an oxymethylene bridge connecting C-8 and C-11 of ring C contributed to increased toxicity.

In one of their experiments, Chuen and Pihie (2004) tested the cytoselectivity of semi purified eurycomanone on non-cancerous breast cells (MCF-10A). The results were encouraging as the semi purified eurycomanone is cytoselective where it inhibited the proliferation of cancer cells (MCF7) but not the normal cells. In contrast to the standard drug tamoxifen, the drug was toxic to both normal and cancer cells. Tee and Lope Pihie, (2005) in their studies on *E. longifolia* fraction also discovered that the fraction only toxic on cancerous cells (MCF-10A) and spare the normal cells (MCF-10A) from the toxic

effects. Cytotoxic effects of root extracts (water, methanol, n-butanol, chloroform) against carcinoma (KB), human prostate cancer cell lines (DU-145), human embryo rhabdomyosarcoma (RD), breast cancer cell lines (MCF-7), ovarian cancer *cell lines* (CaOV-3) and normal cell lines (MDBK) was studied by Nurhanan et al. (2005). All the extracts except water showed high cytotoxic effects on the cancer cell lines and not toxic on normal cell lines. Cytoselectivity of eurycomanone on cancerous cells (CaOv-3, HeLa, HepG2, HM3KO, MCF-7) and normal cells was studied by Mahfudh and Pihie (2008). The results revealed that eurycomanone could act as cytoselective agent as it inhibited the proliferation of cancer cells but not normal cells.

Zakaria et al. (2009) compared the cytoselectivity of Tamoxifen and eurycomanone on normal cells (Chang's liver and WLR-68). It was revealed that Tamoxifen was 12 times more toxic on normal cells as it inhibited 50% cells viability at 1.4 ± 0.31 µg/mL while eurycomanone gave IC50 value of 17 ± 0.15 µg/mL. For WLR-68 cells, eurycomanone gave IC50 value of 20 ± 0.22 µg/mL. This finding supports the fact that eurycomanone has less cytotoxic effects on normal cells. Most of the studies above showed that *E. longifolia* compounds were toxic on parasite or cancer cells, but were relatively non-toxic on normal cells and cell host.

2.3.8 Modulatory and Metabolism

Pan et al. (2014) studied the modulatory effects or interaction of eurycomanone on cytochrome P450 (CYP) isoforms, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2E1, CYP3A4. CYP is needed to metabolize enzyme. However, the activity can be disturbed by other compounds, which can result in treatment failure. Results indicated that eurycomanone did not potently inhibit any of the isoforms investigated, with IC_{50} values greater than 250 µg/mL. Low et al. (2005) discovered the same results in their studies on the bioavailability and pharmacokinetics of eurycomanone in rat plasma following oral and intravenous administration. Higher plasma concentration of eurycomanone was detected after intravenous injection than oral administration despite the oral dose was five times higher. They discussed that the poor oral bioavailability was not because pH instability, but due to poor membrane permeability. Low et al. (2011) studied the bioavailability and pharmacokinetic properties of major quassinoids; 13α(21)-epoxyeurycomanone, eurycomanone, 13α,21-dihydroeurycomanone and eurycomanol in

standardized *E. longifolia* extract following oral and intravenous administration for application in antimalarial activity. They discovered 13α(21)-epoxyeurycomanone and eurycomanone were the only quassinoids that were stable upon oral administration.

2.3.9 Anti Inflammatory Effects

Tada et al. (1991) studied the anti-ulcer on two quassinoids (Pasak bumin A and Pasak bumin B) and they were found to show potent anti-ulcer activity. The anti-ulcer property prompted Ang and Cheang (1999) to investigate the *E. longifolia* fraction on rats of another anti-stress related disorder, which was the anxiolytic effect. The studied anxiolytic effects showed positive effect with the increased of square crossed, inhibit fighting behavior, decreased immobility and faecal pellets, increases number of entries and time spent in the elevated plus maze. There was not much difference of the effect between different fractions (chloroform, methanol, water, butanol) of the extracts, however, the effect was consistent with the effects of diazepam, a drug used for the relief of symptoms related to anxiety disorders.

2.3.10 Ergogenic Effects

Traditionally *E. longifolia* has been used to increase health and general wellbeing. The plant is among the popular herbs used to enhance exercise and sports performance (Chen et al., 2012). In 2010, Muhamad et al. (2010) tested the ergogenic effects, which is the effect of increased performance and stamina in high intensity exercise. The ergogenic effects are signified by athletes' endurance running capacity and physiological responses in the heat. In the study, after supplementation with *E. longifolia* capsules for seven days, it was found unfortunately to have no significant difference on the endurance of the running capacity and physiological responses in the heat compared to placebo controlled. They suggested longer duration of supplementation and higher dosage to evaluate further the potential of *E. longifolia* as ergogenic aids.

E. longifolia is mostly used for its aphrodisiac effects, believed to be contributed by its ability in raising testosterone levels. Shuid et al. (2012) found the testosterone raising ability of *E. longifolia* extracts effect mostly on the regeneration of bone resorption in aged man. The main cause of osteoporosis in men is androgen (e.g.

testosterone) deficiency due to natural aging. Serum testosterone levels were measured in orchidectomised rats, fed with *E. longifolia* extracts capsule. It was reported that supplementing the orchidectomised rats with *E. longifolia* extracts elevated the testosterone levels, reduced the bone resorption marker and up-regulated the gene expression of osteoprotegerin, which is a protein that affect bone resorption.

2.4 Aphrodisiac Property of *Eurycoma longifolia*

E. longifolia gained its notoriety as sexual and virility enhancer (Ang and Sim, 1998). However, the efficacy is based on personal experiences, testimonies and more of a subjective opinion rather than verified scientifically. According to Sandroni, 2001, aphrodisiac can be categorized into three groups according to their mode of action; (1) by increasing libido, (2) by increasing potency, (3) by increasing sexual pleasure. Ang and Sim (1998) started the research pertaining to the aphrodisiac property of this plant. They investigated the potential of this plant in increasing sexual motivation of sexually naive male rats where the rats were administered with different fractions (chloroform, water, butanol) of *E. longifolia* extracts. The successful crossover through electric grids in electrical copulation cage was used to measure the motivation of the male rats to find the receptive females. The result reported that repeated and chronic dosing of EL fractions enabled the rats to cross the electrical grids. The number of mounting, intromission and ejaculation also improved. They also discovered that the results did not show much different between fractions, and they claimed it might be due to the presence of active compounds in more than one fraction.

Ang and Cheang (2001) in their investigation on castrated rats discovered that feeding castrated rats with *E. longifolia* fractions (butanol, methanol, water, chloroform) significantly increased the weight of the rat's laevator ani muscle. However, which fraction gave the best stimulation was failed to be determined. In 2001, Ang et al. noticed that *E. longifolia* extracts (butanol, methanol, water, chloroform), in dose dependent manner gave significant increase in the potency activity which were measured by penile reflexes of treated male rats; quick flips, long flips and erections. The effect of *E. longifolia* on sexual qualities in middle age male rats was observed by feeding the rats with 0.5 g/kg of various fractions (chloroform, water, butanol, methanol) of *E. longifolia* for 12 weeks. After treatment, the hesitation time to meet receptive females in electrical

copulation cage were measured (Ang et al., 2003). It was also observed that there was not much difference on the sexual qualities exhibited by the various fractions of *E. longifolia*. The effects of *E. longifolia* on sexual arousal in sexually sluggish male rats were tested in 2004 by Ang et al. (2004a). The act of yawning and stretching indicated sexual arousal. 200, 400, or 800 mg/kg of various fractions (methanol, butanol, chloroform, water) of *E. longifolia* extracts were fed on the rats. The concentration of 800 mg/kg of the extracts gave the best sexual arousal; however, there was not much difference between fractions.

Zanoli et al. (2009) claimed most previous researches on the aphrodisiac effects of *E. longifolia* were carried out on normal rats, except work done by Ang et al. (2004)a on sexually sluggish male rats. Zanoli et al. argued that studies on sexual disorder should be utilized on sluggish and impotent male rats to mimic human sexual problems. Instead of using *E. longifolia* solvent fractions, they used root powder to avoid any solvent interaction in pharmacological effects. In the test, rats were treated with acute, subacute and sub-chronic level of *E. longifolia*. Copulatory performance during mating, sexual motivation, partner preference and testosterone serum levels were measured. Results showed that acute and subacute dose reduced ejaculation latencies, increased the mounting and ejaculating. However, the motivational behavior is not affected by the treatments.

Testosterone is important for male fertility such as normal spermatogenesis. Hormonal disturbances such as excess of estrogen can cause inhibitory effects on testosterone and disturb testicular function. Abdul Wahab et al. (2010) investigated the effect of *E. longifolia* on testicular histology and sperm count in estrogen-treated male rats. They discovered that *E. longifolia* could heightens testicular function and reverse the effect of excessive estrogen as rats treated with *E. longifolia* showed higher sperm count and mortality. Low et al. (2013,a) showed that quassinoid rich extracts could increase sperm concentration and the amount of testicular testosterone. The quassinoid rich extracts contained highest amount of eurycomanone. In another research, Low et al. (2013,b) investigated the mechanism of action of eurycomanone in increasing spermatogenesis and testosterone steroidogenesis. Results showed that purified eurycomanone significantly increased testosterone production dose-dependently and enhanced testosterone steroidegenesis by inhibiting aromatase conversion of testosterone

to oestrogen and at high concentration may also involve phosphodiesterase inhibition. The increase of testosterone will result in improvement of male fertility.

The aphrodisiac property tests of *E. longifolia* mostly were carried out on rodents. Tambi and Imran (2010), conducted a clinical trial on the effect of standardized, water soluble extracts in managing men with idiopathic infertility, namely men with low sperm concentration with or without low percentage motility and morphology of unknown causes. In the trial, several men were given 200 mg daily of the extracts and follow up semen analysis were conducted. The supplementation resulted in increased sperm concentration, better sperm motility and morphology. There were also spontaneous pregnancies.

All of the researches above showed that *E. longifolia* has the potential to treat male sexual problems. Research conducted by Abdulghani et al. (2012) showed that *E. longifolia* extracts has a promising effect in treating female sexual disorders. They studied the ameliorative effects of *E. longifolia* extracts on reproductive disorders in female rats; an irregular estrous cycle and ovarian cystic follicles. The rats were treated with hormone to induce reproductive disorder. After treatment with the extracts, the reversal effects were shown where fewer rats showed the disorders.

Though famous for sexual enhancing effects, in literatures except for one study by Low et al. (2013,b), the compound in *E. longifolia* that is responsible for the property is surprisingly unknown yet. It is generally very difficult to identify the actual ingredient of a herbal medication that is effective for the treatment of ED (Low and Tan, 2007). As such most of the studies are conducted on extracts only (Ang and Sim, 1998; Ang and Cheang, 2001; Ang et al., 2001; Ang et al., 2003; Ang et al. 2004a; Abdul Wahab et al., 2010; Abdulghani et al., 2012), root powder (Zanoli et al., 2009), and quassinoid rich fractions (Low et al., 2013, a). However, Sambandan et al., (2006) and Asiah et al. (2007) identified a bioactive peptide of 4.3 kDa, labelled as eurypeptide, which enhance synthesis of various androgens. The bioactive peptide (4.3 kDa) (patented: PI 20003988, MAL; 10/362697, USA; 01920972.5, EUROPE and 2002-522919, JAPAN) isolated from *E. longifolia* is a potential phytoandrogen, which has been reported to increase the testosterone level in rat Leydig cells. Therefore, it is a scientific and commercial interest to study this peptide further.

2.5 Bioactive Constituents of *Eurycoma longifolia*

There are wide spectrums of pharmacological activity associated with this plant (Kuo et al., 2014). Recent studies mainly focused on its extract or fraction-based bioactivities while exploration of chemical constituents has not been carried out extensively (Park et al., 2014). Studies of chemical constituents will help better understanding of this activity, thus chemical investigation is necessary. According to Chua et al. (2011), more than 85 compounds have been identified from *E. longifolia*. Majority of these metabolites are from the class of quassinoids, squalene derivatives, biphenylneolignans, beta-carboline alkaloids, tirucallane-type triterpenes and canthine-6-one alkaloids.

This section discusses new compounds that were discovered since year 2000 and onwards and Table 2.2 summarizes constituents from *E. longifolia* that have been isolated. In 2000, Ang et al. isolated three novel quassinoids; eurycolactones A, eurycolactones B and eurycolactones C from the chloroform fraction of *E. longifolia* roots. In 2002, Ang et al. isolated eurycolactones D, eurycolactones E and eurycolactones F. Bedir et al. (2003) discovered new quassinoid type glycoside from the roots of *E. longifolia*, where the C (1)-glycosidation site in the quassinoid framework was encountered for the first time. It was found from the roots of *E. longifolia* by Kuo et al. (2004), four quassinoid diterpenoids; eurycomalide A, eurycomalide B, 13β, 21-dihydroxyeurycomanol, and 5α, 14β, 15βtrihydroxyklaineanone. These quassinoids were reported to be isolated from natural resources for the first time. Also from the roots of *E. longifolia*, Teh et al. (2010), isolated a novel quassinoid; 2,3-dehydro-4α-hydroxy-longilactone. The compound is reported to be the first C19 quassinoid from Simaroubaceae family possessing an unsubstituted vinyl function and C4 methyl group of β configuration in ring A. A phenyl propanoid; scopolin, was isolated from this plant for the first time. Miyake et al. (2010), in their phytochemical investigation of the stems of *E. longifolia* isolated a new tirucallane-type triterpenoid; 23,24,25-trihydroxytirucall-7-en-3,6-dione while two new canthin-6-one alkaloids; 4,9-dimethoxycanthin-6-one and 10-hydroxy-11-methoxycanthin-6-one have also been isolated. Meng et al. (2014) isolated four new quassinoids; 14-hydroxyglaucarubol, 5-iso-eurycomadilactone, eurycomadilactone, and 13-epi-eurycomadilactone. Park et al. (2014) isolated five new quassinoids, eurylactone E, eurylactone F, eurylactone G, eurycomalide D, and eurycomalide E.

Table 2.2:
Chemical constituents isolated from *E. longifolia*

No	Compounds	Source
1	eurycolactones A* eurycolactones B* eurycolactones C*	Ang et al. (2000)
2	eurycolactones D* eurycolactones E* eurycolactones F*	Ang et al. (2002)
3	eurycomaoside*	Bedir et al. (2003)
4	eurycomalide A* eurycomalide B* 13β, 21-dihydroxyeurycomanol* 5α, 14β, 15βtrihydroxyklaineanone* b-sitosterol, stigmasterol, scopoletin, p-hydroxybenzaldehyde, laurycolactone B, methylb-carboline 1-carboxylate, 4, 5-dimethoxycanthin-6-one, eurycomalactone . 6-dehydroxylongilactone	Kuo et al. (2004)
4	9-methoxycanthin-6-one, eurylene, syringic aldehyde, canthin-6-one, 6-hydroxy-5, 6-dehydroeurycomalactone, 6a-hydroxyeurycomalactone, 2, 4-dihydroxy-3-methoxyacetophenone, laurycolactone A, 5-methoxycanthin-6-one, 10-methoxycanthin-6-one, sodium syringate, 8-hydroxy-9-methoxycanthin-6-one, 9,10-dimethoxycanthin-6-one, thymidine, fraxidin, n-pentyl b-carboline-1-propionate, 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl) propan-1-one, ariciresinol, longilactone, l 9-hydroxycanthin-6-one, guanosine, 5-hydroxymethylcanthin-6-one, canthin-6-one 3N-oxide, vanillic acid, canthin-6-one 3N-oxide, vanillic acid, 5-hydroxymethyl-9-methoxycanthin-6-one	Kuo et al. (2004)

*new compound

Table 2.2: (continued)
Chemical constituents isolated from *E. longifolia*

No	Compounds	Source
4	14,15b-dihydroxyklaineanone, pasakbumin C, 5,6-dehydroeurycomalactone, adenosine, protocatechuic acid, nicotinic acid, b-sitosteryl glucoside, picrasidine L, 4-hydroxy-5-methoxycanthin-6-one, b-carboline-1-propionic acid, pasakbumin B, 7-methoxyb-carboline-1-propionic acid, sodiump-hydroxybenzoate, syringic acid, erythro-1-C-syringylglycerol, iandonone, erythro-guaiacylglycerol, eurycomanone, threo-guaiacylglycerol, 1-hydroxycanthin-6-one threo-1,2-bis-(4-hydroxy- 3-methoxyphenyl) propane-1, 3-diol, threo-1-Csyringylglycerol, canthin-6-one 9-O-b-glucopyranoside, 9-hydroxycanthin-6-one 3N-oxide, 9-methoxycanthin-6-one 3N-oxide, 1-hydroxy-9-methoxycanthin-6-one	Kuo et al. (2004)
5	23,24,25-trihydroxytirucall-7-en-3,6-dione* 4,9-dimethoxycanthin-6-one* 10-hydroxy-11-methoxycanthin-6-one*	Miyake et al. (2010)
6	2,3-dehydro-4α-hydroxy- longilactone*	Teh et al. (2010)
7	14-hydroxyglaucarubol* 5-iso-eurycomadilactone* eurycomadilactone* 13-epi-eurycomadilactone*	Meng et al. (2014)
8	eurylactone E* eurylactone F* eurylactone G* eurycomalide D* eurycomalide E*	Park et al. (2014)

2.6 Eurycomanone

Eurycomanone currently is the marker set by Malaysian Standard in determining the quality of *E. longifolia* products. This major compound found in *E. longifolia*, is also known as Pasakbumin-A. It is from quassinoid group, with molecular formula of $C_{20}H_{24}O_9$ and molecular weight of 408.399. Figure 2.3 shows the structure of this compound.

Previous studies have noted its pharmacological effects. Chan et al. (2004) and Kuo et al. (2004) discovered that pure eurycomanone compound showed anti malarial properties. Instead of pure compound, Chan et al. (2005) tested the anti malarial properties of semi synthetic forms of eurycomanone; diacylated 1,15-di-O-isovaleryleurycomanone, 1,15-di-O-(3,3-dimethylacryloyl) eurycomanone, 1,15-di-O-benzoyleurycomanone and the monoacylated 15-O-isovaleryleurycomanone. It was discovered that mono acylated, which is the acylation at the C-15 hydroxyl group showed higher activity. Mohd Ridzuan et al. (2007) and Wernsdorfer et al. (2009) respectively showed that standardized extracts containing eurycomanone possessed anti malarial and anti plasmodial properties.

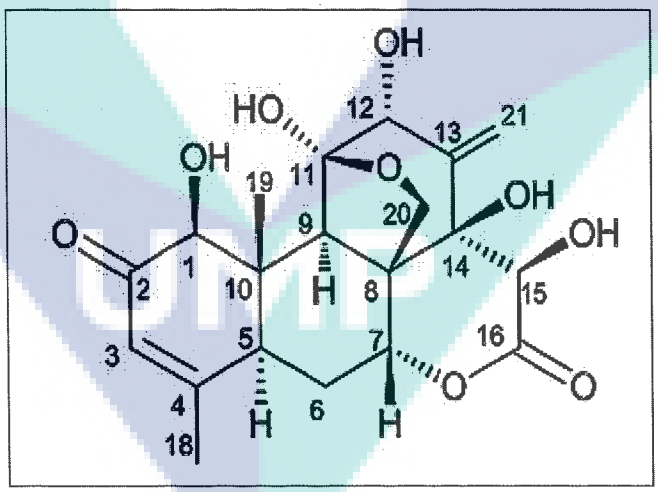

Figure 2.3: Chemical structure of eurycomanone. The basic skeleton consist of three six-atom rings and one lactonic ring.

Source: Wong et al. (2012)

Eurycomanone also showed the ability to suppress cancer cells as shown by Kuo et al. (2004) and Meng et al. (2014) in their studies on breast cancer cells. Wong et al. (2012) showed that eurycomanone inhibited the growth of lung cancer cells in dose dependent manner. Chuen and Lope Pihie studied the effects of semi purified eurycomanone on breast cancer cells, while Tee and Lope Pihie (2005) discovered that the mechanism of inhibition involved the down regulation of Bcl-2, an anti apoptotic protein. In lung cancer cells, the up regulation of p53 and Bax protein was the mode of cell death induced by eurycomanone (Zakaria et al. 2009).

In pharmacokinetics studies, higher plasma concentration of eurycomanone was detected after intravenous injection than oral administration despite the oral dose was five times higher. Low et al. (2005) claimed that poor membrane stability, instead of pH instability contributed to this condition.

Eurycomanone also exerts the ability to induce aphrodisiac effects. Extracts with highest contents of eurycomanone could increase sperm concentration and the amount of testicular testosterone (Low et al. 2013a). Purified eurycomanone was shown to significantly increase testosterone production in a study conducted by (Low et al. 2013b).

2.7 Herbal Industry Overview

Herbal products are categorized as self care and non prescribed, intended and designed for use without the supervision of a medical practitioner (Cranz and Anquez-Traxler, 2014). In terms of economic, the total herbal market sales can be estimated to be around EUR 6 billion (Cranz and Anquez-Traxler, 2014). This is driven by the fact that 70% of world population do not have access to modern medicines. Therefore they rely on traditional treatment including herbal medicines. For example, Ayurvedic, Kampo, Traditional Chines Medicine (TCM) and Unani medicines in India ,Japan, South Asia and Middle East respectively are still used by the majority of people (Pferschy-Wenzig and Bauer, 2015). Since the last few decades, the pharmaceutical industry has focused drug discovery process derived from synthetic and combinatorial chemistry compounds that are more compatible with high throughput screening. However, a remarkably high number of new drug entities are still based of natural products. From the drugs that were newly approved between 1981 and 2010, only 36% were of synthetic

origin, while more than half were inspired by or derived from natural products (Wang and Yu, 2015).

Good manufacturing practices (GMP) are one of the most important tools to ensure that the manufacturing process is carried out by meeting the prescribed standards, quality control measures are properly adopted, so that the finished products are of acceptable quality. However, laws, regulation and guidelines stipulating requirement of GMP of herbal products differ worldwide. For instance, GMP established by EU applies only to pan European regions. In the US, federal laws govern the manufacturing of herbal products as dietary supplements. In China, herbal products consumed according to the Chinese medicine theory are considered as Traditional Chinese Medicines (TCM) and the manufacturing process is regulated as conventional medicines (He et al. 2015).

Rich in genetic resources, good climate and indigenous knowledge has enabled Malaysia to have big potential size in herbal based market (Ibrahim, 2006). Rural herbal entrepreneurship has become another economic growth factor in Malaysia (Murray, 2011). However, herbal industry in Malaysia is facing numerous problems such as skilled manpower, limited cultivation activities, domestic grade without scientific value reports (Paul et al. 2013). For example, in survey conducted by Paul et al. (2013), Malay ethnic groups are lagging behind to other ethnic groups in Malaysia due to poor networking skills in marketing their products.

In Malaysia, The DCA (Drug Control Authority), implemented the phase three registration of traditional medicines on 1 January 1992. The registration criteria for any traditional medicines in Malaysia are limits for heavy metals such as lead, mercury, arsenic, and cadmium, limits of microbial contamination and the absence of steroids and other adulterants, limits of disintegration time, claimed indications, prohibition of herbs with known adverse effects, prohibition of endangered animal species (compliance to both GMP (Good Manufacturing Practice) and GSP (Good Storage Practice) (Ang and Lee, 2006).

2.8 *Eurycoma longifolia* Nutraceutical Products

Natural aphrodisiacs are booming business (Venhuis et al., 2008). The fact that these remedies are easy to obtain and inexpensive compared with prescribed medicines has spawned this profitable industry (Hassali et al., 2012). Aphrodisiac property of *E. longifolia* is contributed by its testosterone enhancing effects. Instead of containing testosterone itself, *E. longifolia* works by promoting the testes to increase the production of testosterone naturally. Current androgen replacement therapy applies supplementation of testosterone gel, injection or pellet. The continuous supplementation can result in the stopping of body natural production system, thus, the natural testosterone promoting effect of *E. longifolia* is much favored treatment. Interestingly *E. longifolia* has been referred to as Malaysia's "homegrown Viagra" (Talbott et al., 2013).

Within the rapidly blooming phytochemical industry in Malaysia, *E. longifolia* has been one of the most important herbs and with high commercial value (Athimulam et al., 2006; Kuan et al., 2007). It has become a potential cash crop which can be developed as a product with lucrative values (Kumaresan and Sarmidi, 2003). In modern dietary supplements, *E. longifolia* can be found in a variety of products intended to improve libido and energy, restore hormonal balance (cortisol/testosterone levels) and enhance both sports performance and weight loss (Talbott et al., 2013). Currently, there are lots of products with *E. longifolia* formulations have been sold in the health food market.

The products can come in the form of raw crude powder, where dried roots are grinded without processed further. It also is prepared in the form of standardized extracts where freeze or spray-dried water extracts are adjusted to defined content of bioactive constituents. In the market, the products can be found in the form of capsules, pill, liquid formulation, pre-mixed coffee and even canned processed drinks (Mohd Effendy et al., 2012). Figure 2.4 shows the examples of *E. longifolia* products available in the market.

The standardized water soluble extracts has been patented in 2004 (US2004/0087493 A1) after having gone extensive animal and human clinical evaluation (Tambi, 2009). The dried roots are priced somewhere between 20 to 25 US dollars/kg while the extracts can have a market value of 26 US dollars per bottle of 60 capsules (Bhat and Karim, 2010).

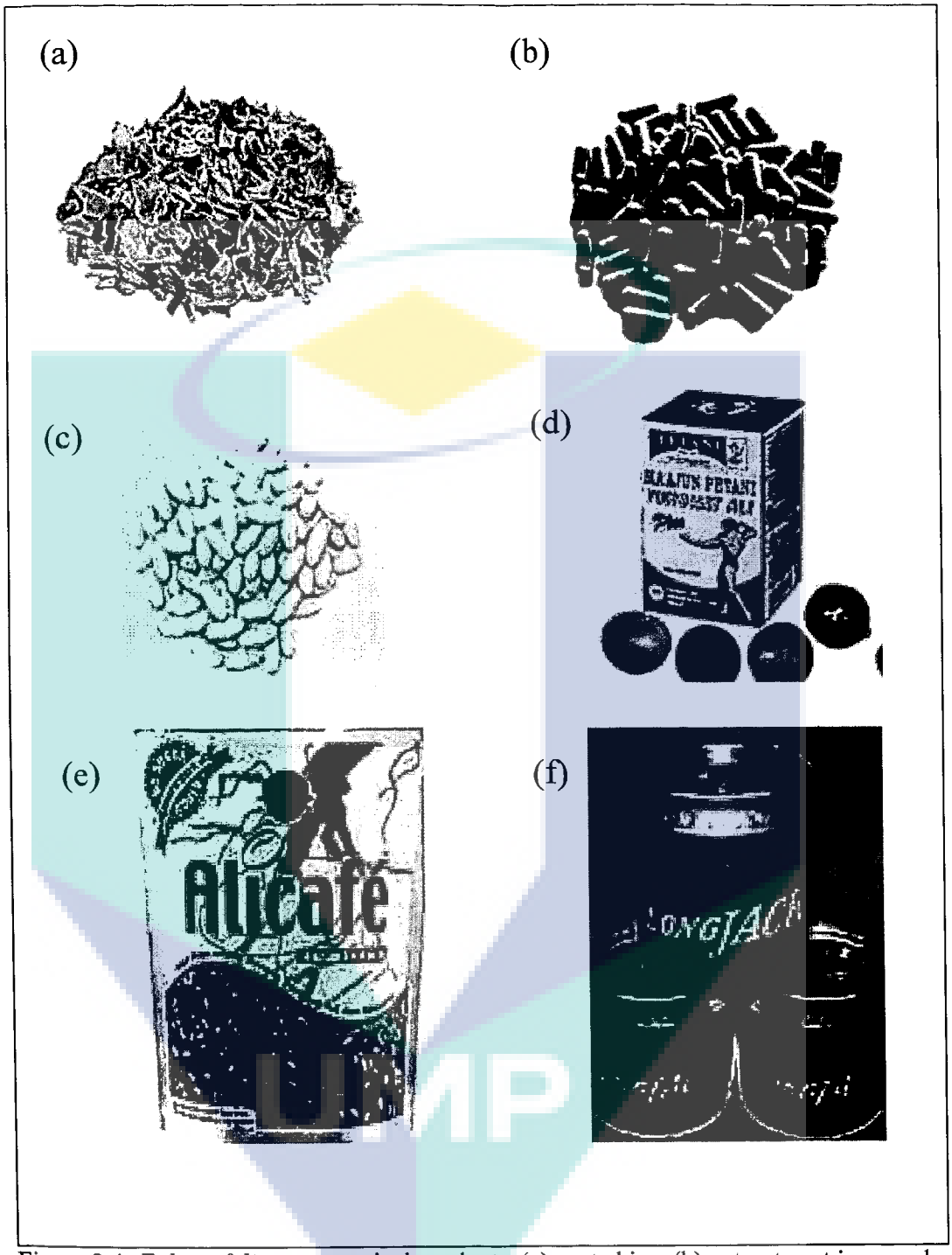

Figure 2.4: *E. longifolia* nutraceutical products. (a) root chips, (b) extracts put in capsule, (c) extracts in tablet form, (d) maajun (e), premixed coffee (f), canned drinks.

2.9 *Eurycoma longifolia* Extraction Method

Herbal product industry is blooming. However, the main pitfall of this industry is the production of the products mainly is based on traditional methods which may lead to high losses and low yield. To overcome the problem, processing method need to be optimized. The most important ingredient in herbal products is the bioactive compound, thus, phytochemical extracts processing method must be developed to achieve maximum yield of bioactive contents. Kumaresan and Sarmidi (2003), studied the effects of processing on the yield of *E. longifolia* water extracts. Particle size, extraction time, solvent ratio were the processing parameters. The results showed that extraction time and solvent ratio need to be increased to produce high yield, while the effect of particle size on the yield was inconclusive. Mohamad et al. (2013) tested several extraction parameters to give higher yield of eurycomanone and maximum yield was achieved at 100 °C, smaller particle size, agitation speed of 400 rpm, solvent to solid ratio of 20 to 1.

Athimulam et al. (2006) modelled and optimized an economically viable water extract production for *E. longifolia* products, with the use of computer aided process design (CAPD) and simulation tools based on existing pilot scale manufacturing setup. Four alternative production schemes were further developed with several debottlenecking and optimization strategies. Spray drying process was recognized as bottleneck in the manufacturing process, thus the drying operation time was reduced as the debottlenecking scheme.

Besides optimizing yield of the product processing, the impact on the environment should be considered. The overall process- yield of *E. longifolia* products was estimated to be 3% of the raw material feed by weight. Significant fibrous residues were generated which pose disposal problems. Kuan et al. (2007) conducted a streamlined lifecycle assessment for the analysis on the environmental impacts on the processing of *E. longifolia* products based on the utilization of fibrous residue from water extracts production. The result showed that using the residue as process fuel was the most environmental friendly option where it produced the least emissions and reduced resources usage per unit. Based on the works done by Athimulam et al. (2006), Kuan et al. (2007) and Tjan et al. (2010) applied graphical technique based on pinch analysis to determine strategies for carbon footprint improvement. The analysis estimated that the

maximum carbon foot print reduction is 1.2 tons, which is 8.8% of the total carbon footprint of the overall process.

2.10 Issues and Solutions Pertaining to *Eurycoma longifolia* Nutraceutical Products

2.10.1 Product Registration

To sell herbal products in Malaysia, the products need to be registered with Drug Control Authority of (DCA) Malaysia. DCA implemented phase three registration of traditional medicines on 1 January 1992. The registration criteria for traditional medicines are limits for heavy metals, limits for microbial contamination and the absence of steroids and other adulterants, limits of disintegration time, claimed indications, prohibition of herbs with known adverse effects, prohibition of endangered animal species and compliance to Good Manufacturing Practice (GMP) (Ang, 2004). However, despite being a prominent growth industry, herbal medicines are not well regulated by DCA (Hassali et al., 2012). Being considered as commodities, herbs are not subjected to strict regulation and people tend to trust natural medicine as they believe it is free of side effects (Venhuis et al., 2008).

Malaysian Standard, an accreditation body in Malaysia, prescribed several required quality of *E. longifolia* commercial extracts. In a document numbered MS2409:2011, the requirements are; minimum amount of eurycomanone from 0.80-1.50 (% w/v), total polysaccharides content of more than 30 (% w/v), total protein contents of more than 20 (% w/v), total glycosaponin content of more than 40 (% w/v). The use of this standard is voluntary rather than been made compulsory by regulatory authorities.

2.10.2 Concentration of Major Phytochemicals

Usually in the market, the concentration of *E. longifolia* in a product is declared as x:y which means x gram of *E. longifolia* is used to produce y gram of extract. However, this declaration cannot be verified (Talbott et al., 2013). There is a need to have a marker to determine the quality of *E. longifolia* products. At present, the products are standardized to eurycomanone, a quassinoid which is the primary component presents in the extracts (Kumaresan and Sarmidi, 2003). However, due to varying planting conditions

such as geographical factor, weather, age during harvesting, the concentration of required phytochemicals might vary quantitatively and not meet the standard required of a product (Kumaresan and Sarmidi, 2003; Low and Tan, 2007; Bhat and Karim, 2010). Quality control for herbal products is more difficult than drugs analysis as herbal products contain large range of active compounds. It will also cause inconsistency of the products between batch to batches. For standardization purpose, consistency of the phytochemicals needs to be ensured.

2.10.3 Contamination

There are external and internal quality issues of herbal medicines. External issues include contamination such as by toxic metals, pesticides, microbes, adulteration with undeclared drugs and misidentification of herbs. Internal issues include complexity and non-uniformity of the ingredients (Zhang et al., 2012). Most of the quality issues of commercial *E. longifolia* products are contamination with heavy metals (Mohd Said et al., 2014). This is of utmost important as the major ingredients of *E. longifolia* products is the root which readily absorbs all toxic ingredients from the soil (Bhat and Karim, 2010). Ang et al. (2004b) using cold vapor atomic absorption spectrophotometer in their screening of 100 products containing *E. longifolia*, discovered that 36% of the products contained between 0.52-5.30 ppm of mercury and the amount do not comply with requirement for traditional medicines in Malaysia whereby mercury content should not exceed 0.5 ppm. If herbal products are not dried properly during preparations, mycotoxins contamination can occur. Ali et al. (2005) determined aflatoxins content using immunoaffinity column (IAC) clean-up and HPLC with trifluoroacetic acid pre-column derivatization and detection by fluorescence detector, which is more accurate and selective than thin layer chromatography (TLC). They modified and evaluated the method to separate aflatoxins from matrices of other chemicals in the samples and found out their method was reliable and selective in determining aflatoxin even at low detection limit in complex matrix of mixed herbs.

2.10.4 Misidentification

Misidentification of one herb with another herb is another external issue. This false authentication usually occurs with incorrect labeling and similar appearance of herbal materials (Zhang et al., 2012). *E. longifolia* has a unique morphology with its long, straight root, so the problem of misidentification with other herbs probably is unlikely.

2.10.5 Adulteration

While contamination can occur unintentionally, adulteration is deliberately done by the manufacturer (Zhang et al., 2012) for cost saving measure. Consumers often are deceived by adding undeclared drugs that is used for erectile dysfunction problem such as sildenafil, vardenafil, tadalafil. Some products even contain none of the actual root, and instead being "spiked" with prescription drugs (Talbott et al., 2013). These drugs selectively inhibit the PDE5 enzyme thus raising cyclic guanoside monophosphate (cGMP) levels causing vasodilating effect. However, these drugs have bad side effects especially for patients with coronary problems, or patients taking antihypertensive drugs that might prolong the half-life of sildenafil (Sandroni, 2001). Bogusz et al. (2006) analyzed an *E. longifolia* product and discovered the product contained sildenafil, an erectile dysfunction treatment drug. Using HPLC-DAD and HPLC–MS, Venhuis et al. (2012) investigated an *E. longifolia* product that was suspected to be adulterated with drug substance. The results are interesting where the capsule content did not show traces of adulterant, but the capsule shell was found to content tadalafil. They used microscopy and RAMAN spectroscopy and the presence of tadalafil was shown throughout the gelatin matrix as particles and dissolved into the matrix. Mohd Said et al. (2014) developed rapid detection of sildenafil analogue in *E. longifolia* products using a new two-tier procedure of the near infrared (NIR) spectra database where this method alleviated the use of reference product or active standard for direct comparison.

2.10.6 Toxicity

E. longifolia products in the market sometimes are mixed with other herbs to improve general wellbeing rather than sexual prowess alone. However, mixing these herbs together might change the cytotoxicity status due to combination of active metabolites. It was in late 1990s that researchers started to pay more attention on its safe

dosage and toxicity profile (Mohd Effendy et al., 2012). Abd Razak et al. (2007) studied the mutagenic effects on the combination of *E. longifolia* and other two herbs; *T. integrifolia*, and *Helmintostachys zeylanica*. They concluded that there is higher toxicity risk if these herbs are consumed together. Razak and Aidoo, (2011) studied the toxicity and mutagenicity of three types of *E. longifolia* products in the market; products containing mixture of *E. longifolia* with other herbs, product which contains only *E. longifolia* and an authenticated *E. longifolia*. They discovered, all extracts, except one product which was mixture of *E. longifolia* and another herb was not mutagenic and they concluded there is a risk of increased cytotoxicity and mutagenicity of extract of *E. longifolia*-based remedies compared to the toxicity of remedies containing solely *E. longifolia*.

Wiart (2012) in a letter to the editors of a publication, claimed that based on several publications that he read, *E. longifolia* can have adverse effects on long term consumption such as cancer, diabetes, obesity, depression and aggressivity, fatal pulmonary hemorrhages, breathing problems, and seizures. He claimed that the effects are caused by raised testosterone level, a well-known effect of *E. longifolia* treatment. It has to be noted here that his readings are not based on testosterone raised by *E. longifolia*, but related to the alarming increased in testosterone level due to synthetic testosterone supplementation. *E. longifolia* does not work by increasing the amount of testosterone directly as it does not contain testosterone, but instead regulates the *de novo* synthesis of testosterone in the body. Chan, (2012) responded to this letter by claiming root aqueous extracts of *E. longifolia* contain very low to negligible concentrations of the bioactive chemical constituents. Thus the toxicity effects from sustained increased levels of testosterone upon oral consumption are overrated and can create a negative impact and suppress the potential benefit of the plant.

2.10.7 Counterfeit Products

Some of the *E. longifolia* herbal products are counterfeit products, which do not even contain any bioactive compounds of *E. longifolia*, so the ability to determine the presence of active ingredients will be useful. Abdul Rahman et al. (2004) developed microcontroller based electronic taste sensing system that is capable of discriminating between liquid samples containing *E. longifolia* and those that do not. An embedded microcontroller controls the overall system, using specially fabricated disposable screen-

printed array of non-specific lipid-membrane. They achieved 100% recognition rate in all samples tested and claimed the system is robust, reliable, flexible and easily applied for other herbal samples.

2.10.8 Phytochemical Contents

As varying planting conditions cause difference in phytochemical contents, Choo and Chan (2002) developed a reversed phase high performance liquid chromatography (HPLC) method with a photodiode array detector to determine three major alkaloids; 9-methoxycanthin-6-one, 3-methylcanthin-5,6-dione and its 9-methoxy analogue in *E. longifolia* obtained from different sources. Besides geographical factor, concentration of metabolites is dependent on the processing temperature. To detect small metabolites, high sensitivity and high mass accuracy tandem mass spectrometer is required to produce highly reliable data. Chua et al. (2011) used LCMS to profile metabolites (quassinoids, alkaloids, triterpene and biphenylneolignans) of *E. longifolia* collected from the state of Perak and Pahang in Malaysia that were extracted at different temperatures (35 °C and 100 °C). Using three LC–MS/MS hybrid systems (QTof, TripleTof and QTrap), quassinoids particularly eurycomanone and its derivatives were detected to be at highest concentration than other metabolites. However, the concentration of canthin-6-one and β-carboline alkaloids was significantly increased when the roots of the plant samples were extracted at 100°C. Extracts that are prepared at 35 °C and 100 °C could be differentiated by a protein; leucine (m/z 679) and a new hydroxyl methyl β-carboline propionic acid respectively. 16-α-o-methylneoquassin could only be detected in the room temperature extracts in small amount. In terms of geographical factor, 3,4ε -dihydroeurycomanone and eurylene could only be detected in the Pahang extracts, while canthin-6-one-3N-oxide could only be detected in the Perak extracts. Concentration of longilactone, chaparrinone, 3,4ε -dihydrochaparrinone and canthin-6-one in the Pahang extracts was significantly higher than Perak extracts at both temperatures.

Quassinoids analysis usually are carried out using LC method using UV, photodiode array or fluorescence detections. However, these methods were not sensitive to detect non-chromophoric constituents; eurycomanol and eurycomanol-2-O-β-D-glucopyranoside that are present in *E. longifolia*. Teh et al. (2011) developed and validated a HPLC method using electrospray ionization (ESI) and atmospheric pressure

chemical (APCI) in positive and negative ion modes for the simultaneous determination of five quassinoid markers; eurycomanone, 13α(21)-epoxyeurycomanone, eurycomanol, eurycomanol-2-O-β-dglucopyranoside, and 13,21-dihydroeurycomanone for standardization of manufactured batches of *E. longifolia* extracts as anti-malarial medicaments. The results showed these product batches varied in range of each constituent concentration probably due to the age of harvesting and growing conditions. In comparing ESI and APCI, positive ion ESI provided the highest response for the test compounds and the use of methanol–water (9:91, v/v) as mobile phase was preferred rather than acetonitrile–water (4:96, v/v).

Volatile compounds will give herbs their own characteristic smell. Usually volatile compounds are analyzed using gas chromatography mass spectrometry (GCMS), which is expensive for routine analysis. Human sensory analysis is limited to subjectivity and sensitivity of the human panels. As alternatives, Shafiqul Islam et al. (2006) used electronic nose, which is simpler and cheaper than GCMS. This electronic nose consisted of chemical imaging and multi-parameter sensing systems. They used lipids and gas chromatography stationary phase materials with different polarities as sensing membranes and developed a quartz crystal microbalance smell sensor array and the volatile compounds were analyzed by the smell sensor and GCMS. The volatile vapors present in the headspace interact with the array sensor and produce a chemical fingerprint or pattern characteristic to the vapor. Based on the analysis, 133 volatile compounds were found, freeze dried extract was found to contain maximum number of 83 compounds while spray dried contained maximum of 28 compounds.

2.10.9 Depletion of *E. longifolia*

In the phytochemical based industry, the raw materials are heavily collected from natural forest. The exploitation can result in the extinction of a plant species. Plucking the leaves, pruning the stems may leave a plant to remain alive, but possessing the roots require the uprooting of the root from the soil which can kill a tree. For *E. longifolia* plant, the root is the most sought part as it contains many bioactive compounds. The indiscriminate practice of uprooting the roots from the habitat can result in depleting natural resources. Moreover, *E. longifolia* is a plant with late maturity, less flowering and bears little fruit, so, the use of seedling are the main mode of plantings. Since this plant

is a valuable resource and has the potential in pharmaceutical industry, there is a need to ensure an adequate supply and sustainability of this plant.

To support conservation of this species, suitable genetic marker would be useful. Osman et al. (2003) assessed genetic variation within and between populations of *E. longifolia*. To investigate the genetic diversity, they applied a genome complexity reduction strategy to identify a series of single nucleotide polymorphisms (SNPs) within the genomes of several *E. longifolia* accessions. That was due to the property of SNPs which were more abundant in the genome and are much more stably inherited. They discovered SNPs could differentiate the plants from different natural populations as SNPs reflect the geographic origins of individual plants. Tissue culture technology through somatic embryogenesis can play important role in preserving plant species. However, the problem with somatic embryogenesis is the quality of the embryo where the developed embryos mostly are asynchronous and malformed (Hussein et al., 2006). In selecting which cultures should be optimized for further regeneration, Hussein et al. (2006) used biochemical marker for early identification of embryogenic cultures. Based on the result, peroxidase, an isozyme was recognized as marker that was closely associated with the regeneration capability of *E. longifolia*.

The main mode of planting of *E. longifolia* is through the plantlet where it is time consuming. Rapid propagation is useful as demand for *E. longifolia* is increasing. Hassan et al. (2012) successfully propagated *E. longifolia* via tissue culture method. They compared the production of *E. longifolia* compounds in roots of tissue culture plantlets and wild plants. Marker compounds from *E. longifolia*, eurycomanone, 9-methoxycanthin-6-one and canthin-6-one compounds were detected in roots of tissue culture plantlets. Rosli et al. (2009) optimized the medium composition of tissue culture to improve the production of 9-methoxycanthin-6-one, which is a potential anti-tumor compound. Medium at pH 5.5, addition of 2% fructose as carbon source, 2.0 mg L^{-1} dicamba and 1×10^{-1} μM phenylalanine resulted in better yield of 9-methoxycanthin-6-one. Iriawati et al. (2014) analyzed the presence of secondary metabolite in the somatic embryo of *E. longifolia*. Results showed that embryogenic callus could produce secondary metabolites, but the amount was lower than non embryogenic callus. Since *E. longifolia* is harvested after 4 to 7 years of cultivation, which is time consuming, phytochemical industry cannot depend on traditional cultivation method. Lulu et al. (2014) optimized the conditions for the production of adventitious roots from *E.*

longifolia in bioreactor culture. Results indicated that compared with cell culture, adventitious root culture is more stable in physicochemical conditions and it facilitates stable secondary metabolite production.

2.10.10 Safety and Efficacy

The popularity of *E. longifolia* based products in herbal or drug form is growing tremendously due to wide spectrum of its pharmacological properties. To ensure the safety and efficacy of those products, the mechanism of adsorption and distribution of the pharmaceutical compounds need to be studied. Tan et al. (2002) developed HPLC analysis of 9-methoxycanthin-6-one, an active compound of *E. longifolia* in rat and human plasma. The result showed that the compound was better absorbed if administered intravenously than orally. Low et al. (2005) discovered the same result in their studies on the bioavailability and pharmacokinetics of eurycomanone in rat plasma following oral and intravenous administration. Higher plasma concentration of eurycomanone was detected after intravenous injection than oral administration despite the oral dose was 5 times higher. They discussed that the poor oral bioavailability was not because pH instability, but due to poor membrane permeability. Low et al. (2011) studied the bioavailability and pharmacokinetic properties of major quassinoids; 13α(21)-epoxyeurycomanone, eurycomanone, 13α,21-dihydroeurycomanone and eurycomanol in standardized *E. longifolia* extracts following oral and intravenous administration for application in antimalarial activity. They discovered 13α(21)-epoxyeurycomanone and eurycomanone were the only quassinoids that were stable upon oral administration.

While other researches utilized quassinoid as the marker to authenticate the quality of *E. longifolia* products, Vejayan et al. (2013) utilized protein as the marker. They detected two distinctive protein markers using 2 dimensional gel electrophoresis (2DE) in a standardized extracts and in several *E. longifolia* products sold in Malaysia. The marker was not detected in selected herbal products devoid of *E. longifolia*.

2.11 Herbal Products Standardization and Marker Development

Difficulty arrived in determining the quality of the herbal products as plants are rich in phytochemicals and herbal remedies cannot be prescribed to patients as they are

no standardization (Heng et al. 2013). In this regard, standardization is needed in order for medicinal plants and the products to be more trustworthy and increase greater securities for consumers.

Standardization can be defined as the requirement to have either a minimum amount, or a range of one or more compounds, in the herbal medicine. As the amount of active constituents in plants can be influenced by various factors, the standardization of herbal medicine should address this problem. The defined amount of the constituent can be determined by calculating and blending the amount from different batches of herbal medicinal products preparation (Charoonratana et al. 2014). The quantity of this compound can be an indicator of the quality of a herbal medicine and can be used during authentication of genuine species, standardization and safety assessment of herbal medicines.

The defined phytochemical can be called as marker compound. According to Li et al (2008), European Medicines Agency (EMEA) categorizes the chemical marker into analytical marker and active marker. Active marker is a compound that contributes towards therapeutic activities while analytical marker acts for analytical purpose only. However, Srinivasan (2006), proposed four categories; active principles, active marker, analytical marker and negative marker. Active principles have known clinical activities, active marker is the compound that contribute towards efficacy, analytical marker has no known efficacy, but serves for analytical purpose while negative marker can indicate toxic properties. Selection of chemical markers is crucial as these chemical markers may lead to discoveries of new drugs (Li et al. 2008).

For *E. longifolia*, eurycomanone is considered as the main marker compound (Pan et al. 2014). Eurycomanone is the marker compound in *E. longifolia* because it is the highest amount of constituent in this plant. However, it is not known which category this marker falls. This is because until now, there is only one publication by Low et al. (2013b) that showed the efficacy of eurycomanone.

The detection of this marker compounds will require the samples to be brought to laboratory and analyzed by equipment. For examples, researches done by Chen et al. (2009), Sun and Chen, (2011) and Charoonratana et al. (2014), utilized liquid chromatography in determining the marker compounds. The detection and determination

of eurycomanone in *E. longifolia* also require the use of liquid chromatography (MS 2409:2011).

In this study, the idea to develop proteomic marker emerged after the work of Vejayan et al. (2013). They detected several proteins that tentatively could be marker towards *E. longifolia* products. Once isolated into homogeneity, the marker then injected into a host animal and antibody will be generated. This monoclonal antibody can be incorporated into biosensor.

Biosensors utilization has the advantage of rapid, real time, sensitive, and the potential to be used as point of care (POC) tests and devices (Cork et al. 2013). If biosensor, a device which combines biological and electrical component to generate measurable signal is used as analytical device, the 'dip and read' approach can be taken, where the biosensors are field-ready and samples can be analyzed on site. Thus quality control can be made easier, without the hassle of samples to be brought to the laboratory, skipping the use of analytical expertise and expensive equipment.

Proteins are remarkable in their ability to form various tertiary structures such as antibodies. These antibodies contain highly specific complimentary determining regions for recognition of various antigens, which provide high affinity and specificity to particular analytes (Dover et al. 2009). As a result, proteins have been explored as ideal probe molecules for biosensors. Proteins also have certain advantages such as high stability, easy modification and high stability (Liu et al. 2015). However, proteins generally do not generate measurable signal directly in response to a binding event, therefore, conjugation with a signal marker is needed to convert the information from the binding of antibody and antigen to be measurable. The biggest disadvantage of antibody based biosensor is the high cost and significant time in developing the antibody (Wu et al. 2011).

2.12 Proteomic Studies of *Eurycoma longifolia*

E. longifolia has a good reputation as sexual enhancer and various scientific literatures have documented the aphrodisiac activities of this plant. However, no chemical constituents have been identified as the compound that is responsible for this property except a work by Low et al. (2013,b), that indicated that eurycomanone from *E. longifolia* could enhance spermatogenesis. This study by Low et al. (2013,b) is recent but strangely

though it is difficult to comprehend this finding convincingly especially when it has been easy to isolate eurycomanone much earlier to be verified for this quality. Most of the other reported studies of aphrodisiac effects were tested on the *E. longifolia* water extracts instead of isolated compounds, mimicking the traditional method where decoction of the water extracts is consumed. To complicate matter, a research collaboration (Sambandan et al. 2006, United States Patent 2006:7132117 B2) between Massachusetts Institute of Technology (MIT) and Forest Research Institute of Malaysia (FRIM) indicated that compound responsible for increasing sexual power is a peptide in a bioactive fraction of water extracts. The water extracts undergo preparative HPLC and size exclusion chromatography to yield a bioactive fraction containing peptide. The 4.3 kDa peptide is called eurypeptide (Sambandan et al. 2006). The protein will activate the CYP17 enzyme (17 alphahydroxylase and 17, 20 lyase) and stimulate dihydroepiandosterone (DHEA) that will act on androgen receptors to initiate the conversion of androstenedione to testosterone. This peptide is highly water soluble and lots of human trials have used the same standardized water extracts (Talbott et al., 2013). Alas, the confusion of the actual active compounds exerting the aphrodisiac activity continues on and remains perplexing.

Furthermore, based on the claims of Asiah et al. (2007) which used Surface Enhance Laser Desorption Ionization Mass Spectrometer (SELDI-MS), aphrodisiac plant was found with 4.3 kDa peptide. Peak of 4.3 kDa were detected by this group in *E. longifolia, Smilax myosotiflora, Rafflesia* sp and *Labisa pumila*. However, the peak was not detected in *Tinospora catappa* even though the plant has been reported to have aphrodisiac activity scientifically. In *E. longifolia*, they detected the peptide in most part of the plant including root, bark, callus and plantlet from tissue culture.

Vejayan et al. (2013) took proteomic approach in determining the quality of the products. From standardized water extracts, using 2 dimensional gel electrophoresis (2DE), two distinctive protein markers were detected. The markers were spots found approximately at molecular weight of 14 kDa at the basic extremities and the protein markers were not detected in products devoid of *E. longifolia*. In this study, the products were categorized as Drug Control Authority (DCA) approved and non-approved. However, no comparison was done between the protein marker and the chemical marker, eurycomanone.

E. longifolia in the wild is disappearing gradually due to indiscriminate harvesting. Tissue culture technology can help overcome this problem. However, somatic embryo often develops asynchronously, and their shapes vary widely with many morphological abnormalities. Biochemical marker will be useful for early identification and selection of somatic embryogenesis in plants and help optimizing the culturing condition. Hussein et al. (2006) used protein as biochemical marker in discriminating cultures to follow the multiplication process. In non embryogenic callus, two protein bands were observed at the Rf of 0.24 and 0.27 while in embryogenic callus, four bands with high intensity were detected at the Rf of 0.24, 0.41, 0.49 and 0.81. However, this protein marker was not utilized for aphrodisiac purpose or ever been characterized mass spectrometrically.

The research of the protein in this plant will be an interesting area to be ventured. The protein characterization may shed a few information. The discovery and development of the protein biomarker will allow fast and reliable screening procedure for aphrodisiac activity in plants (Asiah et al., 2007). Hence, the eventual biosensor developed may be useful in determining the quality of *E. longifolia* products rapidly and reliably. This will enable authentication of many *E. longifolia* products in the market.

2.13 Proteomic Technology

Proteomics deal with the study of proteins and it is typically preceded by electrophoresis process. Protein study includes separation, identification, quantification and sequence analysis of protein.

2.13.1 Protein

Protein is a sequence of amino acids. Protein structure ranges from tens to thousands of amino acid residue and protein with less than 50 amino acids is called a peptide (Berg et al. 2002). Molecular mass of protein is expressed as daltons (Da). 1 Da is equal to 1/12 mass of carbon 12. The polyprotein chain has two ends; the N terminus and C terminus. N terminus is the start of amino acid chain with a free amino group (-NH_2) while C terminus is the end of amino acid chain with a free carboxyl (-COOH) group. Figure 2.5 shows the N terminal and C terminal in an amino acid chain. Protein has different levels of structure; primary, secondary, tertiary and quaternary structure.

Primary structure is the linear sequence of amino acid in polyprotein chain. The secondary structure is defined by the pattern of protein hydrogen bond and it is consisted of beta strand and alpha helix. Tertiary structure is the three dimensional structure of the protein. Quaternary structure is combination of more than one protein chain. Figure 2.6 shows the four structures. Sometimes protein can have carbohydrate or sugar moiety attached to its chain. The attachment process is called glycosylation and there are two types of glycosylation; N-Glycosylation where sugar is attached at amide nitrogen atom of amino acid and O- Glycosylation where sugar is attached at oxygen atom of amino acid. About 40% proteins applied in therapeutics are glycosylated (Spiro, 2002).

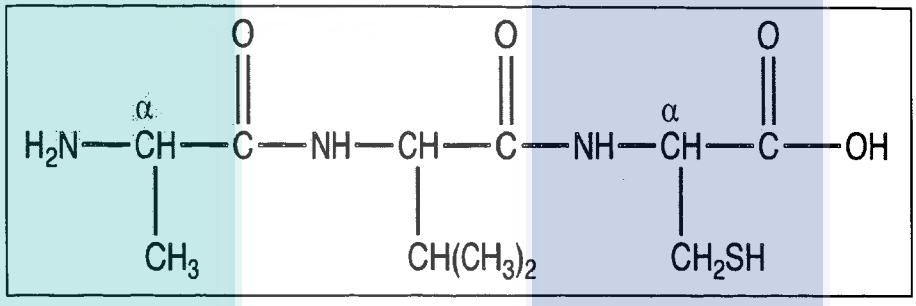

Figure 2.5: N terminal (-NH2) and C terminal (-COOH) in an amino acid chain Source: www.ochempal.org/index.php/alphabetical/m-n/n-terminal/

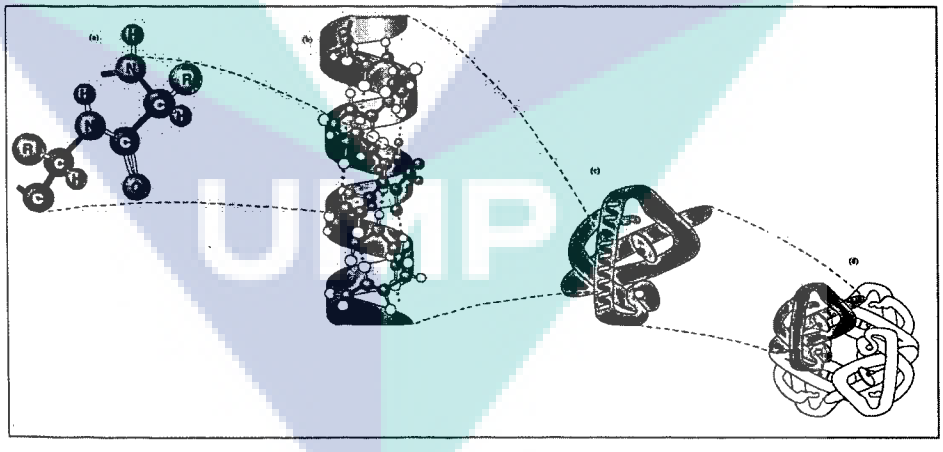

Figure 2.6: Four structures of protein. Primary structure (a), sequence of amino acid linked together by a protein bond, forming a polyprotein. Secondary structure (b), which is folded version of linear polyprotein. Tertiary structure (c), which is several secondary structure folded together. Quaternary structure (d), where several tertiary structures folded together.

Source: www.mun.ca/biology/desmid/brian/BIOL2060/BIOL2060-03/CB03.html

2.13.2 Protein Quantification- Bradford Assay

Bradford protein assay is one of simple colorimetric method to determine total protein concentration in a sample. Bradford assay is recommended for general use, especially in assessing protein concentration for gel electrophoresis (Caprette, 1995). In the method, Coomassie Brilliant Blue G-250 dye will bind to protein. When binding to protein occur in acidic solution, the absorbance of the dye shifts from 465 to 595 nm, causing visible color change. The color will change from red to blue. The higher the protein concentration, the more intense will be the color of the test reaction.

2.13.3 Protein Purification

For a detail functional and structural characterization of a protein, isolation or purification of the protein is an essential first task (Camper and Viola, 2009). The purification of protein from a protein mixture involves well defined chromatography techniques. In size exclusion chromatography (SEC), as the name implies, protein will be separated according to molecular size. In this form of chromatography, samples with various protein sizes are passed through porous particle in a column. Smaller size protein will move slower as it penetrates deep into the pores. While larger protein does not enter the pores. There are two types of SEC; gel permeation chromatography which utilizes aqueous mobile phase and hydrophilic stationary phase and gel filtration chromatography which utilizes non aqueous mobile phase and hydrophobic stationary phase. This form of chromatography is commonly used in semi preparative step (Hong et al, 2012).

In ion exchange chromatography (IEX), protein is separated based on its affinity to ion exchanger. There are two forms of IEX; cation exchange and anion exchange. Cation exchange has negatively charged functional group and will bind to positively charged protein. Anion exchange has positively charged functional group and will bind to negatively charged protein. Separation is optimized by adjusting the pH of buffer and salt concentration (Moustafa and Morsi, 2013).

After ion exchange chromatography, and before protein can be analyzed in subsequent step, normally protein will undergo buffer exchange and removal of salt buffer that might interfere in next analysis such as gel electrophoresis. This process can be carried out by dialysis, gel filtration and diafiltration. In dialysis, sample is put in a

membrane bag of defined porosity, and placing the bag in a buffer. Large molecule that cannot pass through the pore will be retained in the bag. However, this method is quiet slow. In gel filtration, the principle is the same as size exclusion chromatography. Gel filtration is easier to run than dialysis. In diafiltration, using gas or pump to pressurize membrane chamber, sample will be separated according to molecular size (Philips and Signs, 2005).

2.13.4 Protein Detection and Visualization

Protein can be visualized or detected by gel electrophoresis method. In gel electrophoresis, proteins are embedded in a matrix (gel) and separated in an electric field (electrophoresis), thus the term is gel electrophoresis. Gel electrophoresis has several advantages, high resolution, cell lysates or tissue extracts can be applied to gels directly, and minimal loss of hydrophobic protein. Protein separation usually is carried out using 1 dimensional gel electrophoresis or 2 dimensional gel electrophoresis (2DE). Normally, the purpose of gel electrophoresis is to determine the homogeneity of fractionated protein. For instance, a single band and single spot in SDS PAGE and 2DE gel respectively can indicate the presence of single protein.

In 1 dimensional gel electrophoresis, SDS PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) is often applied. The most commonly used gel electrophoresis is called Laemmli method, after U.K. Laemmli, the first person to publish a scientific paper employing SDS PAGE (Caprette, 1996). This gel is chemically inert, can be made to different concentration and pore size by adjusting the percent of total acrylamide (%T) and the cross linker concentration (%C). When electrical field is applied, protein will be separated based on their differences in migrating through a sieving matrix, which is a gel. Higher molecular mass protein will travel slower in the gel than the lower molecular mass. Tertiary structure of protein need to be destroyed to make it linear and separated by size. SDS detergent along with detergent such as dithiothreitol (DTT) or 2-Mercaptoethanol (B-ME) is added to break down disulphide bonds of proteins. SDS has a negative charge (Sulphate), thus SDS will render the protein to be negative in charge, therefore the protein will migrate towards positive pole in electric field. Protein can be viewed as bands after migration (Brunelle and Green, 2014). In SDS PAGE, protein mobility depends on molecular mass, while in native PAGE, the mobility

is dependent on charge of the protein and the conformation. In native PAGE, SDS and reducing agent such as DTT and B-ME will be left out (Arndt et al., 2012).

In 2 dimensional gel electrophoresis (2DE), protein is separated by two steps, independently. First dimension separates protein by isoelectric point (pI). In an IPG strip, protein isoelectric focusing takes place with pH gradient and high concentration of urea. IPG strip can exist in different length (7-30 cm) and pH range; wide (3-10), medium (3-6, 4-7, 7-11), narrow (6.3-.8.3, 4.5-5.5) and ultra-narrow (4.9-5.3) (Pomastowski and Buszewski, 2013). In the electrical field, protein will migrate along the pH range until it carries no net charge. Seconds dimension separates protein by molecular mass in SDS PAGE, in a direction 90 degrees from the first dimension. Proteins will be viewed as spots and the resulting spot patterns are usually oriented according to the Cartesian convention. The low, acidic isoelectric will be oriented to the left and the low molecular mass will be towards the bottom of the gel. Many applications can be carried out such as different gel formats, pH ranges, detection method and reducing or no reducing condition, depending on research purpose. Technique of 2DE is gaining popularity due to the possibility of coupling it with numerous analytical techniques (Pomastowski and Buszewski, 2013).

Once protein has been separated in gel, protein can be viewed using staining method such as silver staining and Coomassie staining. In Coomassie staining, the sulphonic acid groups of the dye bind to protein positive amine group. There are two Coomassie dyes, R-250 and G-250. R-250 is the most sensitive, can detect as little as 0.1 ug of protein. G-250 is the less sensitive, but the procedure is more convenient and rapid (National Diagnostic, 2011). The rationale of silver staining is protein binds to silver ions (Chevallet et al., 2007) then reduced to build up visible image of finely divided silver metal.

2.13.5 Protein Characterization

Digital image of 2DE can be analyzed using specialized software such as PDQuest (Biorad), Dymension (Syngen) and Image Master (GE Healthcare). This software will conduct protein profiling, spots identification, data normalization, and statistical analysis if required (Pomastowski and Buszewski, 2014).

In mass spectrometry (MS), the identification of protein is based on mass to charge ratio (m/z). Prior analysis, protein will be digested using proteases such as trypsin. This very sensitive method will give information on the composition, structure of protein. MS can give accurate values of protein molecular masses until the level of picomole. Matrix assisted laser desorption/ionization (MALDI) is one ionization technique in MS. A nanosecond laser radiation will cause desorption and ionization of analytes. The advantages of MALDI are high throughput and sensitivity, accuracy, can be automated and ease of operation while surface enhancer laser desorption/ionization (SELDI) is variation of MALDI. SELDI has the advantage of requiring only small amount of samples and being rapid.

2.13.6 Protein Sequencing

In determining the sequence of amino acids in a protein, usually MS/MS data are submitted and matched against existing data in protein database such as Mascot, Sequest, Tandem, and MS-Blast. When the protein of interest is a new protein, *de novo* sequencing, which is sequencing without database is carried out (Tannu and Hemby, 2007). *De novo* sequencing is a process of mapping amino acids from a protein. This method involves deducing sequence of protein from MS/MS spectra computationally. Using LC MS in general, protein are separated in a column according to mass to charge ratio, the protein then fragmented and the resulting ions are detected by second mass analyzer, forming MS/MS spectrum (Hennrich et al., 2010). There are six possible fragment ions; a, b, c, x, y, z. a, b, c ions have the charge retained on the N terminal of the fragment while x, y, z on the C terminal of the fragment, the ions are labeled in Figure 2.7. The most common cleavage is at the protein bond, giving rise to y and b ions. The mass different between two adjacent ions is indicative of a particular amino acid residue. Table 2.3 shows the mass of each amino acid. The sequence is then deduced from the spectrum.

The advantage of *de novo* sequencing method is the derived amino acid partial sequence can also be used to identify post translation modification (Hughes et al., 2010). However, MS/MS spectrum is complex to be interpreted and sometimes can lead to ambiguous sequence determination (Wattenberg et al. 2002). Contaminants, missing fragment ions can cause *de novo* sequencing to figure out partially correct sequence spectrum (Campbell and Vestal, 2002). Similar mass residue could also contribute to this problem. As indicated in Table 2.3, leucine and isoleucine have similar mass residue

which is 113.16. Normally several sequencing method such as *de novo* sequencing, comparing againts databases, Edman degradation, and protein derivatization are combined to get complete sequence.

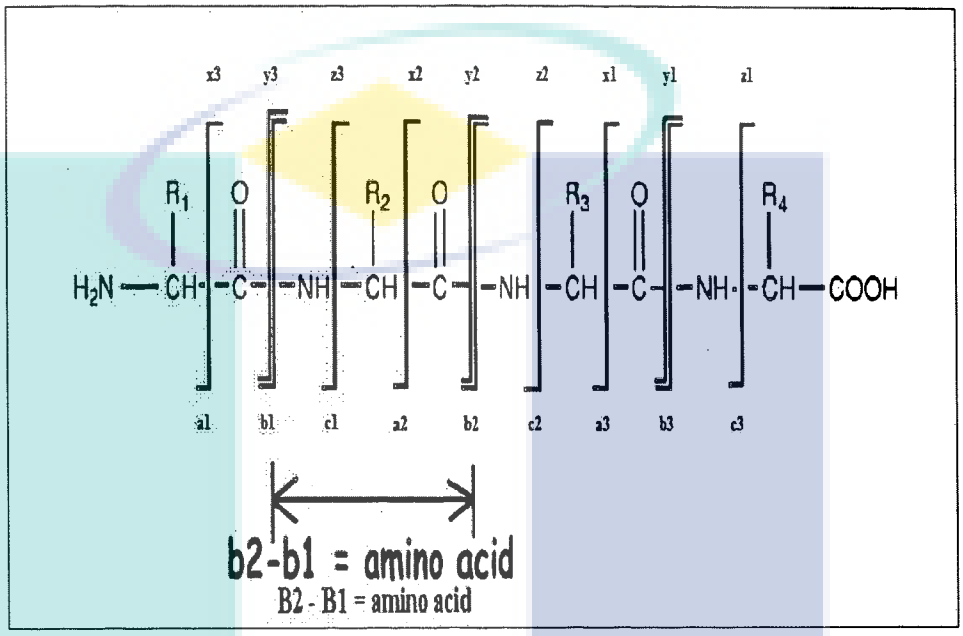

Figure 2.7: Six possible fragment ions for an amino acid. a, b, c ions are retained on the N terminal while x,y,z are retained on the C terminal.

Source: www.astbury.leeds.ac.uk/facil/MStut/mstutorial_files/tandem1.jpg

Table 2.3:
Mass of each amino acid residue

Symbol	Mass (Da)
Ala A	71.0
Arg R	156.1
Asn N	114.0
Asp D	115.0
Cys C	103.0
Gln Q	128.1
Glu E	129.0
Gly G	57.0
His H	137.1
Ile I	113.1
Leu	113.1
Lys K	128.1
Met M	131.0
Phe F	147.1
Pro P	97.1
Ser S	87.0
Thr T	101.0
Trp W	186.1
Tyr Y	163.1
Val V	99.1

CHAPTER 3

MATERIALS AND METHOD

3.1 Sample Sourcing

A total of 50 *E. longifolia* products, in various pharmaceutical dosage forms, both registered and unregistered with National Pharmaceutical Control Bureau (NPCB) Malaysia, were sourced from Malaysian and international market. Any products bearing the scientific (*E. longifolia*) and common name (Tongkat Ali) were chosen randomly from the market. Those products were categorized into; Malaysian Registered Products (MRP), Malaysian Unregistered Products (MUP) and International Products (IP), sourced from Amazon.com. Two herbal products without *E. longifolia*, but were promoted as having aphrodisiac effect, were bought as negative control. Eight samples of premixed coffee, allegedly containing *E. longifolia* were sourced from various food courts in Kuantan, Pahang. *E. longifolia* root was sourced from Bentong Pahang, while roots of Tongkat Ali Merah and Tongkat Ali Hitam were sourced from Sungai Buloh, Selangor.

3.1.1 Sample Preparation

For products in capsule form, the capsule shells were removed, then the powdery content was weighed. Meanwhile, products that were in tablets form were crushed until they turned powdery prior weighting process.

3.2 Water Extraction of Raw Materials

Root powders of 50 g were put in 600 mLdeionized water. The sample then was boiled under reflux for five hours, followed by filtration with Whatman No 1 filter paper and freeze drying process. Figure 3.1 shows the Soxhlet apparatus set up for the water extraction process. Other raw samples; root of Tongkat Ali Merah and Tongkat Ali Hitam were extracted using this method.

Figure 3.1: Soxhlet apparatus set up for water extraction of raw samples

3.3 Freeze Dry Method

Samples were put in -80 °C for three days prior freeze drying process. Using freeze dryer (Labconco, US), freeze drying was carried out at -80 °C with pressure of 0.002

mBar. Process was discontinued when the samples appeared powdery and the bottom of the flask containing the samples was not cold to the touch.

3.4 HPLC Analysis of Eurycomanone

3.4.1 Eurycomanone Standard Preparation

Eurycomanone (ChromaDex, USA) standard stock solution (2.5 mg/mL) was prepared by dissolving 5 mg of the reference standard in 2 mL of water. The stock solution was stored at 4 °C and remained stable for at least one month. The working standard solution was prepared by diluting the standard stock solution to give serial concentration of 2.5, 0.5, 0.25, 0.125, 0.06250 and 0.01325 mg/mL. Calibration graph was plotted using Microsoft Excel 2007 based on linear regression analysis of the integrated peak areas (y) versus concentrations (x, mg/mL). The peak area values were the average values of three replicates injection. The limit of detection (LOD) and limit of quantitation (LOQ) were determined from the standard solution and calculated using Microsoft Excel 2007.

3.4.2 HPLC Analysis

HPLC (Waters, US) with photodiode array detector and XBridge column (Supelcosil 5 µm, 250 mm X 4.6 mm) was used. Absorbance was taken at 238 nm. The mobile phase consisted of isocratic mixture of water and acetonitrile (Fisher Scientific, UK) (86:14) (v:v) with a flow rate of 0.8 mL/min. For eurycomanone standard, 10 µL of was injected into the HPLC system and calibration curve was generated from the eurycomanone standard that has been serially diluted. For products analysis, 5 mg of each sample were dissolved in 1.5 mL water and filtered through 0.45 µm nylon membrane filter. Then, 10 µL of each sample was injected into the HPLC system. Contents of eurycomanone of each product were counted using the Equation 2.1;

$$\%\frac{weight}{volume} = \frac{weight\ of\ eurycomanone\ calculated\ (mg)}{volume\ of\ sample\ injected\ (ml)}\ \%$$

Equation 2.1: Eurycomanone contents calculation

3.5 Protein Quantification

3.5.1 Materials

Coomassie Brilliant Blue G-250 (Biorad, US), ethanol (Merck, US), phosphoric acid (Merck, US), deionized water, albumin standard (Thermo Scientific, UK).

3.5.2 Bradford Assay

Assay was done according to Bradford (1976) with slight modification. Briefly, sample of 10 mg sample was dissolved in 1 mL deionized water. Then 100 µL of sample solution was mixed with1mL Bradford reagent. The solution then incubated in the dark for 20 minutes and UV absorbance was read at 595 nm using UV spectrophotometer (Shimadzu). Stock solution of Bovine Serum Albumin (BSA) (2 mg/mL was diluted to produce BSA concentration of 10, 8, 6, 4 and 2 µg/mL. Protein contents of the samples was determined by standard curve of the BSA.

3.6 Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS PAGE)

3.6.1 Materials

Sodium dodecyl sulphate (Plusone, GE Healthcare, Sweden), deionized water, acrylamide (Plusone GE Healthcare, Sweden), bis(N,N-methylene bisacrylamide) (Biorad, US), tris (Plusone GE Healthcare, Sweden), glycine (Plusone GE Healthcare, Sweden), hydrochloric acid (Fisher), tetramethylethylenediamine TEMED (Plusone GE Healthcare, Sweden), ammonium persulphate APS (Biorad, US), tris (Plusone GE Healthcare, Sweden), bis(N,N- methylene bisacrylamide) (Biorad, US), dithiothreitol DTT (Plusone GE Healthcare, Sweden), unstained protein molecular weight marker (Thermo Scientific, UK), Coomassie tablet (Plusone GE Healthcare, Sweden), acetic acid (Fisher, UK), methanol (Merck, US), sodium thiosulphate (Merck, US), silver nitrate (Merck, US), formalin (Merck, US).

3.6.2 Gel Casting

A comb was put into assembled gel sandwich. A mark at the glass plate was put using marker about 1 cm below the teeth of the comb. This was the level to which separating gel would be poured. The comb was removed and resolving gel solution was put into. The polymerizing gel solution was covered with 0.1% SDS to create a smooth top of the gel. Approximately after 30 minutes or after the gel has solidified, the 0.1% SDS covering solution was poured and the polymerized gel was rinsed with distilled water. Then stacking gel solution was put on top of the resolving gel in the caster. Gel comb was immediately put on top of the stacking gel to produce sample well. It was estimated that the gel solidified after 20 minutes

3.6.3 Gel Electrophoresis

Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS PAGE) was done according to the method of Laemmli and Favre (1973). Electrophoresis was carried out using mini vertical slab gel system. (Biorad, Laboratories, Hercules, CA, USA), with stacking gel 8% and resolving gel 15% of 1 mm thickness. Into 1 mL sample buffer, 50mg DTT was added. Then 20 µg protein sample was dissolved in 200 µL sample buffer. Into sample well, 5 µL of the sample solution was pipetted together with bromophenol blue as tracking dye. Low molecular weight marker that acted as reference also was incorporated into one of the well in the gel. The molecular weight of the protein marker is in Figure 3.2. The electrophoresis was run for about 1 hour and half. After migration completed, protein bands were detected using Coomassie Brilliant Blue staining. The gel then was photographed and stored in 10% acetic

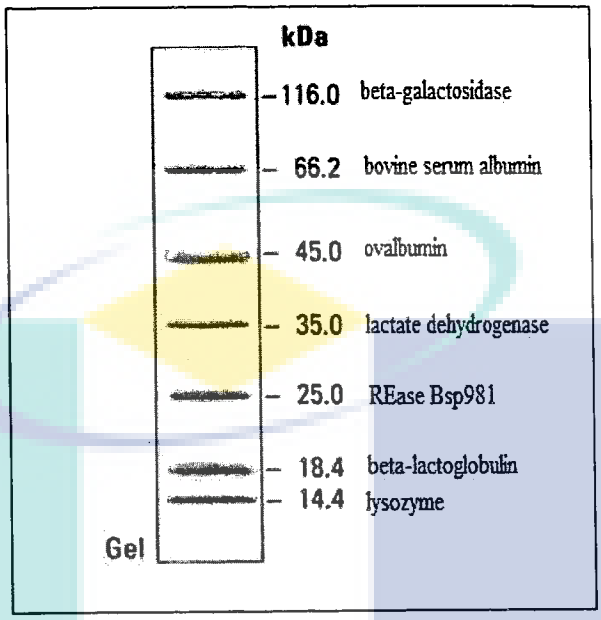

Figure 3.2: Molecular weight of protein marker

Source: http://www.thermoscientificbio.com/protein-electrophoresis/unstained-protein-molecular-weight-marker/

3.6.4 Coomassie Blue Staining

Coomassie tablet was dissolved in 10%, 2L acetic acid solution. The solution then was filtered using Whatman filter paper no 1. Staining solution was heated until boiling prior putting the gel in. Gel was put in staining solution for 10 minutes with gentle shaking. Repeated destaining steps using 10% acetic acid were carried out until the gel turned into transparent color.

3.6.5 Silver Staining

Gel was fixed in 1L solution containing deionized water, 120 mL glacial acetic acid (Fisher, UK), 500 mL 96% (v/v) ethanol (Merck, US), 500 µL 35% (w/v) formaldehyde (Merck, US) overnight. After that, gel was washed in 1L solution containing water and 20% (v/v) ethanol (Merck, US) for 2 minutes, three times. Gel was put in sensitizing solution containing 200 mg sodium thiosulfate (Merck, US) in 1L of deionized water for 2 minutes. Gel later was washed 2 times with deionized water for 1

minute. For staining, gel was put in 1L staining solution containing deionized water, 0.2% (w/v) silver nitrate, Merck, US), 760 µL formaldehyde (Merck, US) for 20 minutes. After that, gel was washed twice with deionized water for 30 seconds. For development, gel was put in 1L developing solution containing deionized water, 60 g sodium carbonate (Merck, US), 500 µL formaldehyde, 4 mg sodium thiosulfate for 5 minutes. Gel later was put in 1L stopping solution containing 12% (v/v) glacial acetic acid, for 10 minutes. Gel was stored in 1% (v/v) acetic acid (Merck, US).

3.7 2 Dimensional Electrophoresis (2DE)

3.7.1 Materials

Sodium dodecyl sulphate (Plusone, GE Healthcare, Sweden), deionized water, acrylamide (Plusone GE Healthcare, Sweden), bis(N,N-methylene bisacrylamide) (Biorad, US), tris (Plusone GE Healthcare, Sweden), glycine (Plusone GE Healthcare, Sweden), hydrochloric acid (Fisher), tetramethylethylenediamine TEMED (Plusone GE Healthcare, Sweden), ammonium persulphate APS (Biorad, US), tris (Plusone GE Healthcare, Sweden), bis(N,N- methylene bisacrylamide) (Biorad, US), dithiothreitol DTT (Plusone GE Healthcare, Sweden), unstained protein molecular weight marker (Thermo Scientific, UK), Coomassie tablet (Plusone GE Healthcare, Sweden), acetic acid (Fisher, UK), methanol (Merck, US), sodium thiosulphate (Merck, US), silver nitrate (Merck, US), formalin (Merck, US), IPG strips pH 3-10, 7 cm (Biorad, US), IPG strips pH 3-10, 17 cm (Biorad, US), mineral oil (Biorad, US), ampholyte (Biorad, US), urea (Plusone, GE Healthcare, Sweden), CHAPS (Plusone, GE Healthcare, Sweden), Wick paper (GE Healthcare, Sweden), agarose (GE Healthcare, Sweden). bromophenol blue (Biorad, US), agarose (Plusone GE Healthcare, Sweden).

3.7.2 Rehydration Buffer

Urea of 12 g, 0.5 g chaps, 125 µL ampholyte, and traces of bromophenol blue were dissolved in 15 mL deionized water and the solution volume was brought to 25 mL. The solution then was kept in 1 mL aliquot before being kept in -20 °C.

3.7.3 Equilibration Buffer

SDS of 4 g, 6.7 mL tris HCl pH 8.8, 72.07 g urea, 68.97 mL glycerol, and traces of bromophenol blue were dissolved in 150 mL deionized water and the solution volume was brought to 200 mL.

3.7.4 Agarose Sealing Solution

Trace of bromophenol blue, 25 mL electrophoresis buffer, 125 mg agarose IEF were put together and stirred. The solution was then heated in microwave oven for 2 minutes, left to cool, and kept in refrigerator.

3.7.5 2DE First Dimension - Rehydration and Iso Electric Focusing

2.5 µL ampholyte and 30 mg DTT was put into 1 mL rehydration buffer. For 7 and 17 cm IPG strip, 20 µg and 60 µg protein samples was put into rehydration solution. Then 125 µL (7cm) and 300 µL (17 cm) of the solution was pipetted along the edge of a channel in rehydration tray. IPG strip cover then was peeled, and the strip was put gel side down onto the sample solution with the '+' marked on the IPG strip was positioned at the end of the tray marked '+'. After that, IPG strip was overlaid with mineral oil to prevent drying during rehydration process. Rehydration was set for 16 hours using iso electric focusing apparatus (Biorad). The isoelectric focusing for 7 and 17 cm IPG strip were programmed with the following protocol in Table 3.1 and Table 3.2 respectively.

Table 3.1:
Isoelectric focusing programs for 7 cm IPG strip

Voltage mode	Voltage (V)	Duration(hr)
Step and hold	200	1
Gradient	1000	1
Gradient	5000	4
Step and hold	5000	1

Table 3.2:
Isoelectric focusing programs for 17 cm IPG strip

Voltage mode	Voltage (V)	Duration(hr)
Step and hold	200	1
Gradient	1000	1
Gradient	8000	4

3.7.6 2DE Second Dimension- Equilibration Process

DTT of 200 mg was added into 10 mL equilibration buffer. IPG strip was put into equilibration tray with the gel side up. Equilibration buffer of 2.5 mL (7cm) and 6 mL (11cm) then was put into the equilibration tray containing the IPG strip. The tray then was put on orbital shaker and shaken for 10 minutes. The equilibration solution in the tray then decanted. In a container, 250 mg iodocetamide was put into 10 mL equilibration buffer. Then 2.5 mL (7cm) and 6 mL (11cm) of the solution was put into the tray containing the IPG strip. The tray then shaken for 10 minutes using orbital shaker.

3.7.7 2DE Second Dimension- SDS PAGE

Gel for second dimensional separation was prepared according to the method in section 3.6.2 minus the stacking gel. IPG strip that has been rehydrated and equilibrated was put horizontally on top of gel. Into a paper wick, 5 µL of protein molecular marker was absorbed. The paper wick was then put beside the IPG strip. Agarose sealing solution was poured on top of resolving gel to secure the IPG strip and paper wick in place. Electrophoresis was run at 150 V.

3.7.8 2DE Gel Image Analysis

Gels were analyzed using Image Master™ 2D Platinum 7.0 software (GE Healthcare, Sweden). Differences between corresponding spots were analyzed in each set of gels. Gel of purified protein was used as reference gel.

3.7.9 Excipient Incorporation into *E. longifolia* Extracts

About 50 mg of each maltodextrin and cyclodextrin were dissolved in 50 mL anhydrous ethanol and 50 µg protein containing *E. longifolia* extracts were added into each solution. The solution then stirred using magnetic stirrer and ethanol was evaporated using rotary evaporator. The blend then dried at 40 °C for 3 hours.

3.8 Protein Purification

3.8.1 Materials

Hi Trap Desalting 5mL column (GE Healthcare, Sweden), Q Sepharose HP gel beads (GE Healthcare, Sweden), tris (Plusone, GE healthcare, Sweden), hydrocloric acid HCl (Fisher, UK), Natrium Chloride NaCl (Merck, US), AKTA Prime (Amersham, Sweden), AKTA explorer (Amersham, Sweden).

3.8.2 Size Exclusion Chromatography

Protein sample in *E. longifolia* crude extracts was pre fractionated by size exclusion chromatography using AKTA Prime Protein Purification System (GE Healthcare), absorbance at 280 nm, 20 cm X 3 cm column, with Sephadex G25 Superfine (cross linked dextran, 15-88 µm particle size, GE Healthcare). 100 mg crude extract was dissolved in 1 mL deionized water. Sample was loaded into 500 µL sample loop. With 2 mL/min flow rate, column was equilibrated with 5 columns volume of water. Elution was carried out until 5 columns volume and fraction collector was programmed to collect 2 mL per fraction. All fractions were then freeze dried.

3.8.3 Ion Exchange Chromatography

AKTA Explorer, XK16 column (10 mm bed height, 0.75 mm internal diameter) with Q Sepharose High Performance beads (24 to 44 µm particle size, 6% cross linked agarose, GE Healthcare) were used in ion exchange chromatography to separate protein in mixture. About 50 mg sample was dissolved in 1 mL loading buffer, 20 mM Tris HCl, pH 8.7. With flow rate of 1.5 mL/min, column was equilibrated with 3 columns volume of binding buffer, 20 mM Tris HCl, pH 8.7. Sample was loaded into 500 µL sample loop. Column was washed with 3 columns volume of binding buffer to get rid of unbound material. Elution was carried out in stepwise gradient mode; starting by 60% of elution buffer (0.5 M NaCl in 20 mM Tris HCl, pH 8.7) in 1 column volume, followed by 65% of the elution buffer in 1 column volume, then 70% in 1 column volume, followed by 90% in 1 column volume. Second washing step using 100% elution buffer, 2 columns volumes of washing was carried out to ensure all components elute from column. Fractions with size of 2 mL were collected at first washing and elution steps.

3.8.4 Desalting and Buffer Exchange

AKTA Explorer and 5 mL HiTrap Desalting column (cross linked dextran, 15-88 µm particle size, 16 mm internal diameter, GE Healthcare) were used in desalting and buffer exchange process. Sample was loaded into 500 µL sample loop. With 2.5 mL/min flow rate, column was equilibrated with 5 columns volume of water. Elution was carried out until 5 columns volume and fraction collector was programmed to collect 2 mL per fraction. All fractions then freeze dried.

3.9 MALDI TOF MS Analysis

3.9.1 Linear Mode

Analysis was done using MALDI TOF MS Kratos Axima (Shimadzu, Japan), operating in positive ion linear mode in the mass range of m/z 200- 2000 with resolving power of 10^6 and the laser energy of 15 µJ per laser shot.

3.9.2 Reflectron Mode

Gel piece in 1.5 mm diameter was excised from gel. The gel piece then was washed with 400 µL of 10 mM ammonium bicarbonate (Merck, USA) solution containing 50% (v/v) acetonitrile (Merck, USA) for 30 min at room temperature, under vigorous shaking. The supernatant then removed and gel piece was dried for 15 min in vacuum concentrator. The gel then incubated in a 150 µL reduction solution containing 10 mM DTT (GE Healthcare, Sweden), 100 mm ammonium bicarbonate (Merck, USA) at 56 °C for 30 min. After removing the reduction solution, 100 µL alkylation solution containing 50 mm iodocetamide (Biorad, USA), 100 mm ammonium bicarbonate was added for 30 min under room temperature in the dark. Then, 5 µL trypsin solution (Promega, Madison) and 10 ng/µL in 5 mM ammonium bicarbonate/5% acetonitrile was added and incubated for 5 hr at 37.8 °C. The reaction then stopped by adding 1 µL of 1% TFA (Fisher, UK). The sample then stored at 5°C overnight for better extraction of proteins. One µL of sample supernatant was mixed with 2 µL of matrix solution (5 mg α- cyano-4-hydroxycinnamic acid in 40% (v/v) acetone (Merck, USA), 50% (v/v) acetonitrile, 9.9% (v/v) water and 0.1% (w/v) TFA in water). 1 µL of the mixture then deposited onto the MALDI target.

Analysis of MALDI TOF MS was performed in Kratos Axima (Shimadzu, Japan) system. Sample was dissolved in 5% acetonitrile and 0.5% trifluoroacetic acid. Mass Spectrometer was in reflectron mode. Positive ionization was conducted at 20 kV. The data were analyzed using Kompact software system. Protein identification was performed by searching for plant proteins in latest version of Swissprot database using Mascot search engine.

3.10 *De Novo* Sequencing

De novo sequencing analysis was carried out as service provided by Proteomics International, Australia. Briefly, the method was; protein samples in 2DE gels were cleaved appropriately and trypsin digested. Proteins were further extracted using solid phase extraction (Strata-X, Phenomenex). Proteins were analyzed by electrospray ionization mass spectrometry using Agilent 1260 infinity HPLC system, coupled to an Agilent 6540 mass spectrometry. Protein was loaded onto a C18 column 300 SB, 5 µm

(Agilent) and separated with a linear gradient of water/acetonitrile/0.1 % formic acid (v/v). MS/MS spectra were analyzed using PEAKS Studio Version 4.5 SP2 (Bioinformatics Solutions) and manual interpretation.

3.11 Statistical Analysis

Data were analyzed using Microsoft Excel 2007. All samples were analyzed in triplicates. The data was expressed as mean ± standard deviation (SD). Differences between the mean values were determined using the Student's t test. P values of less than 0.05 were considered as statistically significant.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Sample Requisition

In order to analyze the quality of *E. longifolia* products using chemical and protein marker that will be discussed further in subsequent sections, *E. longifolia* products in the market were sampled. The products were categorized into International Products (IP) and local products. The local products were further divided into Malaysian Registered Products (MRP), Malaysian Unregistered Products (MUP) and Beverages (B). Hologram at the packaging was used to identify Malaysian Registered Products (MRP) and the registration number was keyed in at Ministry of Health website to check the validity. Table 4.1 provides the number of the products that were obtained for this study. In total, 50 *E. longifolia* products were collected. There were 16 Malaysian Registered Products (MRP), 14 Malaysian Unregistered Products (MUP), 8 Beverages and 12 International Products (IP). Briefly, 76% of the samples in this study were acquired in Malaysia, while 24% were acquired internationally.

Samples were collected from multiple sources such as pharmacies, night markets, jamu shops, food courts and even online stores. The samples were selected on the basis of bearing 'Tongkat Ali' or '*Eurycoma longifolia*' at the ingredient level. In attempts to make the sampling as random as possible, samples were picked upon recommendation by the sellers at the selected premises. The products was coded according to the turn it was sampled.

Table 4.1:
Sample requisition of *E. longifolia* products

International products		**Local products**	
Source: Amazon.com	12	Malaysian Registered Products	16
		Malaysian Unregistered Products	14
		Beverages	8
	12		**38**
	Total:	**50**	

These products were obtained in modern dosage forms; root powder or freeze dried extracts in capsule, pills, maajun and premixed coffee. Traditionally, herbal medicine is taken in the form of decoction. However, decoction is easily degraded, susceptible to bacteria and has unpleasant taste. Therefore, the dosage is developed into dosage such as tablets and capsules as they have the advantage of elegance in appearances, accurate dosage, masking the unpleasant taste, chemically and physically stable (Charoonratana et al. 2014).

Unlike conventional drug that consists of single compound, normally herbal medicine consists of multiple putative active ingredients, while the remaining ingredients in the preparation just act as carrier (Charoonratana et al. 2014). The details of the Malaysian Registered Products, Malaysian Unregistered Products, Beverages and International Products can be found in Table 4.2, Table 4.3, Table 4.4 and Table 4.5 respectively and the picture of the products is in Appendix A. It was noticed that other herbs that were often mixed with *E. longifolia* were herbs that are perceived to have aphrodisiac property such as Ubi Jaga (*Smilax myosotiflora*), Maca (*Lepidium meyenii),* Horney Goat Weed (*Epimedium grandiflorum*) and Ginseng. It is interesting to note that some of the products were named as 'Tongkat Ali' even though Tongkat Ali or *E. longifolia* was not the main constituent, reflecting the situation that manufacturers were milking the popularity of *E. longifolia.* A product which listed a plant species, *Smilax*

myosotiflora (Ubi Jaga) as ingredient was chosen as negative control. This species is also said to confer aphrodisiac property (Wan et al. 2016).

Table 4.2:
List of Malaysian Registered Products (MRP)

No	Code	Name	Dosage Form	Formulation
1	C1	Tongkat Ali Plus	Capsule	Multiple constituents
2	C2	Eury Goal Tongkat Ali	Capsule	Single constituent
3	C3	Jamu Tongkat Ali	Capsule	Multiple constituents
4	C6	Herba Tongkat Ali Plus	Capsule	Multiple constituents
5	C7	Nu Prep Lelaki	Capsule	Single constituent
6	C8	Tongkat Ali Maca Plus	Capsule	Multiple constituents
7	C9	Hurix Tongkat Ali	Capsule	Multiple constituents
8	C20	Tongkat Ali Hitam Plus	Capsule	Multiple constituents
9	C21	Fenugreek Tongkat Ali	Capsule	Multiple constituents
10	C24	Ubi Jaga Tongkat Ali	Capsule	Multiple constituents
11	C25	Jamu Vigor For Men	Capsule	Multiple constituents
12	C26	Ubi Jaga Plus	Capsule	Multiple constituents
13	C27	Formulations For Men	Capsule	Multiple constituents
14	C28	Ubi Jaga Tongkat Ali	Capsule	Multiple constituents
15	C35	Jabbar 101	Capsule	Multiple constituents
16	C38	Kapsul Tongkat Ali	Capsule	Single constituent

Large portion of the products in this study were Malaysian products. This is partly because *E. longifolia* is indigenous to Malaysia (Bhat and Karim, 2010) and it can be said that Malaysia is one of the major producer of *E. longifolia* products, so the sampling could signify the products that are consumed by people worldwide.

Table 4.3:
List of Malaysian Unregistered Products (MUP)

No	Code	Name	Dosage Form	Formulation
1	C4	Black Jack	Capsule	Multiple constituents
2	C10	Tanduk Rusa	Capsule	Multiple constituents
3	C11	Tongkat Ali Ginseng	Capsule	Multiple constituents
4	C15	Ramuan 4 Jenis Akar	Capsule	Multiple constituents
5	C16	100% Tongkat Ali & Ubi Jaga	Capsule	Multiple constituents
6	C17	Ranjang Besi dan Tongkat Ali	Capsule	Multiple constituents
7	C18	Ramuan Akar Kayu	Capsule	Multiple constituents
8	C19	Tongkat Ali	Capsule	Single constituent
9	C22	Kapsul Tongkat Ali Hitam	Capsule	Multiple constituents
10	C23	Orang Kampong Tongkat Ali	Capsule	Multiple constituents
11	C29	Wild Tongkat Ali	Capsule	Single constituent
12	C30	Urat Madu	Capsule	Multiple constituents
13	C31	Jamu Sakti	Capsule	Multiple constituents
14	C42	Maajun Pak Tani	Maajun	Multiple constituents

Table 4.4:
List of *E. longifolia* Beverages (B)

No	Code	Name	Dosage Form	Formulation
1	D1	Power Root Ali Café	Coffee powder	Multiple constituents
2	D2	Kopi Jantan	Coffee powder	Multiple constituents
3	D3	Capuccino Tongkat Ali	Coffee powder	Multiple constituents
4	D4	Mahkota Dewa	Coffee powder	Multiple constituents
5	D5	Tongkat Ali with Ginseng	Coffee powder	Multiple constituents
6	D6	Tongkat Ali and Cordiseps	Coffee powder	Multiple constituents
7	D7	Jus 2 Tongkat	Liquid	Multiple constituents
8	D8	Healthy Man Coffee	Coffee powder	Multiple constituents

Table 4.5:
List of International Products (IP)

No	Code	Name	Dosage Form	Formulation (comstituent)	Manufacturing Country
1	C5	Pure Tongkat Ali 200:1	Capsule	Single	Thailand
2	C12	Tongkat Ali Root Extract	Capsule	Single	Indonesia
3	C13	Unleash Your Beast	Tablet	Multiple	USA
4	C14	Pure D	Capsule	Single	USA
5	C32	Testosterone Booster	Capsule	Multiple	USA
6	C33	Longjack	Capsule	Multiple	USA
7	C34	Tongkat Ali Doctor Formulated	Capsule	Single	USA
8	C36	*Eurycoma Longifolia* Jack Extract	Capsule	Single	USA
9	C37	Make My Pepper Big	Capsule	Multiple	USA
10	C39	Unleash Power	Tablet	Multiple	USA
11	C40	Herb Natural Tongkat Ali	Capsule	Single	Thailand
12	C41	Indonesia Tongkat Ali	Capsule	Single	Indonesia

Tongkat Ali Merah and Tongkat Ali Hitam are local names for *Goniothalamus* sp and *Polyalthia bullata* respectively. These plants bear the same local name (Tongkat Ali) with *E. longifolia*, due to the same root morphology, which is the long straight root, but differ in color. The root color of *E. longifolia* is slightly yellow while *Goniothalamus* sp and *Polyalthia bullata* are red (Merah) and black (Hitam) respectively. Both of these plants roots were also sampled and tested to determine whether they have the same marker(s) as *E. longifolia.*

4.2 Water Extraction of Raw Materials

Water extraction in laboratory scale was carried out on *E. longifolia* root and the freeze dried extracts acted as positive control in this study. Results from this extract would serve as reference. The root powder was boiled for 5 hours. Water extraction method was chosen because it could mimic the traditional preparation method and *E. longifolia* products in the market are also available in the form of freeze dried water extracts (Bhat and Karim, 2010; Mohd Effendy et al., 2012) besides chipped roots. For consistency, it is very important to use the extraction process based on the traditional preparation method of the herbal medicine considering the fact that the purpose is to investigate the possible compounds that are ingested by consumers (Charoonratana et al. 2014). Vejayan et al. (2013) also used water extraction method in analyzing several *E. longifolia* commercial products.

4.3 Analysis of Eurycomanone Marker

Herbal medicines comprise of myriads of compounds and the amount might vary, so determining the quality of herbal products is a common problem. To achieve consumer higher level of trust towards herbal products, standardization should be carried out. This is also to confirm the accuracy of the label claim in a product. Standardization is where a minimum amount or a range of one or more compounds in herbal medicine is determined (Charoonratana et al. 2014). These compounds can be called as chemical marker (Li et al. 2008).

Eurycomanone is the chemical marker in this study. Known also as Pasak bumin A (Zakaria et al. 2009), eurycomanone is a quassinoid with molecular formula of $C_{20}H_{24}O_9$. It appears as white cubic crystals once isolated in pure form (Chuen et al. 2004). This section adapts a parameter provided by Malaysian Standard in determining the quality of *E. longifolia* freeze dried extracts. Malaysian Standard (MS249:2011) set the quality based on several criteria; eurycomanone level, total polysaccharide, total protein and total glycosaponin and based on the standard, the recommended minimum level of eurycomanone in *E. longifolia* freeze dried extracts is from 0.8 to 1.5 w/v (%).

To serve as reference compound, eurycomanone standard analysis was run and the calibration curve is in Figure 4.1. It shows good linearity ($R^2 = 0.999$) between the peak area and concentration at the selected wavelength. The values of limit of detection

(LOD) and limit of quantitation (LOQ) were 0.0227 and 0.0690 respectively, which indicates that the method is highly sensitive in detection of the compound.

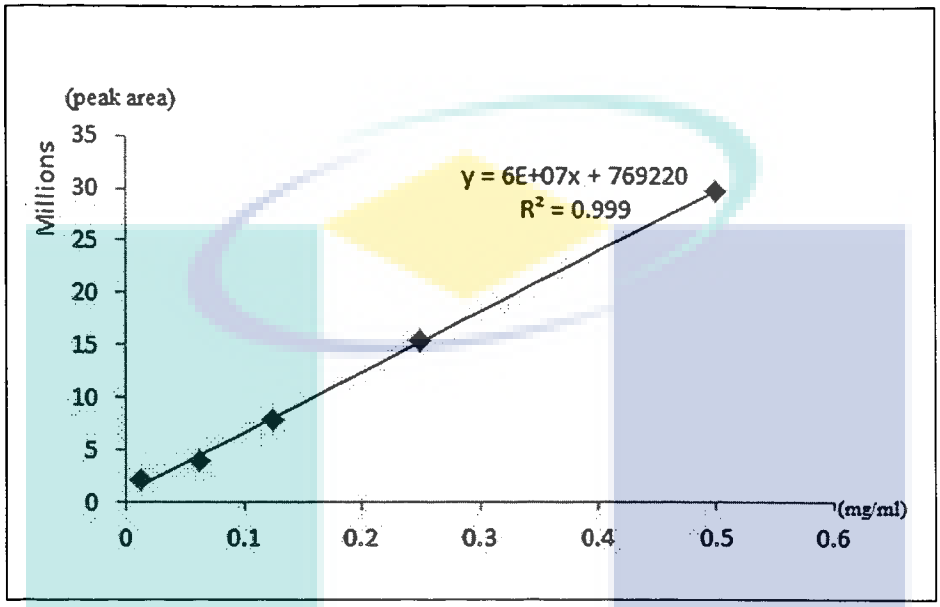

Figure 4.1: Calibration curve of eurycomanone standard from HPLC analysis. Absorbance was taken at 238 nm

High Performance Liquid Chromatography (HPLC) is the analytical tool and this equipment has the advantage of simplicity and high sensitivity. The method development for this analysis was carried out using freeze dried extracts and the chromatogram is in Figure 4.2 (b). After several optimizations through comparisons of different solvents, solvent ratio and gradient profile, good HPLC profile was achieved using water and acetonitrile as the mobile phase. The analysis time was less than 10 minutes. It can be said here that good profile was achieved as best analysis is best separation, shortest analyzing time and lowest column pressure (Song et al. 2007).

Figure 4.2 (a) shows the HPLC profile of eurycomanone standard. It can be seen that eurycomanone elutes approximately at retention time of 7.08 min. HPLC profile of the freeze dried water extract is in Figure 4.2 (b). Eurycomanone can be detected in the chromatogram and it appears as the compound which gives the highest signal. The result is similar with studies conducted by Chua et al. (2011), Teh et al. (2011), Hassan et al, (2012), Al Salahi et al. (2013) and Low et al. (2013a) where they observed eurycomanone

was the most abundant compound in *E. longifolia* based on HPLC profiles. Chromatogram for negative control, which was a product which contained *Smilax myosotiflora* (Ubi Jaga) as constituent is in Figure 4.2 (c). No peak was detected at retention time of 7.0 min.

Figure 4.3 shows the chromatograms of (a) Tongkat Ali Merah and (b) Tongkat Ali Hitam. No eurycomanone peak which elutes at approximately 7.0 min can be observed in the chromatograms. Eurycomanone was absent in Tongkat Ali Merah and Tongkat Ali Hitam indicating that these plants have different phytochemical, thus verification of these plant using eurycomanone marker could not be utilized.

The abundance of eurycomanone in all products was calculated based on calibration curve of eurycomanone standard in Figure 4.1. From Table 4.6, it can be observed that freeze dried *E. longifolia* extracts in this study has the highest content of eurycomanone than the rest of the products. Eurycomanone level was calculated based on weight of eurycomanone in a sample volume. Even though a product claims to be 100% *E. longifolia* extracts, normally in a product, other non-herbal substance will be added to act as filler, carrier or stabilizer hence the lesser amount of eurycomanone in the product compared to freeze dried extract.

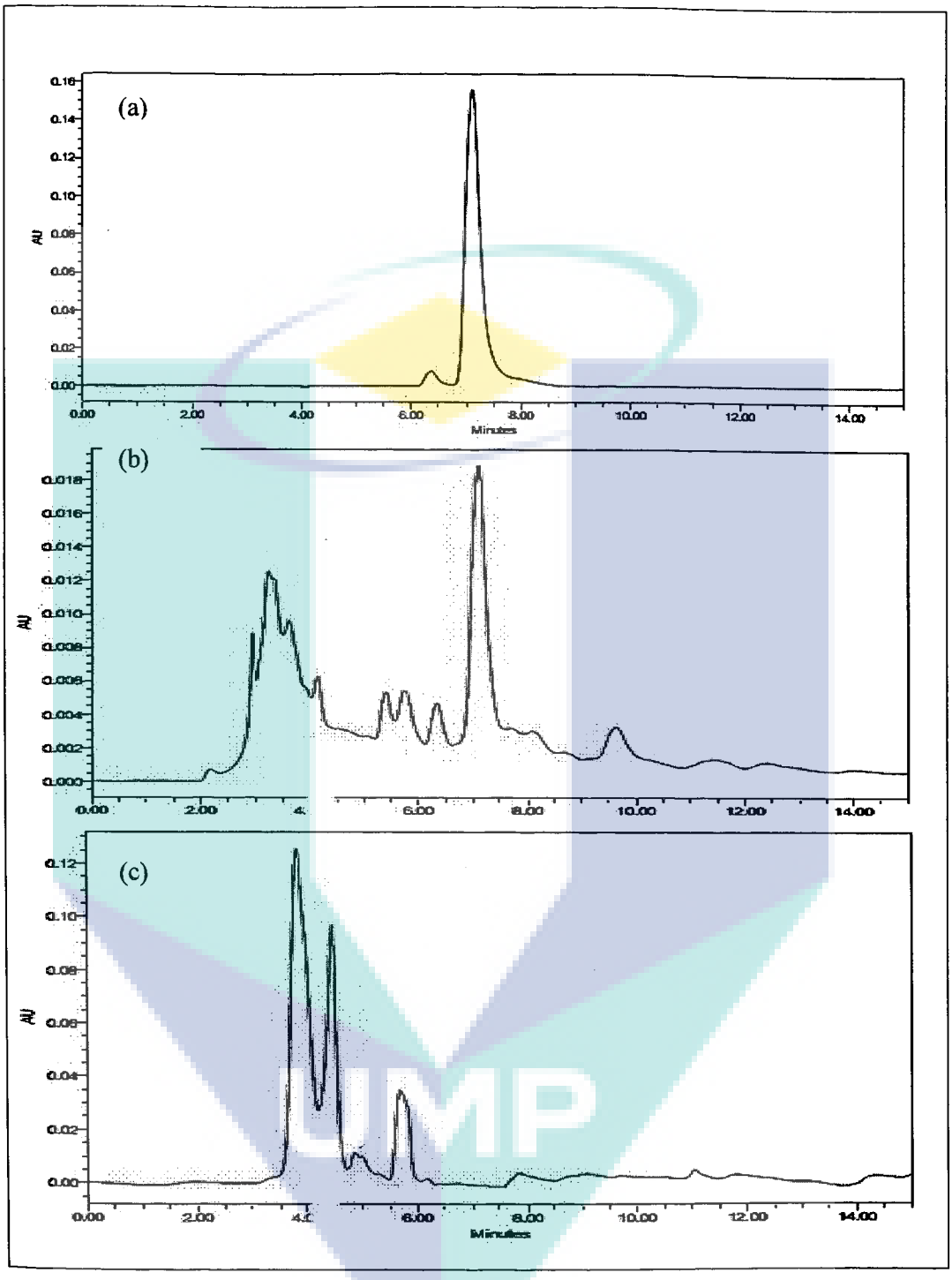

Figure 4.2: HPLC chromatogram of (a) eurycomanone standard, (b) freeze dried extracts, (c) negative control using water: acetonitrile, (86:14, v:v) as mobile phase, absorbance at 238 nm.Eurycomanone elutes at 7.08 min and can be detected in freeze dried extracts while no eurycomanone peak was detected in negative control, a product containing Ubi Jaga as single constituent.

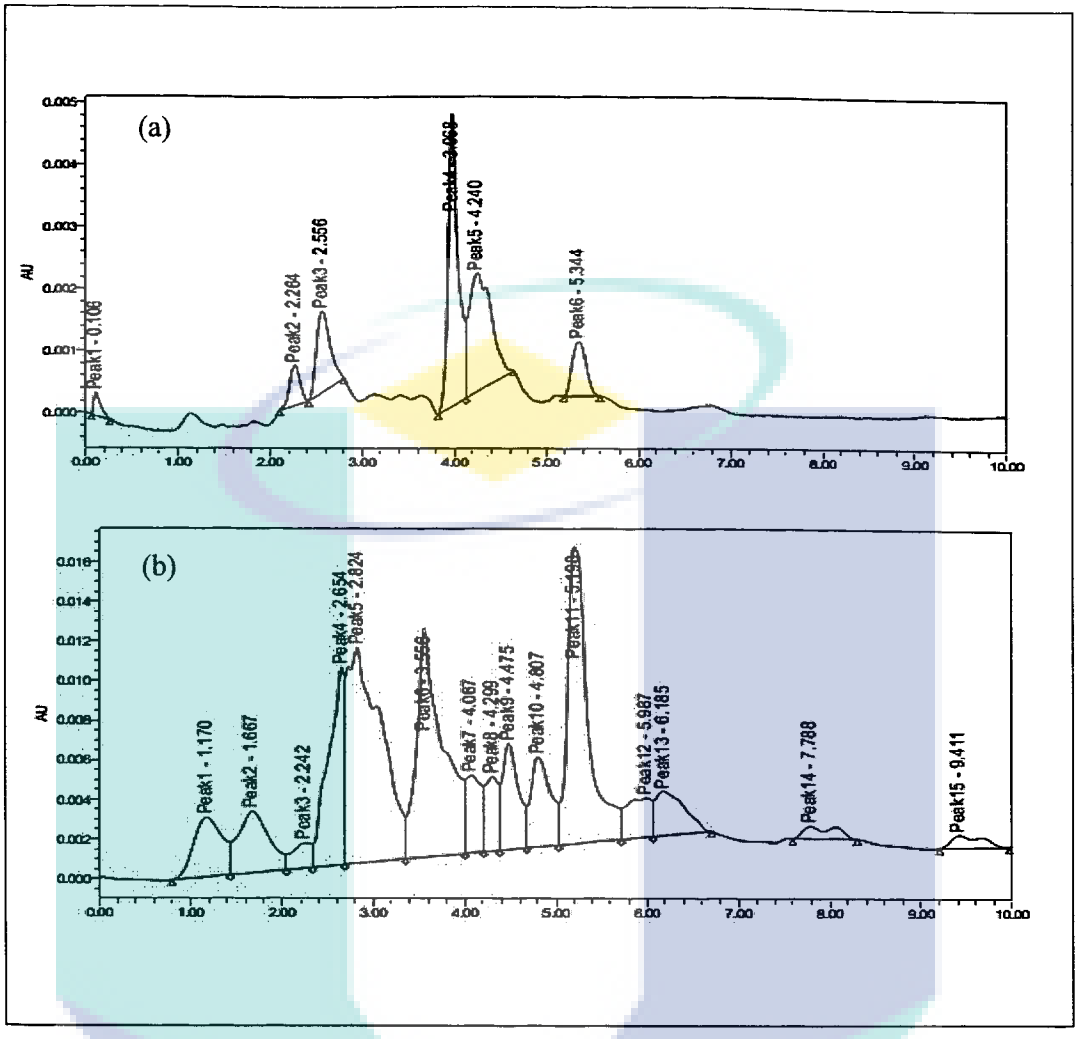

Figure 4.3: HPLC chromatograms of (a) Tongkat Ali Merah, (b) Tongkat Ali Hitam, using water: acetonitrile, (86:14, v:v) as mobile phase, absorbance at 238 nm. No eurycomanone peak which elutes at 7.0 ± can be detected in the chromatograms.

Besides eurycomanone which is from quassinoid, the majority groups in *E. longifolia*, which are the compounds from canthin-6-one alkaloids, β-carboline alkaloids, squalene type triterpene, and biphenylneolignans groups are also present in this plant. Based on the chromatogram of freeze dried extract in Figure 4.2(b), it can be observed that other compounds are present besides eurycomanone. Using HPLC, Hassan et al. 2012 detected compounds from canthin-6-one alkaloids; canthin-6-one and 9-methoxycanthin-6-one. However, those compounds were detected at different wavelength (366 nm) than the wavelength where eurycomanone was detected (245 nm). Furthermore, in the analysis, the standards of those two compounds were required. In quality control work, it

is not feasible where the HPLC analysis requires more than one standard of pure compound and the compounds need to be detected at different wavelength.

Chua et al. (2011) screened 56 quassinoids, 16 canthin-6-one alkaloids, 4 β-carboline alkaloids, 6 squalene type triterpene and 3 biphenylneolignans compounds in *E. longifolia* extract using a triple (Time of Flights) TOF mass spectrometer integrated to Ultra-Fast Liquid Chromatography (UFLC). They also managed to detect derivatives of eurycomanone; 13α(21)-epoxyeurycomanone, 12,15-diacetyl-13α(21)-epoxy-eurycomanone, 12-acetyl-13,21- dihydroeurycomanone, 15-acetyl-13α(21)-epoxyeurycomanone, 3,4ε-dihydroeurycomanone and 13, 21-dihydroeurycomanone. These compounds present in small amount in the plant and the detection require high end and high sensitivity. Some of these compounds also are not chromophore, thus cannot be detected by UV light in HPLC analysis.

A total of 50 *E. longifolia* products were analyzed for eurycomanone content using HPLC. Table 4.6 lists the properties of all samples including sample category, code name, dosage form, herb contents and eurycomanone contents. Some of the products contained *E. longifolia* as single constituent while some of the products were mixture of several herbs. Appendix B shows the chromatogram of all products, and eurycomanone can be seen to elute in the range from 7.02 to 7.15 min. From HPLC profiles in Figure 4.2 (b) and Appendix B, it can be seen *E. longifolia* freeze dried extracts and several products (C7, C14, C19, C29) that claimed to have *E. longifolia* as single ingredient have almost the same chromatographic profiles. However, there are some products (C5, C12, C36) that claimed to contain 100% *E. longifolia* but no eurycomanone peak was detected in the HPLC profiles. These results could suggest that regulating the quality of *E. longifolia* based on a marker, and in this case, eurycomanone can discriminate low quality or fake *E. longifolia* products.

Of all the products, product coded as C7 showed the highest level of eurycomanone. This product was a single formulation where *E. longifolia* was the only ingredient. Table 4.6 shows that 6 (C7, C2, C29, C41, C19 and C14) out of 10 or 60% products with the highest eurycomanone content consisted of products which contained solely *E. longifolia*. It can be generalized that single formulation has been found with higher amount of eurycomanone compared to those mixed herbal products. Product that contained multiple herbs would have more constituents relatives to product with single

formulation. The presence of more constituents may have reduced the concentration of *E. longifolia*.

It is interesting to note that only 4 (C14, C34, C40, C41) out of 12 international products or only 33.3% contained eurycomanone. *E. longifolia* plant is from South East Asia, and most people worldwide especially in the western world need to obtain the products from online sources. The International Products (IP) in this study were acquired from a reputable online shopping website; amazon.com. Search of *E. longifolia* in the website generated thousands of hits, indicating that most people outside of the plant endogenous region obtain the products through this website. This situation is a major concern because international products are relatively more expensive than local products but result from this study illustrates those international products of *E. longifolia* showed poor quality in terms of eurycomanone level whereby eurycomanone can be indicative of the presence of *E. longifolia* altogether.

E. longifolia premixed coffee is famous among Malaysian men. This type of coffee is easily available in eating outlet. Last year, 40000 sachets of fake *E. longifolia* premixed coffee were seized (The Star, 2014). This situation could reflect the high demand towards the product. However, in this current study, no eurycomanone was detected in any of eight premixed coffees tested. It is possible that the amount of *E. longifolia* that was put in the products was too little and beyond detection level. There is also possibility that no *E. longifolia* was added at all in the products. Due to this, unfortunately, it can be concluded that no benefits from *E. longifolia* can be obtained in consuming these beverages.

Table 4.6:
Eurycomanone levels in samples

No	Sample	Herbs content (as stated at the label of the packaging)	Eurycomanone level (w/v (%) ± SD)
1	Freeze dried *E. longifolia* extracts (Positive control)	*Eurycoma longifolia*	15.15 ± 0.20
2	Ubi Jaga (Negative control I)	Ubi jaga	nd
3	Gali Gali (Negative control II)	*Cornus officinalis, Turnera diffusa, Ptychopetalum olacoides, Piper retrofractie*	nd
1	Nu Prep Lelaki (C7) [MRP]	*Eurycoma longifolia*	8.48 ± 0.08
2	Ubi Jaga Tongkat Ali (C28)[MRP]	*Eurycoma longifolia, Striga asiatica, Allium sativum, Angullia angullia, Zingiber officinale, Smilax myositiflora*	3.82 ± 0.21
3	Fenugreek Tongkat Ali plus (C21) [MRP]	*Eurycoma longifolia*, Horny Goat weed, Fenugreek, *Trigonella foenum graecum, Epimedium brevicornum*	3.53 ± 0.15
4	Tongkat Ali Plus (C1) [MRP]	*Eurycoma longifolia, Epimedium Sagittatum*	3.33 ± 0.37
5	Wild Tongkat Ali (C29) [MUP]	Tongkat Ali	3.24 ± 0.06
6	Tongkat Ali Ginseng (C11) [MUP]	Tongkat Ali, Ginsing	2.95 ± 0.26

(C*): product code [MRP]: Malaysian Registered Product, [MUP]: Malaysian Unregistered Product [IP]: International Product n.d: not detected

Table 4.6 (continued):
Eurycomanone level in samples

No	Sample	Herbs content (as stated at the label of the packaging)	Eurycomanone level (w/v (%) ± SD)
7	Eury Goal Tongkat Ali Capsule (C2) [MRP]	*Eurycoma longifolia*	2.27 ± 0.07
8	Indonesia Tongkat Ali (C41) [IP]	*Eurycoma longifolia*	2.12 ± 0.48
9	Tongkat Ali (C19) [MUP]	Tongkat Ali	1.96 ± 0.32
10	Pure D *Eurycoma* longifolia (C14) [IP]	*Eurycoma longifolia*	1.72 ± 0.30
11	Formulations For Men (C27) [MRP]	*Eurycoma longifolia, Tribulus terrestris, Piper nigrum, Whitania somnifera, Eugenia aromatic, Centella asiatica*	1.66 ± 0.30
12	Ubi Jaga Plus (C26)[MRP]	*Eurycoma longifolia, Gonoiothalamus flubus, Smilax myostiflora, Smilax artaboltys, Freycinetia acuminata, Helminthostach zeylanica, Globia panicoides, Globia pendula.*	1.03 ± 0.24
13	100% Tongkat Ali and Ubi Jaga (C16) [MUP]	Tongkat Ali, Ubi Jaga	0.99 ± 0.43
14	Herb Natural Tongkat Ali (C40) [IP]	*Eurycoma longifolia*	0.84 ± 0.06

(C*): product code [MRP]: Malaysian Registered Product, [MUP]: Malaysian Unregistered Product [IP]: International Product n.d: not detected

Table 4.6 (continued):
Eurycomanone level in samples

No	Sample	Herbs content (as stated at the label of the packaging)	Eurycomanone level (w/v (%) ± SD)
15	Herba Tongkat Ali Plus (C6) [MRP]	*Eurycoma longifolia, Artaboltrys sp, Smilax myostiflora, Podocarpus neriifolius, Helminthostachs zeylanica Acalypa indica*	0.75 ± 0.37
16	Tongkat Ali Doctor Formulated (C34) [IP]	*Eurycoma longifolia*	0.55 ± 0.26
17	Ramuan Akar Kayu (C18) [MUP]	Tongkat Ali, Ranjang besi, Ranjang tembaga, Gajah beranak, Akar Pawang, Akar Gamat.	0.40 ± 0.20
18	Ubi Jaga Tongkat Ali Plus (C24) [MRP]	*Eurycoma longifolia, Smilax myosotiflora, Nigella sativa, Allomorphia malacensis, Trigonella foenum graecum, Zingiber minus, Leptospermum flavenscens, Acorus calamus, Coriandum sativum, Piper longum Trachyspermum ammi.*	0.36 ± 0.14
19	Jamu Sakti (C31) [MUP]	Tongkat Ali, Ubi Jaga, Tongkat Ali Hitam	0.31 ± 0.19
20	Ranjang besi, Tongkat Ali (C17) [MUP]	Tongkat Ali, Ubi jaga, Ranjang besi, Ranjang tembaga, Gajah beranak	0.26 ± 0.15
21	Ramuan 4 Jenis Akar (C15) [MUP]	Tongkat Ali, Ubi jaga, Ranjang besi, Akar pawang	0.22 ± 0.09

(C*): product code [MRP]: Malaysian Registered Product, [MUP]: Malaysian Unregistered Product [IP]: International Product n.d: not detected

Table 4.6 (continued):
Eurycomanone level in samples

No	Sample	Herbs content (as stated at the label of the packaging)	Eurycomanone level (w/v (%) ± SD)
22	Kapsul Tongkat Ali (C38) [MRP]	*Eurycoma longifolia*	0.19 ± 0.33
23	Orang kampong Tongkat Ali (C23) [MUP]	*Eurycoma longifolia, Pimpinella anisum, Cuminum cyminum, Zingiber officinale, Coriandum sativum, Alpina galanga, Curcumae zadoaria,* Garlic, *Stipegus variegatus, Piper nigrum,*	0.16 ± 0.17
24	Black Jack (C4) [MUP]	*Eurycoma longifolia, Polyathia bullata*	0.08 ± 0.22
25	Tongkat Ali Maca Plus (C8) [MRP]	*Eurycoma longifolia,* Maca, Ginger, Rice Bran, Black seed	n.d
26	Hurix's Tongkat Ali Plus (C9) [MRP]	*Eurycoma longifolia, Epimedium brevicornum, Actinolitum, Cynomorium songaricum, Cistanche deserticola, Cuscuta chinensis Cistanche Deserticola*	n.d
27	Tongkat Ali Hitam Plus (C20) [MRP]	*Eurycoma longifolia, Nigella sativa, Foeniculum vulgare, Globia pendula, Curcuma domestica, Nigris fructus, Languas galangal*	n.d

(C*): product code [MRP]: Malaysian Registered Product, [MUP]: Malaysian Unregistered Product [IP]: International Product n.d: not detected

Table 4.6 (continued):
Eurycomanone level in samples

No	Sample	Herbs content (as stated at the label of the packaging)	Eurycomanone level (w/v (%) ± SD)
28	Jamu Vigor For Men (C25) [MRP]	*Eurycoma longifolia, Freycinetia malaccensis, Curcuma xanthorriza*	n.d
29	Jamu Tongkat Ali (C3) [MRP]	*Eurycoma longifolia, Smilax myostiflora, Tinospora crispa,Ficus callicarpa, Nigella sativa, Zingiber officinale, Piper cubeba*	n.d
30	Jabbar 101 (C35) [MRP]	*Eurycoma longifolia, Cominum cyminum, Nigella sativa, Smilax myostiflore, Acorus calamus, Pimpinella anisum*	n.d
31	Tanduk Rusa (C10) [MUP]	*Eurycoma longifolia*, Ganoderma, *Hypocampus pantrocinum, Yohimbea*, Panax ginseng, *Syngnathoides biaculeatus*	n.d
32	Kapsul Tongkat Ali Hitam (C22) [MUP]	*Eurycoma longifolia, Zingiber officinale, Eugenia aromatica, Acorus calamus, Nigella sativa, Coriamdrum sativum, Piper longum, Trachyspermum ammi, Pimpinella anisum, Crodon caudatum*	n.d

(C*): product code [MRP]: Malaysian Registered Product, [MUP]: Malaysian Unregistered Product [IP]: International Product n.d: not detected

Table 4.6 (continued):
Eurycomanone level in samples

No	Sample	Herbs content (as stated at the label of the packaging)	Eurycomanone level (w/v (%) ± SD)
33	Urat Madu (C30) [MUP]	*Eurycoma longifolia*, *Miristica* fragrans, *Zingiber* sp, *Curcumae*, yohimbin	n.d
34	Pure Tongkat Ali 200:1 Extract (C5) [IP]	*Eurycoma longifolia*	n.d
35	Tongkat Ali Root Extract (C12) [IP]	*Eurycoma longifolia*	n.d
36	Unleash Your Beast (C13) [IP]	Tongkat Ali, Maca, Ginseng (Siberian/Korean)	n.d
37	Testosterone Booster (C32) [IP]	Tongkat Ali, Horney goat weed, Saw palmetto, orchic substance, Wild yam Sarsaparilla, nettle root	n.d
38	Longjack (C33) [IP]	Tongkat Ali, sarsaparilla, pumpkin powder, *Muira puama*, oat straw, nettle, cayenne paper, astragalus, Catauba licorice, *Tribulus terrestris* , orchic oyster	n.d
39	Unleash Power (C39) [IP]	Tongkat Ali, panax ginseng, horney goat weed *Xanthoparmelia scabrosa*, *Cnidium monieri* *Mucuna pruriens*, guarana, wild yam, maca	n.d

(C*): product code [MRP]: Malaysian Registered Product, [MUP]: Malaysian Unregistered Product [IP]: International Product n.d: not detected

Table 4.6 (continued):
Eurycomanone level in samples

No	Sample	Herbs content (as stated at the label of the packaging)	Eurycomanone level (w/v (%) ± SD)
40	*Eurycoma longifolia* jack extract (C36) [IP]	*Eurycoma longifolia*	n.d
41	Make My Pepper Big (C37) [IP]	Tongkat Ali, Maca, Ginseng (Siberian/Korean)	n.d
42	Maajun Pak Tani (C42) [MUP]	*Eurycoma longifolia, Angelica sinensis Parkia roxburghil, Curcuma xantthorria,* honey *Boswellia carteri, Nigella sativa, Piper nigrum Cuminum cyminum, Codonopsis pillosula*	n.d
43	Power Root Ali Cafe (D1) [B]	Ginseng, Tongkat Ali	n.d
44	Kopi Jantan (D2) [B]	Tongkat Ali, ubi jaga, ganoderma	n.d
45	Capuccino Tongkat Ali (D3) [B]	Tongkat Ali,	n.d
46	Mahkota Dewa (D4) [B]	Tongkat Ali, mahkota dewa	n.d
47	Tongkat Ali with Ginseng (D5) [B]	Tongkat Ali, ginseng	n.d

[MRP]: Malaysian Registered Product, [MUP]: Malaysian Unregistered Product [IP]: International Product [B]: Beverages n.d: not detected

Table 4.6 (continued):
Eurycomanone level in samples

No	Sample	Herbs content (as stated at the label of the packaging)	Eurycomanone level (w/v (%) ± SD)
48	Tongkat Ali and Cordiceps (D6) [B]	Tongkat Ali, cordiseps,	n.d
49	Jus 2 Tongkat (D7) [B]	Tongkat Ali, ubi jaga, Tongkat Samad, stevia,	n.d
50	Healthy Men Coffee (D8) [B]	Tongkat Ali, guarana, maca,	n.d
	Tongkat Ali Hitam Extracts		n.d
	Tongkat Ali Merah Extracts		n.d

[MRP]: Malaysian Registered Product, [MUP]: Malaysian Unregistered Product [IP]: International Product [B]: Beverages n.d: not detected

Table 4.7 shows that 24 or 48% of these products contained eurycomanone while no eurycomanone was detected in 26 products. The large part of products that did not contain eurycomanone consisted of Beverages (B) and International Products (IP).

Table 4.7:
Summary of products with (+) and without (-) eurycomanone

Sample	(+) Eurycomanone	(-) Eurycomanone
MRP	10	6
MUP	10	4
IP	4	8
Beverages	0	8
Total	**24**	**26**

Malaysian Standard (2011) stated that good quality of *E. longifolia* herbal products should contain minimum level of eurycomanone at the range of 0.8 to 1.5 w/v (%). Table 4.6 lists the level of eurycomanone in all products from the highest amount to the lowest. Out of 24 products that contained eurycomanone, 11 of the products (C7, C28, C21, C1, C29, C11, C2, C41, C19, C14, C27) had eurycomanone level above 1.5 w/v (%). Three out of 24 products (C26, C16, C40) showed eurycomanone level above 0.8 but below 1.5 w/v (%), while 10 out of 24 products (C6, C34, C18, C24, C31, C17, C15, C38, C23, C4) showed eurycomanone level below 0.8 w/v (%).

Some products (C11, C19 and C16) (MRP) showed high level of eurycomanone even though the product did not register with Drug Control Authority (DCA), Ministry of Health (MOH) of Malaysia. This is partly because the processing steps of *E. longifolia*

products are so simple, even the cottage industry can get involve easily and probably unaware of the necessities to register their products.

It can be observed that some of the products (C3, C8, C9, C20, C25 and C35) (MUP) were devoid of eurycomanone even though the products have been registered with herbal regulatory body. Thus it can be generalized that product registration offers assurance on product safety but not the quality.

Eurycomanone was chosen as chemical marker for *E. longifolia* because it is the major compound of this plant (Pan et al. 2014), thus it can signify and quantitate the level of *E. longifolia* in a product. The most abundant compound in a herb, does not necessarily be the characteristic compound with the bioactive property. However, it can be applied for qualitative and quantitative analysis of herbal products (Li et al. 2008).

Chemical marker can be categorized into analytical marker and active marker. Active marker is the marker that contributes to therapeutic effect while analytical marker serves only for analytical purpose. However, all markers can be applied in evaluation and standardization of herbal products. (Li et al. 2008). In this study, it is not particularly clear how to categorize eurycomanone. This is because, in all published reports of *E. longifolia* pharmaceutical effects, only one literature reported the therapeutic effects of eurycomanone; Low et al. 2013 (b) which indicated that eurycomanone could increase spermatogenesis. Surprisingly though, researches on *E. longifolia* spanned from early 70s, but this major compound which have been isolated has never been proven to produce the aphrodisiac activity until today.

Evaluation of herbal products quality is a challenging task since the composition of phytochemicals varies greatly from batch to batch (Charoonratana et al. 2014). The great variability can be caused by genetic factors so even plant from the same species will produce unequal amount of composition. Geographical or ecological factors such as climates, temperatures, lights, rainfalls, altitudes, soil quality, humidity, seasonal changes will also influence the composition of phytochemicals in plants. Other factors that can lead to differences in quality are extraction and manufacturing processes such as cleaning, drying, grinding, storage, packaging, transport. Study by Kumaresan and Sarmidi, (2003) showed that extraction parameter influenced the quantity of phytochemical extracted from *E. longifolia* while Chua et al. (2011) showed that *E. longifolia* from different geographical terrains contained different metabolites profiles. *E. longifolia* usually is

harvested at mature age. But it is possible for manufacturers to harvest the root at earlier time to fulfil the demands toward *E. longifolia* products. In this regards, quality control of *E. longifolia* products need to be more rigorous and improved.

4.4 Analysis of *Eurycoma longifolia* Products Using Protein Marker

4.4.1 Protein Content

Next sections deal with protein analysis in *E. longifolia* products and gel electrophoresis was one of the analytical methods. Protein in all products was quantified using Bradford assay prior to gel electrophoresis. Bradford assay has been the method of choice due to the high sensitivity, linearity and speed of analysis (Redmile-Gordon et al. 2013). Bovine Serum Albumin (BSA) acted as reference protein standard. The protein content was measured based on calibration curve of BSA protein standard in Figure 4.4.

Table 4.8 lists the protein contents of all products. From the table, it can be observed that Beverages products (D1-D8) have higher protein content than the rest of the products. These products contained relatively more non herbal constituents compared to other products from different category. The non herbal ingredients were mostly milk and creamer, which are generally good source of protein. The results of these protein contents would be clearly manifested in gel electrophoresis analysis that will be discussed in the next section. Products with relatively higher protein contents would show multiple protein bands or band with higher color intensity.

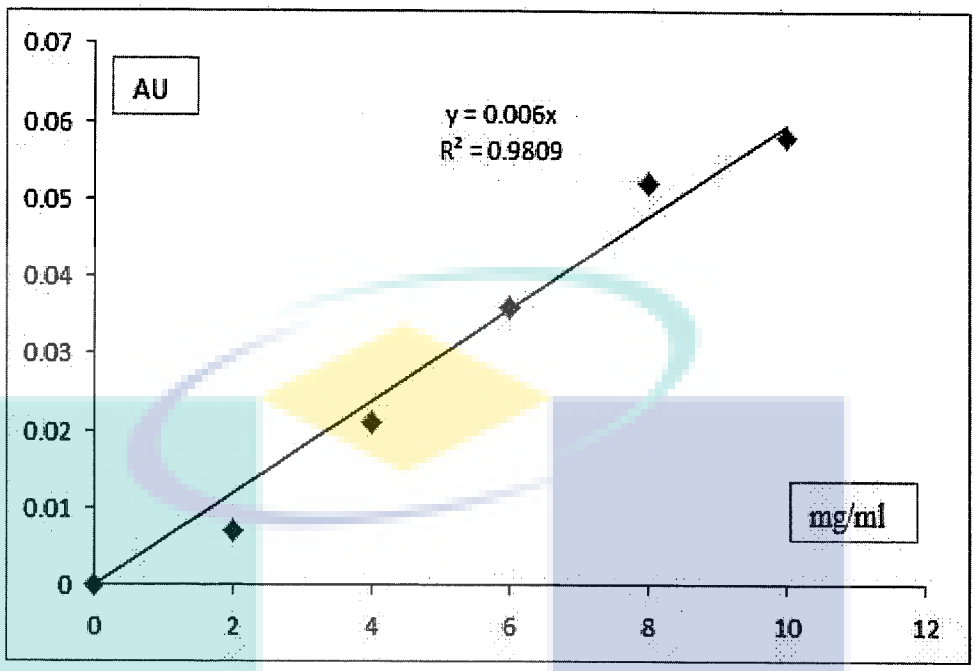

Figure 4.4: Calibration curve of Bovine Serum Albumins (BSA) standard. Absorbance was taken at 595 nm using UV Visible Spectrophotometer.

Table 4.8:
Protein contents in *E. longifolia* products

No	Sample Code	Protein content µg/mg ± SD	amount used in 2DE and SDS PAGE (mg)	Non-herbal constituents (listed at packaging label)
1	E.L root extracts	72.76 ± 0.03	0.275	n.l
2	T.A. Hitam	1.12 ± 0.04	20.212	n.l
3	T.A.Merah	0.72 ± 0.31	23.416	n.l
4	C1	3.14 ± 0.44	1.440	n.l
5	C2	6.20 ± 0.27	3.220	n.l
6	C3	62.60 ± 0.53	0.310	n.l
7	C4	2.51 ± 0.07	1.304	n.l
8	C5	10.49 ± 0.05	1.907	n.l
9	C6	21.23 ± 0.41	0.942	n.l
10	C7	9.38 ± 0.15	2.133	n.l
11	C8	19.11 ± 0.89	1.046	rice bran
12	C9	15.78 ± 0.24	1.267	n.l
13	C10	2.66 ± 0.31	7.530	n.l
14	C11	11.81 ± 0.46	1.690	n.l
15	C12	39.00 ± 0.21	0.512	n.l
16	C13	35.98 ± 0.33	0.550	zinc oxide, maca, argenine
17	C14	17.58 ± 0.42	1.1378	n.l
18	C15	20.01 ± 0.59	0.990	n.l
19	C16	17.42 ± 0.13	1.140	n.l
20	C17	14.08 ± 0.64	1.420	n.l
21	C18	5.41 ± 0.71	3.698	n.l
22	C19	11.65 ± 0.64	1.710	n.l
23	C20	96.07 ± 0.78	0.208	n.l
24	C21	2.41 ± 0.34	1.611	n.l
25	C22	59.91 ± 0.57	0.330	n.l
26	C23	121.49 ± 0.68	0.164	n.l
27	C24	53.28 ± 0.63	0.375	n.l
28	C25	59.27 ± 0.28	0.330	n.l
29	C26	72.13 ± 0.53	0.277	n.l
30	C27	30.86 ± 0.33	0.648	n.l
31	C28	51.23 ± 0.68	0.390	n.l
32	C29	9.90 ± 0.09	2.020	n.l

TA: Tongkat Ali, nl; not listed

Table 4.8 (continued):
Protein content in *E. longifolia* products

No	Code	Protein content µg/mg ± SD	Amount used in 2DE and SDS PAGE(mg)	Non-herbal constituents (listed at packaging label)
33	C30	30.95 ± 0.48	0.633	n.l
34	C31	4.83 ± 0.47	4.135	spices
35	C32	13.46 ± 0.56	1.380	boron amino chelate, calsium carbonate, microcrystalline cellulose, stearic acid,
36	C33	1.86 ± 0.12	10.770	boron, oat straw, pumpkin powder
37	C34	5.77 ± 0.37	3.460	rice flour, gelatin, silicon dioxide, magnesium stearate
38	C35	7.52 ± 0.57	2.650	n.l
39	C36	25.14 ± 0.25	0.795	n.l
40	C37	6.41 ± 0.42	3.110	n.l
41	C38	10.54 ±0.04	1.890	n.l
42	C39	4.83 ± 0.51	4.145	n.l
43	C40	54.03 ± 0.72	0.370	n.l
44	C41	67.78 ± 0.63	0.464	n.l
45	C42	13.56 ± 0.57	1.470	n.l
46	D1	44.03 ± 0.33	0.454	non dairy creamer, natrium cassinate, emulsifier,
47	D2	1.00 ± 0.41	19.928	creamer, sugar, coffee maltodextrin
48	D3	135.62 ± 0.68	0.147	creamer, sugar, coffee
49	D4	91.65 ± 0.35	0.210	creamer, sugar, coffee
50	D5	20.22 ± 0.47	0.980	sucrose, creamer, coconut
51	D6	69.91 ± 0.36	0.286	sugar, creamer, coffee powder, food conditioner
52	D7	1.46 ± 0.08	8.450	mollasses, stevia, distilled water
53	D8	63.56 ± 0.54	0.310	sugar, instant coffee non dairy creamer
54	NC1	21.97 ± 0.21	0.910	n.l
55	NC2	17.21 ± 0.11	1.162	n.l

4.4.2 Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS PAGE)

As previous section deals with chemical marker which is the eurycomanone, this section is about developing protein marker in authenticating quality of *E. longifolia* products. Sodium dodecyl sulfate polyacrylamide gel electrophoresis or SDS PAGE is a simple, one dimensional gel electrophoresis technique where protein was separated according to molecular size. In this study, SDS PAGE was performed to obtain an overview about the protein profile in *E. longifolia* and the products.

Analytical SDS PAGE showed that only one protein band was observed in *E. longifolia* extracts. After analysis in several gels, it is evident from Figure 4.5 that the band appeared as a thick band. As the protein band from the extract did not match to any protein standard, calibration curve (Figure 4.6) of log molecular weight of the protein standard against migration distance (Rf) was generated. Protein from *E. longifolia* extracts had Rf of 0.83. Extrapolation of the graph revealed that the log molecular weight of 0.83 Rf was 1, which means that the molecular weight of the protein could be 10.0 kDa.

Theoretically, products that claim to be containing *E. longifolia* should show the same band in protein profiling by SDS PAGE. It is proven in Figure 4.7. It can be observed that products C14, C41, C34 which contained *E. longifolia*, as evidenced from the presence of eurycomanone in Table 4.6, also showed the same distinctive band as the extracts. Products C41 and C34, which contained solely *E. longifolia* displayed the same protein profile as the extracts while product C14, which contained multiple herbs including *E. longifolia,* showed multiple bands.

However, from Figure 4.7, it can be seen that product C29, which listed *E. longifolia* as single ingredient showed multiple protein band. It is assumed that, besides *E. longifolia*, this product contained undisclosed ingredient such as filler or carrier, and the protein containing carrier contributed to the multiple bands.

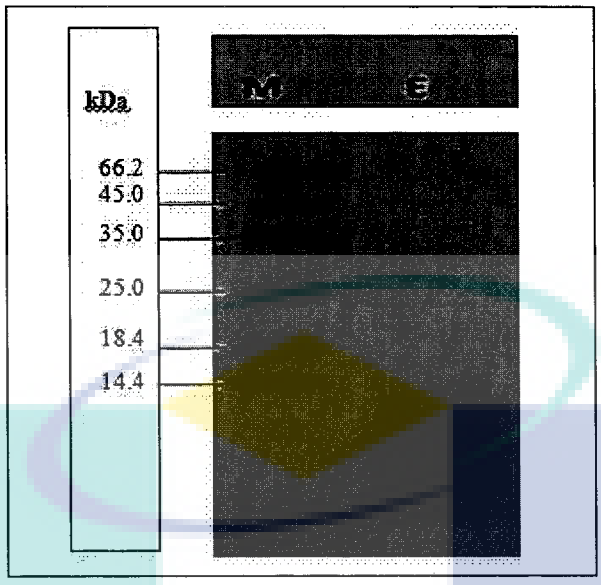

Figure 4.5: Coomassie stained, SDS PAGE gel of low molecular weight marker (M) and freeze dried extracts (E). Single protein band can be observed in the extracts.

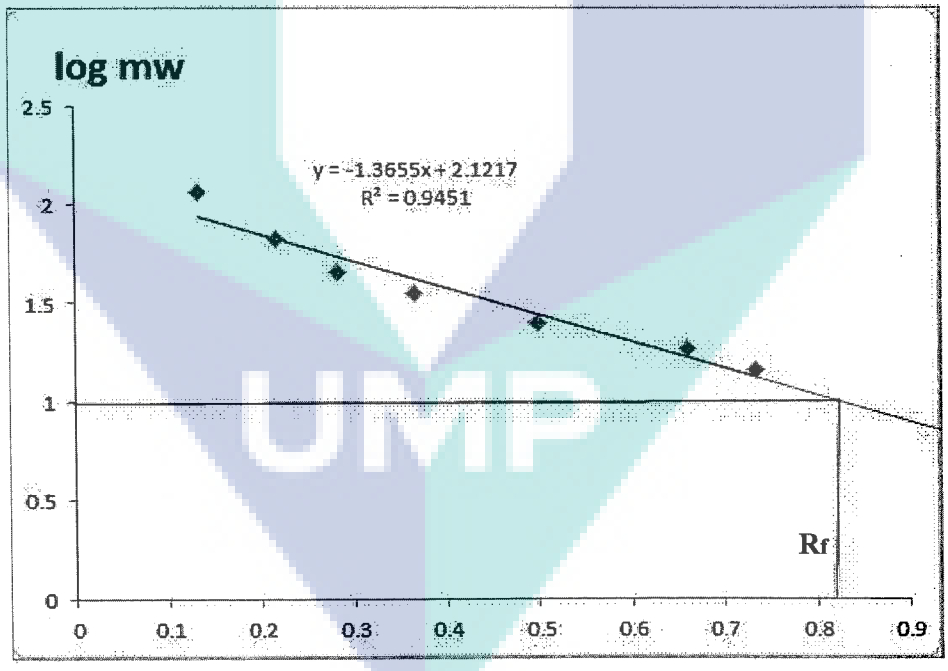

Figure 4.6: Calibration curve of log molecular weight (log mw) of protein standard againts migration distance (rf). Migration distance of protein from *E. longifolia* extracts is 0.83, with log mw of 1.

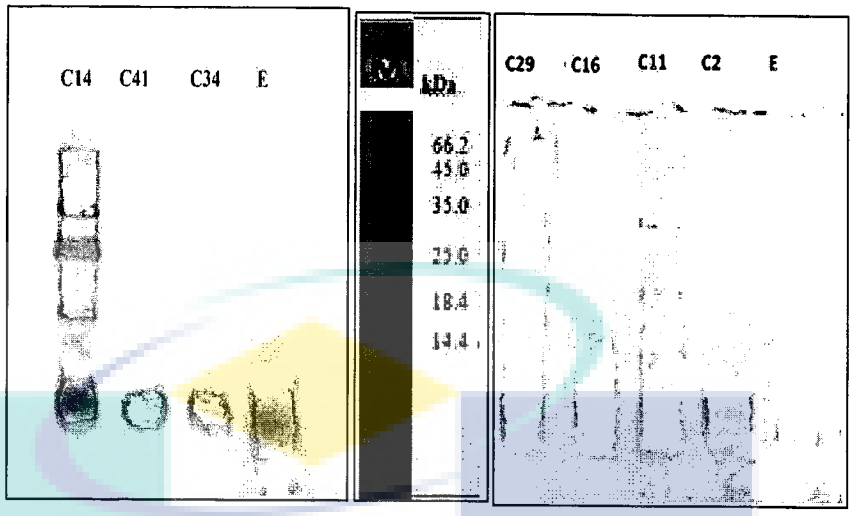

Figure 4.7: Coomassie stained, SDS PAGE gels of crude extracts and products containing *E. longifolia*. A protein band, which have the same molecular weight with the extracts (E), can be observed in products coded as C14, C41, C34, C29, C16, C11, C2. M is low molecular weight marker.

Negative control products, NC1 and NC2 showed a very dissimilar pattern with the extracts. Figure 4.8(a) indicates clearly that the products did not have the same distinctive band as the extracts. Other products (C37, C36, C35, C10, C9) in Figure 4.8 (b) that were proven did not contain *E. longifolia* based on the absence of eurycomanone, also did not show the same band as the extracts.

All beverages products (Figure 4.8 (c), which did not contain eurycomanone, did not have the same band as the extracts. However, as these products were higher in protein content as revealed by Bradford assay, multiple protein bands can be observed in the gels. Tongkat Ali Hitam and Tongkat Ali Merah showed the absence of the single protein band.

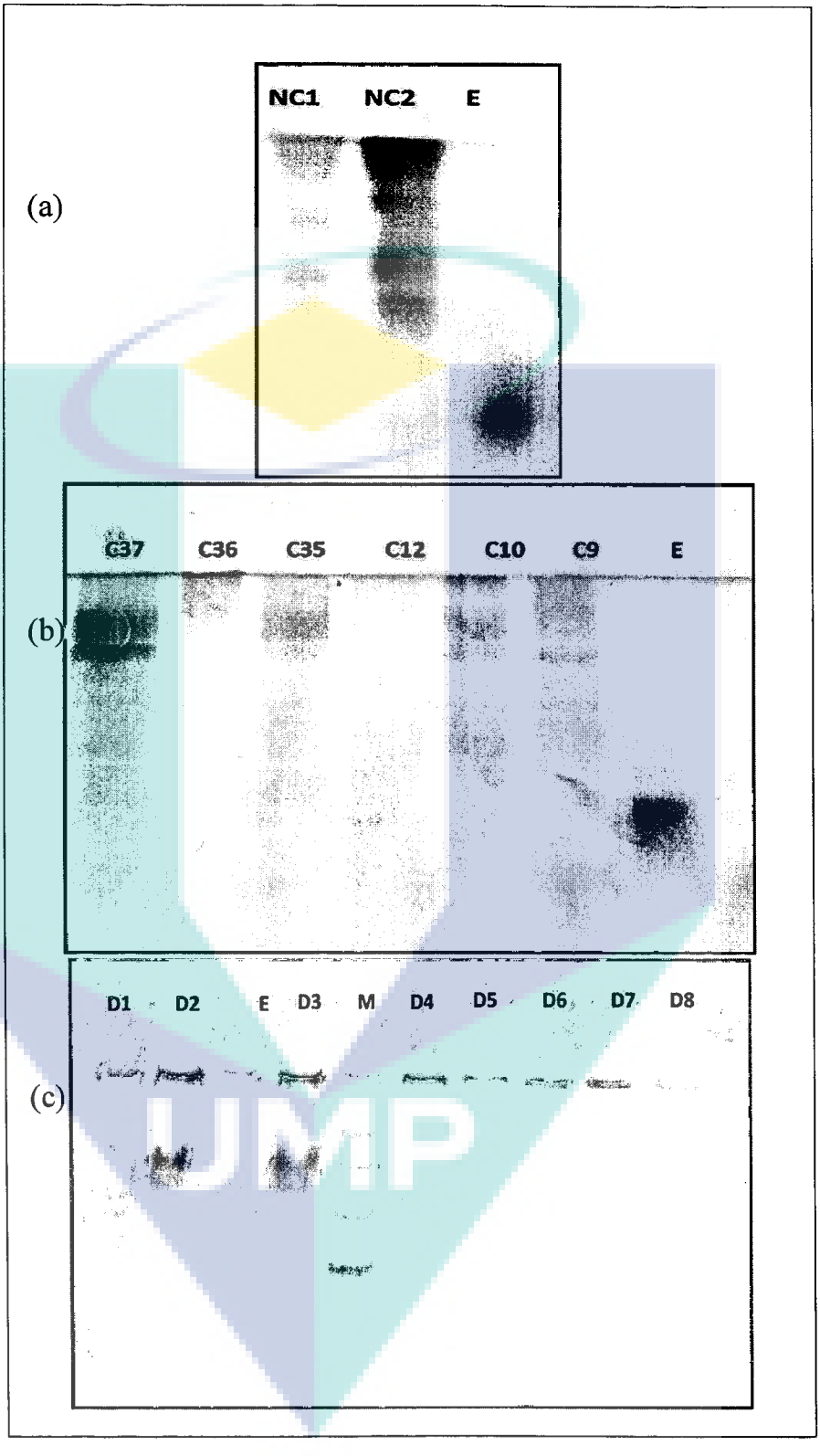

Figure 4.8: Coomassie stained, SDS PAGE gels of *E. longifolia* products. No band with similar molecular weight of the extracts (E) can be observed in the products (a; NC1, NC2), (b; C37, C36, C35, C12, C10, C9), (c; D1, D2, D3, D4, D5, D6, D7, D8).

Protein profiles for the rest of the products are in Appendix C. The analytical SDS PAGE in this study was carried out on the crude of the products without any protein extraction. Thus it can be seen that lots of artifacts formation and streaking effect in the protein band profiles. The clarity and the appearance of the bands might be disrupted by the non protein constituents such as lipids and nucleic acids that were not removed from the samples.

From this analytical SDS PAGE of the protein in the extracts and the products, it can be concluded here that products that contained *E. longifolia* would have signature protein band in SDS PAGE at 10.0 kDa in size. The results showed that this analysis could discriminate products that did not contain *E. longifolia*, based on the presence of the distinctive protein band. Since SDS PAGE is only one dimensional separation of protein, 2 dimensional gel electrophoresis (2DE) was carried out to further characterize the protein.

4.4.3 2 Dimensional Electrophoresis (2DE)

Previous sections were on eurycomanone which is the chemical marker for *E. longifolia* and protein profiling by SDS PAGE. SDS PAGE results indicated the presence of single band in *E. longifolia* crude extracts which possibly could indicate the presence of single protein. Therefore, attempts were made to discover more about the protein in *E. longifolia* using 2 Dimensional Gel Electrophoresis (2DE). This is due to the ability of 2DE analysis to separate protein based on protein iso electric point besides protein molecular weight. Furthermore, the resolving power of 2DE is superior to any other protein separation techniques (Gopalakrishnan et al., 2015). It also has the ability to resolve similar but differentially modified forms of a given protein (Jensen, 2004). Furthermore, Vejayan et al. (2013), found a protein that could be a marker in *E. longifolia* products using 2DE. This section focuses on the analysis of *E. longifolia* products using 2DE and the possibility of utilizing protein as marker in the products.

The products were separated using 2DE with 7 cm IPG strip of pI 3 -10 and stained with Coomassie blue. 2DE analysis in this study was commenced on crude extracts which served as positive control and protein spots that appeared in the crude extracts were made

as reference. A product without *E. longifolia,* but listed *Smilax myosotiflora* as single ingredient was the negative control.

Figure 4.9 is representative of 2DE analysis of *E. longifolia* crude extracts. It could be observed that after several runs, four reproducible spots appeared in the gel. The four spots were denoted as A, B, C and D and the four spots indicated that the protein are similar in size but differ in iso electric point. The spots migrated with similar distance as protein band in SDS PAGE where the protein molecular weight was estimated to be 10.0 kDa.

Crude extracts of *E. longifolia* was also tested on 17 cm IPG strip. The profile was the same as using 7 cm IPG strip where four spots could be observed (Figure 4.10), but the separation of the spots was more defined in 17 cm strip gel. However, since both strips gave almost the same profile, the rest of the products were analyzed using 7 cm strip, while 17 cm strip was used in protein characterization analysis.

Gels were stained with Coomassie stain as silver staining yielded no spots (Figure 4.11). Based on literatures, it can be said that the proteins in *E. longifolia* are probably glycosylated. Silver stain dye are known to be less sensitive for detection of highly glycosylated protein (Harvey et al. 1997; Moller and Poulsen, 2009).

The protein is also said to be glycoprotein based on the diffused band and spot appearance in SDS PAGE and 2DE gel (Figure 4.9). According to Zhu and Zhou, (2005), protein with high sugar content tends to have anomalous migration behavior and the band will become diffuse and wider than protein with low sugar content. This is due to carbohydrate units have greater hydration in water solution because of their hydrophilic properties, which could cause the protein band to have expansive structure in the gel. This was proven by the gels in Figure 4.12. In the figure, gel labelled as A, B, and C are gels of crude extracts, crude extracts incorporated with maltodextrin, and cyclodextrin respectively. Maltodextrin or cyclodextrin are carriers that usually added into a product to reduce hygroscopicity and increase photostability of the product (Goula, and Adamopoulos, 2008; Jamrogiewicz et al. 2014). As can be observed from those gels, preparing the crude extracts with maltodextrin and cyclodextrin, which are carbohydrate polymers, caused the protein spots to have more expansive or streaking structure compared to protein spots without the carbohydrate polymer.

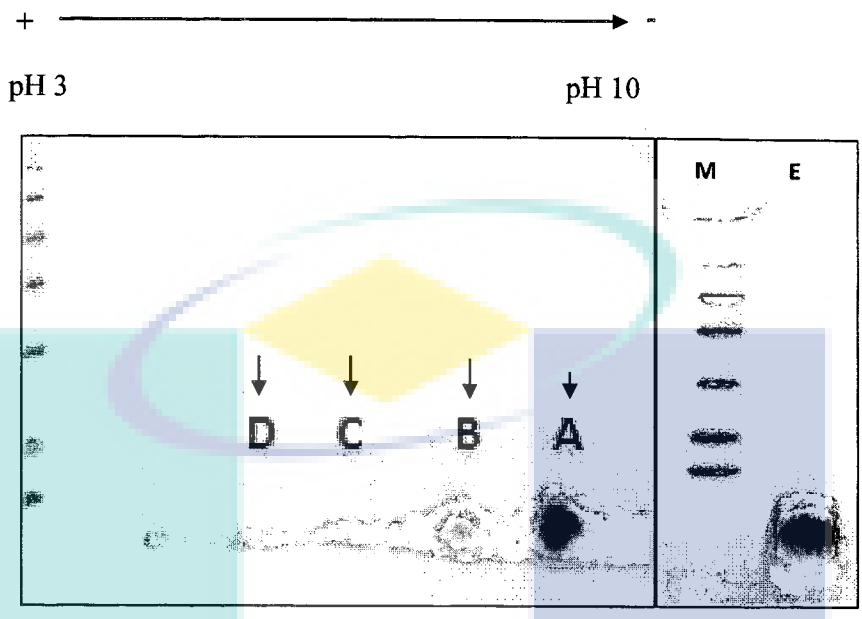

Figure 4.9: Coomassie stained, 2DE (left) and SDS PAGE (right) gels of crude extracts. 2DE gel displays the presence of four protein spots (A, B, C, D) with same molecular weight but different in iso electric point. Gel in SDS PAGE shows one protein band.

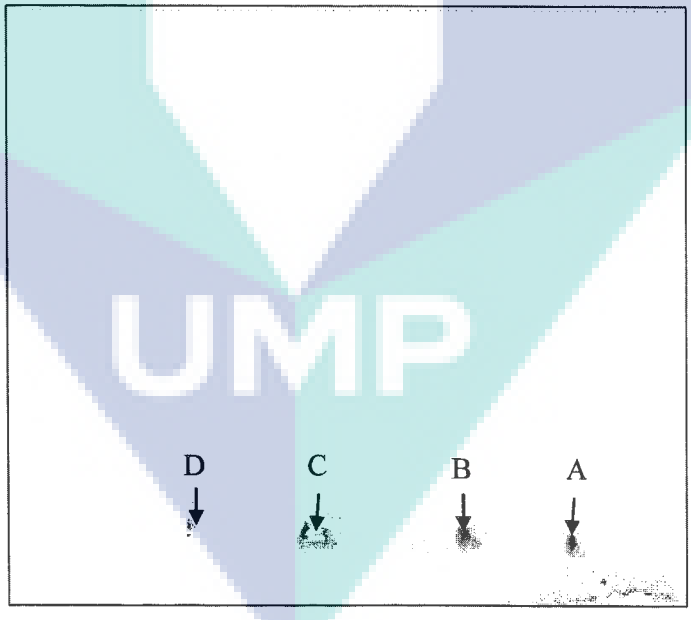

Figure 4.10: Coomassie stained, 2DE gel of 17 cm IPG strip. Defined separation of four spots (A, B, C, D) can be observed.

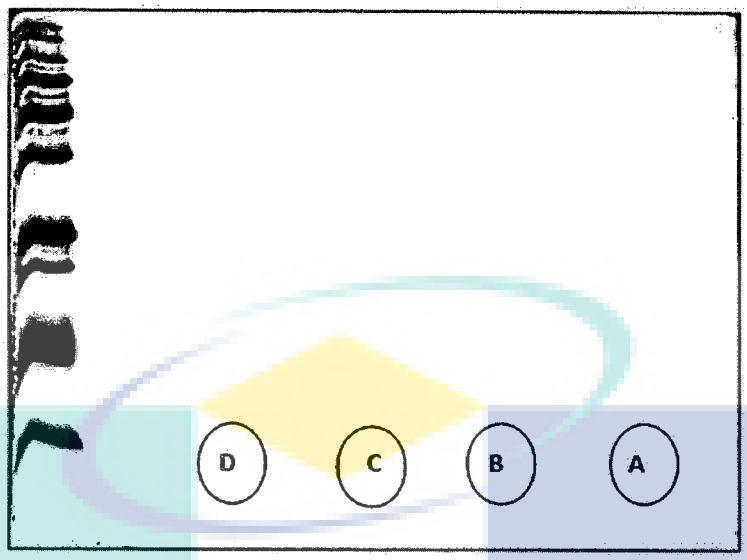

Figure 4.11: Silver stained, 17cm strip, 2DE gel of crude extracts. No protein spots can be observed. Circles are where the protein markers (A, B, C, D) should be spotted.

The four protein spots in 2DE gel were of the same size and different pI. It can be deduced here that the protein could be isoform. Protein isoform is caused by post-translational modifications, where native protein undergoes change of surface charge or conformation and it can be manifested by change in pI (Jensen 2004). A series of protein spots with the same molecular weight but of differing pI suggests that the protein undergoes post-translational modifications (Kwon et al., 2006).

Techniques of iso electric focussing itself can cause protein modifications and it can be minimized by increasing solubility, reduction, and distrupt the non covalent interation by the use of detergent, DTT and urea (Du and Mcmanus, 2011). In this study, all of these ingredients were included, thus it can be deduced here that the pI heterogeinity observed in *E. longifolia* protein is probably caused by post translational modification.

Post translational modification can be phosphorylation, glycosylation, acetylation and myristilation (Jensen 2004). Based on the diffused band and spots in gels as discussed above, probably the protein in *E. longifolia* undergoes post translational modification by glycosylation. Detection methods such as metabolic labelling, affinity enrichment, affinity tagging, mass tagging, can be applied to confirm the type of modification. Unfortunately in this study there was no such detection methods carried out but it would be interesting to venture into this in future studies.

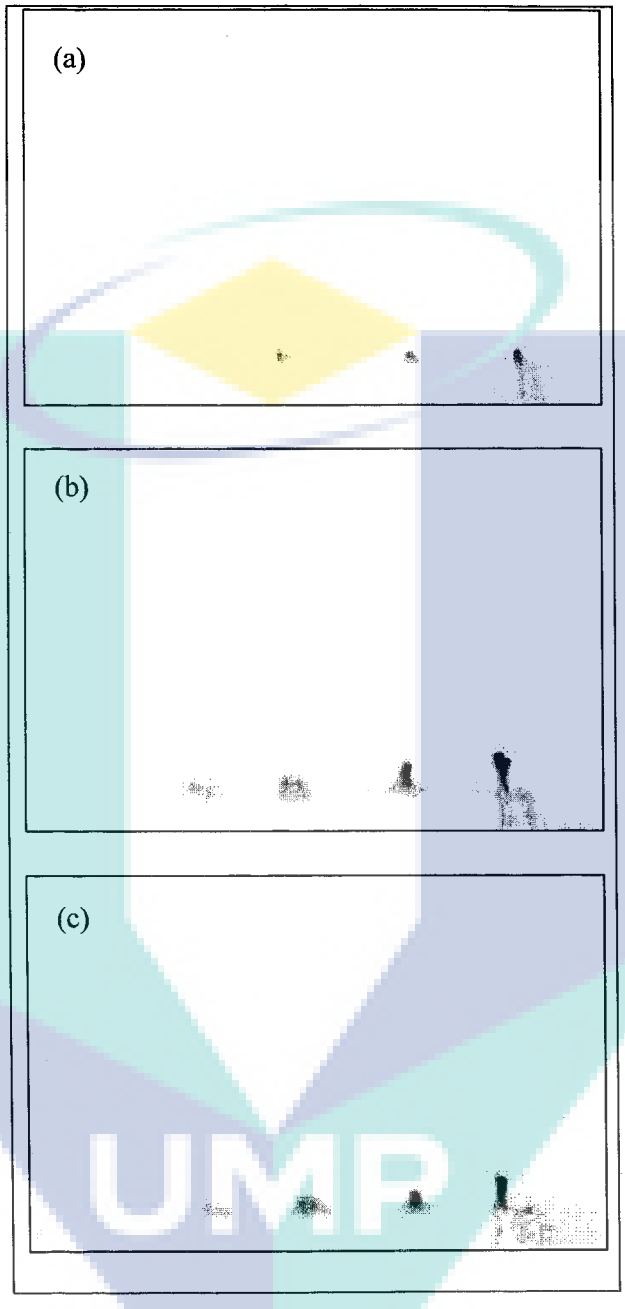

Figure 4.12: Coomassie stained, 17cm strip, 2DE gel of (a) crude extracts, crude extracts incorporated with excipients (b) maltodextrin, (c) cyclodextrin. Spots in gel b and c appear to be expanded upon incorporation with the excipients.

For negative control (Figure 4.13), there was the absence of the signature four spots. Figure 4.14 shows the 2DE gel of a product (C2) which contained *E. longifolia* as proven by eurycomanone analysis. Spot A, B and C can be observed clearly, but spot D is absence. This product contained only *E. longifolia* as listed at the product label, so no other protein spot besides spots that are characteristic of *E. longifolia* could be seen. While a product, (C28, Figure 4.15) is a product which contained *E. longifolia* and multiple other herbs. It can be seen from the 2DE gel that the product had a spot that is characteristic of *E. longifolia* which is spot A. However, as this product contained multiple herbs, other protein spots could also be observed in the gel. Figure 4.16 indicates the gel of a product (C5) that did not contain *E. longifolia* as proven by eurycomanone analysis. The gel showed other protein spots but there is no defined spot(s) of *E. longifolia* protein.

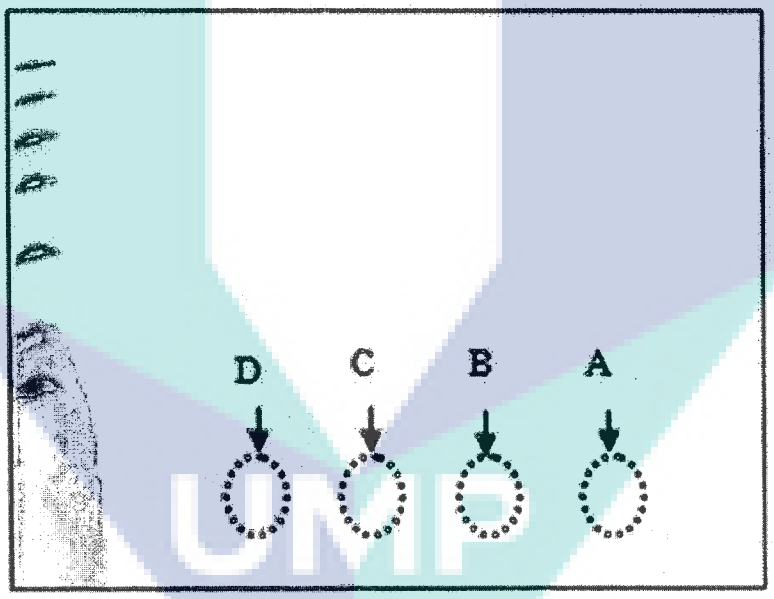

Figure 4.13: Coomassie stained, 7 cm IPG strip, 2DE gel of negative control. The dotted circles are area where the protein marker should present.

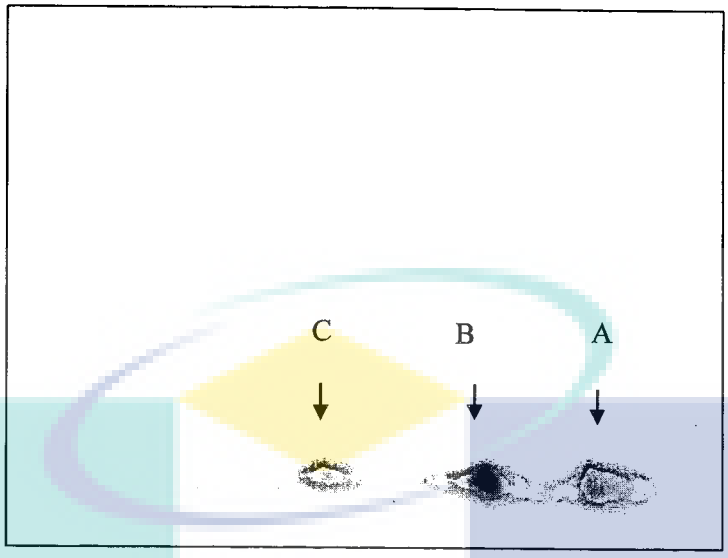

Figure 4.14: Coomassie stained, 7 cm IPG strip, 2DE gel of a product (C2) which contained only *E. longifolia*. No other protein spots present besides *E. longifolia* signature protein spots (A, B, C).

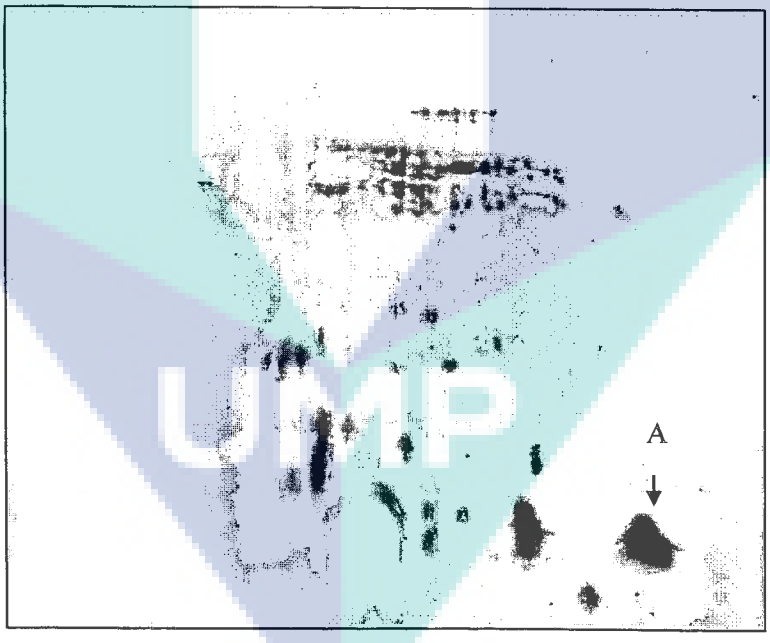

Figure 4.15: Coomassie stained, 7 cm IPG strip, 2DE gel of a product (C28) which contained *E. longifolia* and other herbs. Besides spot from *E. longifolia* (A), other protein spots can be observed.

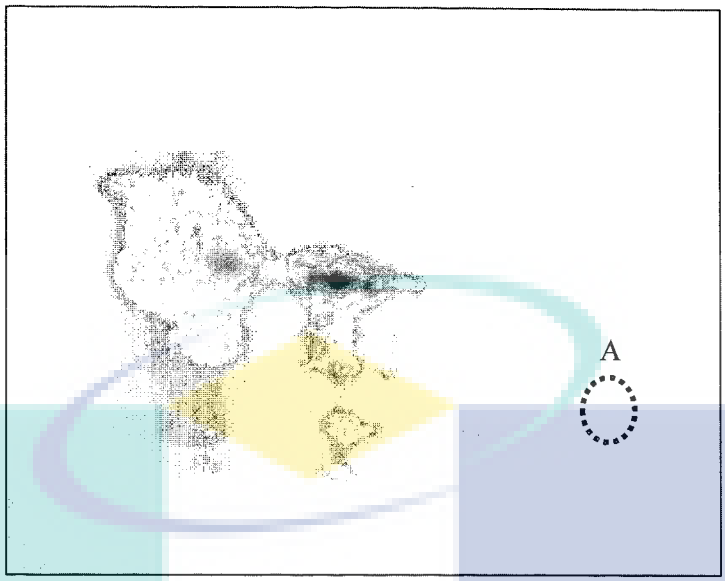

Figure 4.16: Coomassie stained, 7 cm IPG strip, 2DE gel of a product (C5) which did not contain *E. longifolia*. The dotted circle is where Marker A should present.

Tongkat Ali Hitam and Tongkat Ali Merah showed the absence of protein markers (Figure 4.17). This indicated that these two plants, with the same root morphology and local name, have different profile than *E. longifolia* roots.

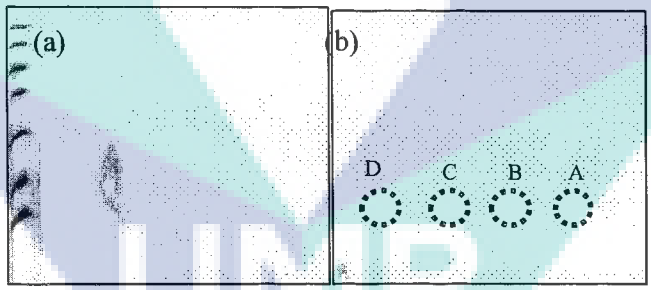

Figure 4.17: Coomassie stained, 2DE gels of (a) Tongkat Ali Hitam, (b) Tongkat Ali Merah. The dotted circles are where the protein marker(s) should present.

All beverages products showed the absence of the protein marker. These products, which mostly consisted of premixed coffee, were also devoid of eurycomanone, indicating that no *E. longifolia* was incorporated into these products or the concentration was too low to be detected.

Figures of the gels for the rest of the commercial products are in Appendix D. The streaking and smearing of protein spot can be observed in almost entire products. Products that contained multiple herbs were more prominent to have the problems. 2DE analysis was carried out on the crude of the products, without protein extraction process. The presence of non protein molecules such as salt, DNA and phenolic compounds might disturb the iso electric focusing process (Lee and Chang, 2009). It was also noticed that some products faced water solubility problem. This is probably because, in the manufacturing process, the herbs in some products probably were only dried and grinded, without the extraction and freeze drying process. Insoluble particles can also disturb the 2DE analysis.

Four protein spots; A, B, C and D could be observed in crude extracts. It displayed all of the spots probably because it had the highest *E. longifolia* contents as proven by eurycomanone analysis. Other products were lower in *E. longifolia* as the content in the volume was somehow diluted by the presence of compounds such as fillers and other herbs. In most of the products, only spot A and B, were more distinctive. Both spots appeared at molecular weight below 10.0 kDa, at basic extremities of the gel. However, Spot or Marker A was chosen as the ultimate marker as it was more prominent and consistently present. Marker A would be isolated, characterized further and will be discussed in next section.

4.5 Protein Marker Purification

The current research studied the potential of this protein to be utilized as marker for *E. longifolia* products. Based on the results of 2 dimensional gel electrophoresis (2DE) in section 4.4.3, isolation and characterization of Marker A are essential tasks. This section reports the successful isolation of Marker A.

Typically, protein purification involves initial capture, intermediate purification, and a final polishing step (Camper and Viola, 2009). In this current study, the initial capture was size exclusion chromatography (SEC), intermediate purification was ion exchange chromatography (IEX) while the final polishing steps was desalting chromatography.

First, the protein portion in *E. longifolia* extracts was pre fractionated from the non protein portion using size exclusion chromatography. The separation on SEC depends on the molecular mass of interest. The higher the molecular mass, the faster a solute will be eluted (Liang et al., 2006). Protein, which generally bigger in size in relatives to non protein molecule, will not interact with the pore of the gel in SEC column and will be eluted earlier compared to the non protein constituents.

As these proteins in *E. longifolia,* which consisted of four proteins, are similar in sizes as indicated by 2DE results (Figure 4.9), these proteins cannot be separated by SEC, but it was just to eliminate the interference of non protein materials prior isolation steps. The mobile phase of size exclusion chromatography in this study was deionized water. So, the utilization of SEC was also to avoid artifacts formation, protein denaturation and structural changes that have the potential to occur during protein extraction using organic solvent.

When the *E. longifolia* freeze dried extracts was subjected to size exclusion chromatography, three peaks were obtained as can be seen in chromatogram generated in Figure 4.18. The three peaks were denoted as P1, P2, and P3. Each of the three fractions were collected, pooled, freeze-dried and analyzed by SDS PAGE to detect the presence of protein. Conductivity peak can also be observed from the chromatogram. This might be due to very small constituents or ions that were present in the extracts.

Figure 4.19 (a) shows the gel from SDS PAGE analysis. A protein band can be observed in lane denoted as p1, which corresponds to peak P1, indicating the presence of protein in P1. However, no bands were observed in lane p2 and p3, which correspond to P2 and P3, indicating the absence of protein in the fractions. Protein is bigger in size compared to other constituents in plants, hence the protein eluted first, in peak P1 compared to other solutes in peak P2 and P3.

The protein band in lane p1 has the same electrophoretic profile as the band in crude extracts before size exclusion chromatography (SEC) (Figure 4.19 b). This is because the protein in *E. longifolia* is similar in sizes, so no separation could be obtained in SEC.

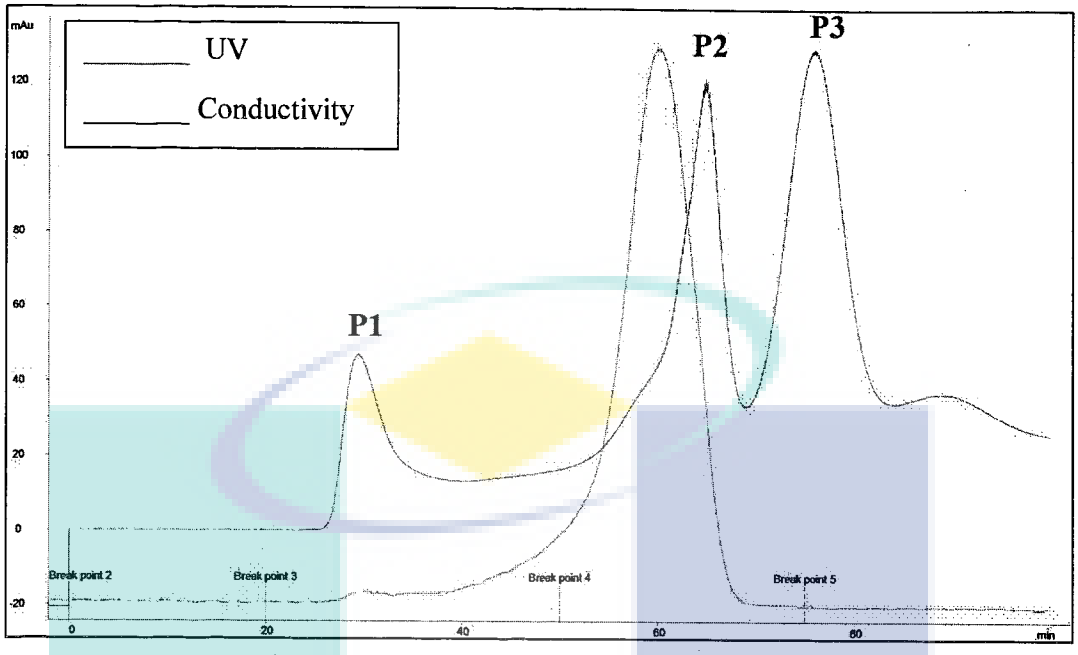

Figure 4.18: Chromatogram from size exclusion chromatography, using AKTA Prime system, with deionized water as mobile phase. Three peaks (P1, P2, P3) can be observed.

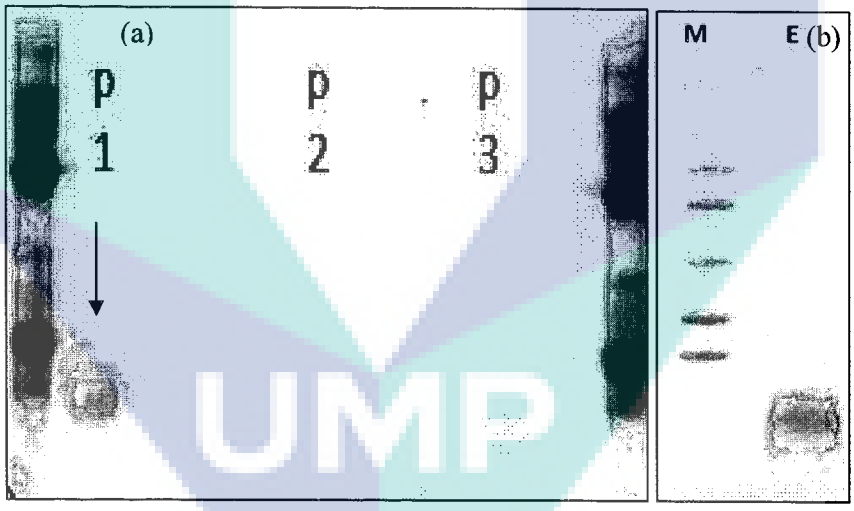

Figure 4.19: (a) Coomassie stained, SDS PAGE analysis of the fractions from size exclusion chromatography (SEC). Presence of protein band in lane denoted as p1 indicates that fraction P1 from SEC contains protein.(b) Coomassie stained SDS PAGE gel of *E. longifolia* crude extracts before SEC.

After size exclusion chromatography, the proteins in fraction P1 were further resolved on ion exchange chromatography (IEX) to separate the proteins into single constituent. These proteins, which showed one band in SDS PAGE but four spots in 2DE, would ideally be separated into four peaks in IEX.

In ion exchange chromatography, protein is separated by ionic interaction and pH. The method is based on the use of an ionic column and a salt gradient elution. In order to explore optimum condition for a good resolution between these proteins, several elution profiles were carried out and tested, namely several pH of mobile phases, salt concentration and elution modes. Anion exchange chromatography was the form of IEX in this study.

Figure 4.20 shows the elution profiles upon anion exchange chromatography. This separation is considered as satisfactory because it can be observed in the profile that the proteins were separated into four peaks, which probably correspond to four protein spots in 2DE profiles (Figure 4.9). Those four peaks in the chromatogram were denoted as I, II, III and IV. Green line in the chromatogram indicates buffer salt or eluting buffer concentration (%), while brown line indicates conductivity. Peak I eluted at the unadsorbed fraction. Peak II and III both eluted at eluting buffer 65% while peak IV eluted at eluting buffer 70%.

After ion exchange chromatography, fraction I, II, III and IV underwent desalting chromatography. Desalting, as the word implies, is to remove buffer salts that is used during ion exchange chromatography. Desalting chromatography also is based on size exclusion chromatography where smaller protein will be separated from the bigger molecule. In this study, protein which is relatively bigger in size than salt, will be eluted earlier.

During desalting process, buffer exchange is also occur, where the buffer used in ion exchange process is exchanged for intended buffer for subsequent analysis. In this study, since the samples would be freeze dried and analyzed by 2DE, the intended buffer was water.

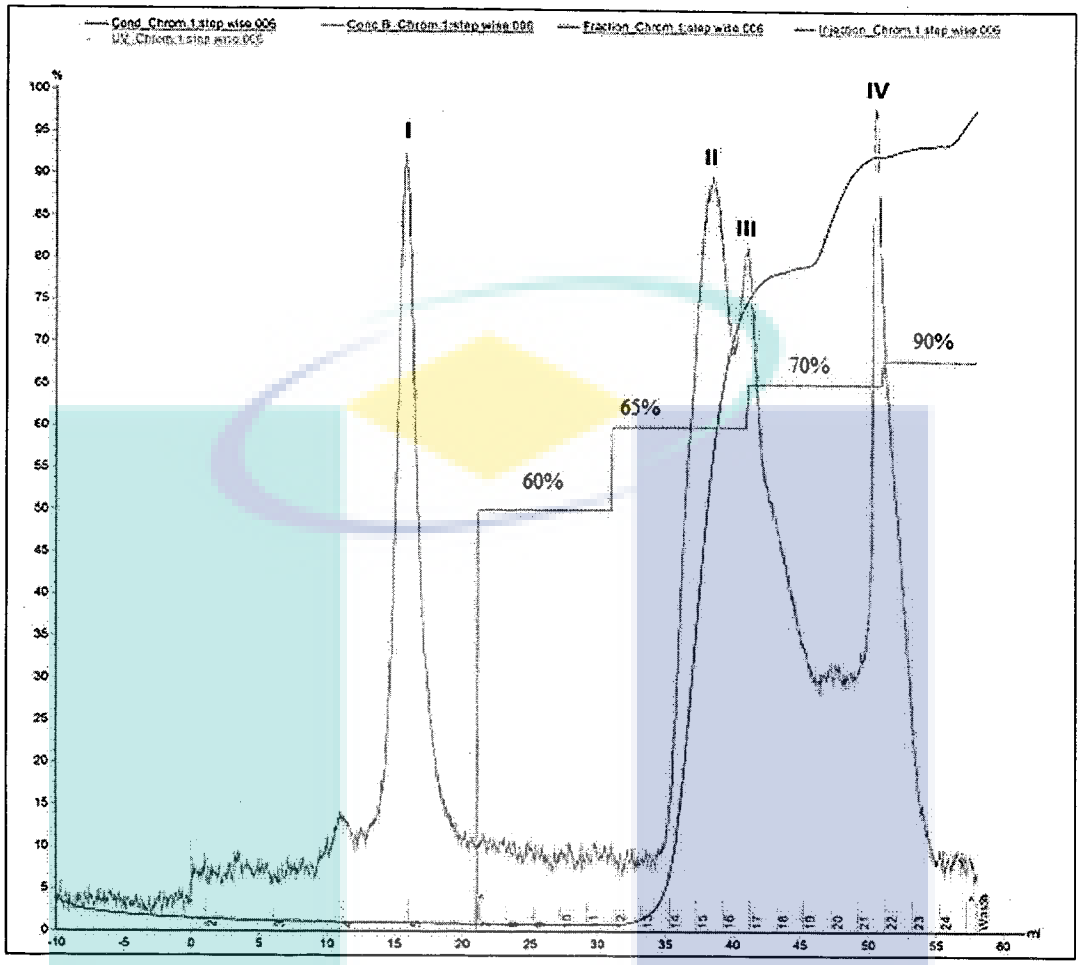

Figure 4.20: Elution profiles of anion exchange chromatography from AKTA Explorer system, with 0.5 M Tris HCL pH 8.7 as binding buffer and 0.5 mM NaCl in 0.5 M Tris HCL pH 8.7 as elution buffer. Four peaks; I, II, III, and IV can be observed. Green line indicates concentration of elution buffer, brown line indicates conductivity.

After chromatographic separation of protein, homogeneity and purity test was carried out. The purity of each protein fractions (I, II, III and IV) were tested using 2 dimensional gel electrophoresis (2DE) instead of Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE) because the proteins in *E. longifolia* are similar in sizes as proven in Section 4.4.2.

From 2DE analysis of fraction IV, it can be said that isolation of a protein marker has been achieved based on the presence of a single spot in electrophoretic profiles in Figure 4.21 (b). It is apparent that the single spot has the same isoelectric point and molecular weight as Marker A in the extract in Figure 4.21 (a). In the 2DE gel, Marker A spot was at the most negative extremities of the gel. While in anion exchange

chromatography, negatively charged molecules are attached to a positively charged solid support in a column. Fraction IV was the most tightly bound to the column as it eluted the latest. It could be implied here that it was the most negative compared to other fraction.

Three dimensional views from 2DE software (Image Master 2D Platinum 7) also revealed the presence of single spot (Figure 4.22). Thus it can be concluded here that Marker A was purified. Fraction I, II, and III were also analyzed in 2DE analysis, but no protein spot could be observed.

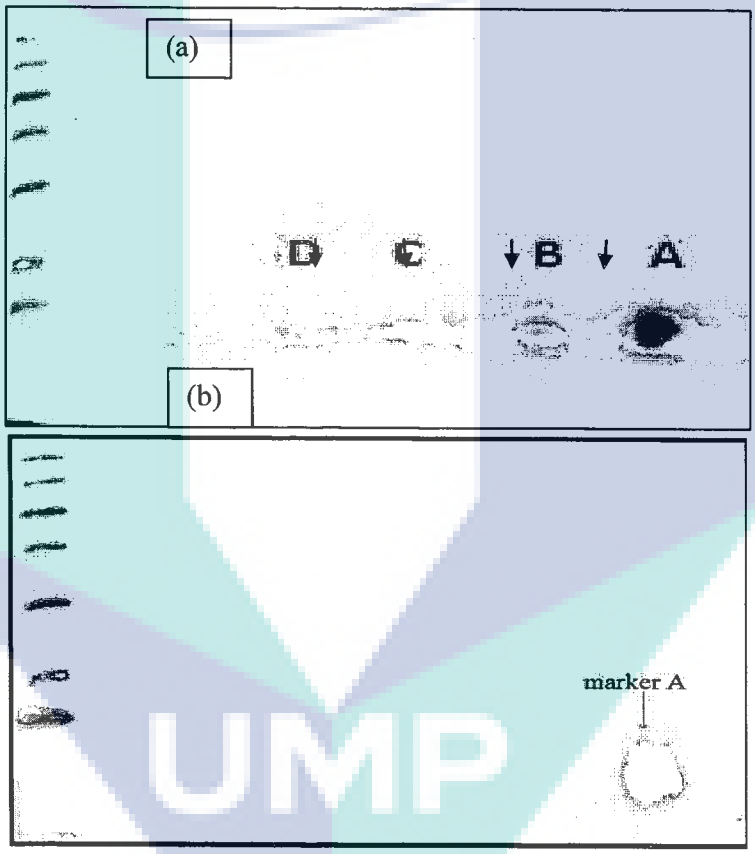

Figure 4.21: (a) 2DE profile of *E. longifolia* crude extracts, with the presence of 4 protein spots (A, B, C, D). (b) 2DE profile of fraction IV. A single spot, which has same electrophoretic profile as spot A, can be observed.

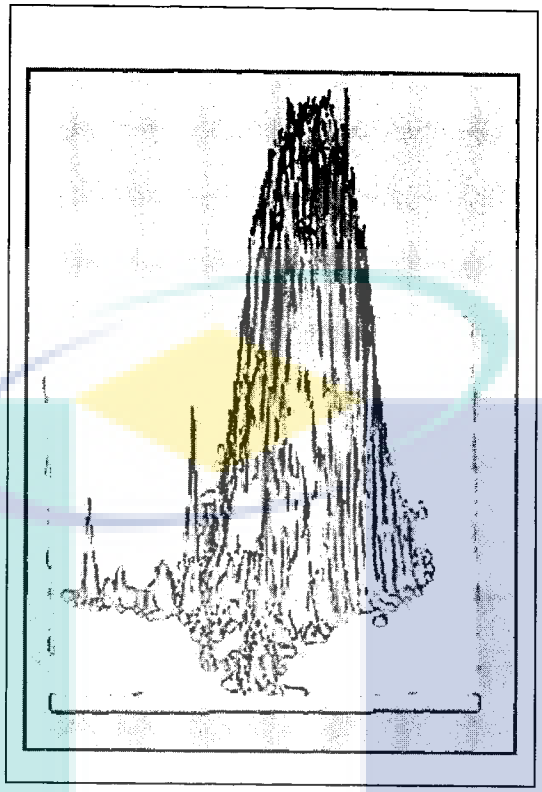

Figure 4.22: 3 dimensional views of 2DE gel of purified Marker A, using Image Master, 2D Platinum 7 software. The single peak viewed corresponds to single spot in 2DE gel.

4.6 Eurycomanone and Marker A Comparison

After isolation of Marker A, attempts were made to compare the protein marker and the chemical marker, eurycomanone. Quantification of Marker A in samples was done using 2DE software, which was Image Master 2D Platinum 7 (IMP7). The gels were first viewed in 3 dimensional views, where each protein spot was converted to peak, where the height of the peak indicates the intensity of the protein. Example of 3 dimensional views obtained from the software is in Figure 4.23.

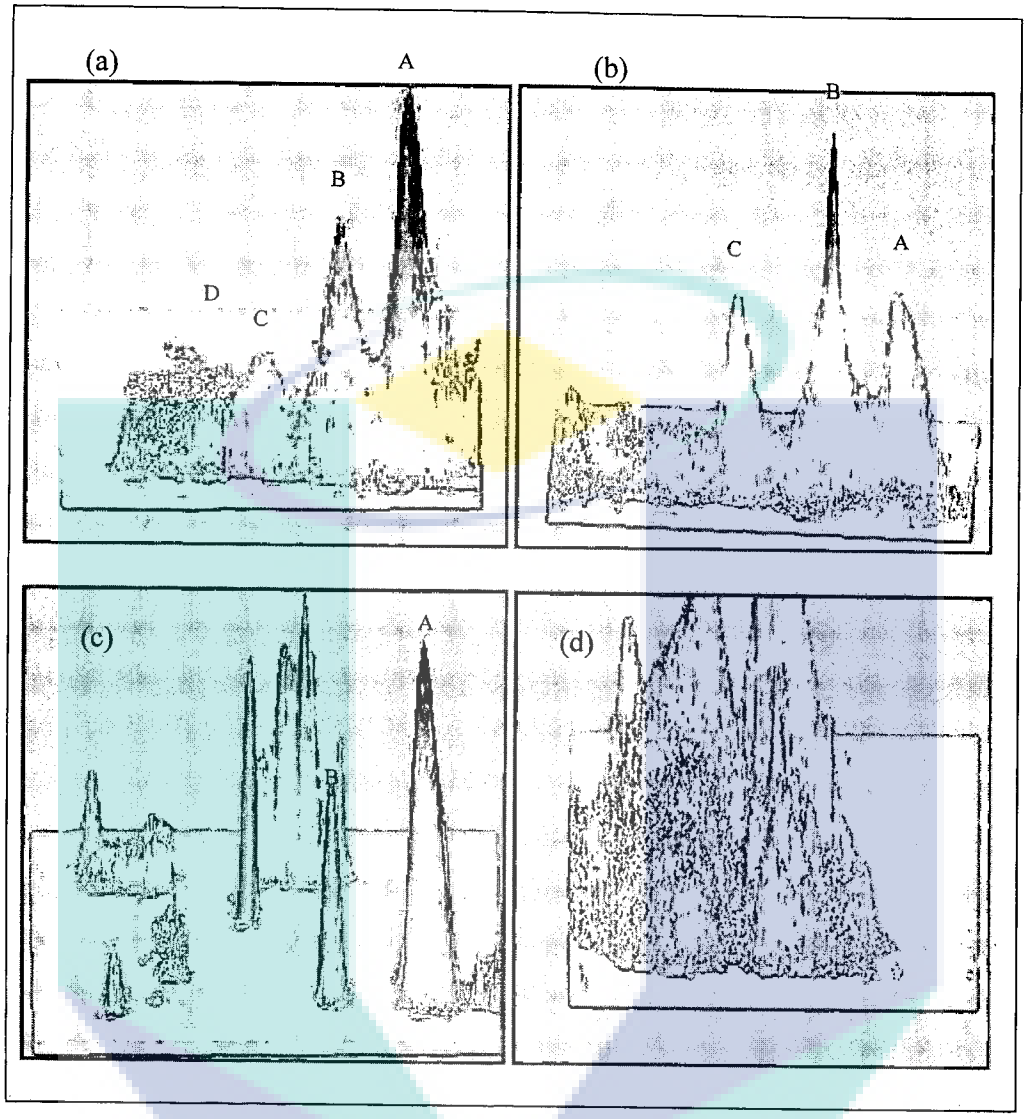

Figure 4.23: 3D view using Image Master, 2D Platinum 7 software. The height of the peak indicates intensity of respective spots. (a) *E. longifolia* freeze dried extracts, (b) sample with single formulation (C2), (c) sample with multiple formulations (C34), sample with the absence of protein marker (C5).

To quantify Marker A in all samples, normalization of the gels of the products against gel of purified Marker A was conducted. The amount was quantitated based on fold level differences with Marker A. For example, Figure 4.24 from the IMP7 software analysis shows that the amount of Marker A found in sample C34 differs by 0.525 fold relative to purified Marker A. The amount of purified Marker A that was put in the 2DE was 20 µg, thus sample C34 was estimated to contain 10.5 µg Marker A. The fold differences for all samples containing Marker A are listed in Table 4.9. Samples that are

not listed in the table concluded no match against Marker A. Table 4.10 lists the contents of eurycomanone and protein marker in samples that were tested.

Table 4.9:
Spot intensity and fold differences of Marker A and samples

No	Sample	Intensity	Fold difference with Marker A	µg
1	Marker A	52.6134	1.000	20.0
2	Crude Extracts	34.9879	0.665	13.3
3	C2	11.3119	0.215	4.3
4	C6	21.5715	0.410	8.2
5	C11	11.8380	0.225	5.1
6	C14	21.8346	0.415	8.3
7	C15	11.3119	0.215	4.3
8	C16	21.7823	0.395	7.9
9	C17	16.0471	0.305	6.1
10	C18	24.4652	0.465	9.3
11	C19	30.7788	0.585	11.7
12	C23	7.6289	0.145	2.9
13	C24	6.8397	0.130	2.6
14	C26	6.3136	0.120	2.4
15	C27	22.3607	0.425	8.5
16	C28	14.9948	0.285	5.7
17	C29	21.5715	0.410	8.2
18	C31	14.7318	0.280	5.6
19	C34	27.6220	0.525	10.5
20	C38	18.9408	0.360	7.2
21	C40	27.0959	0.515	10.3
22	C41	26.5698	0.505	10.1

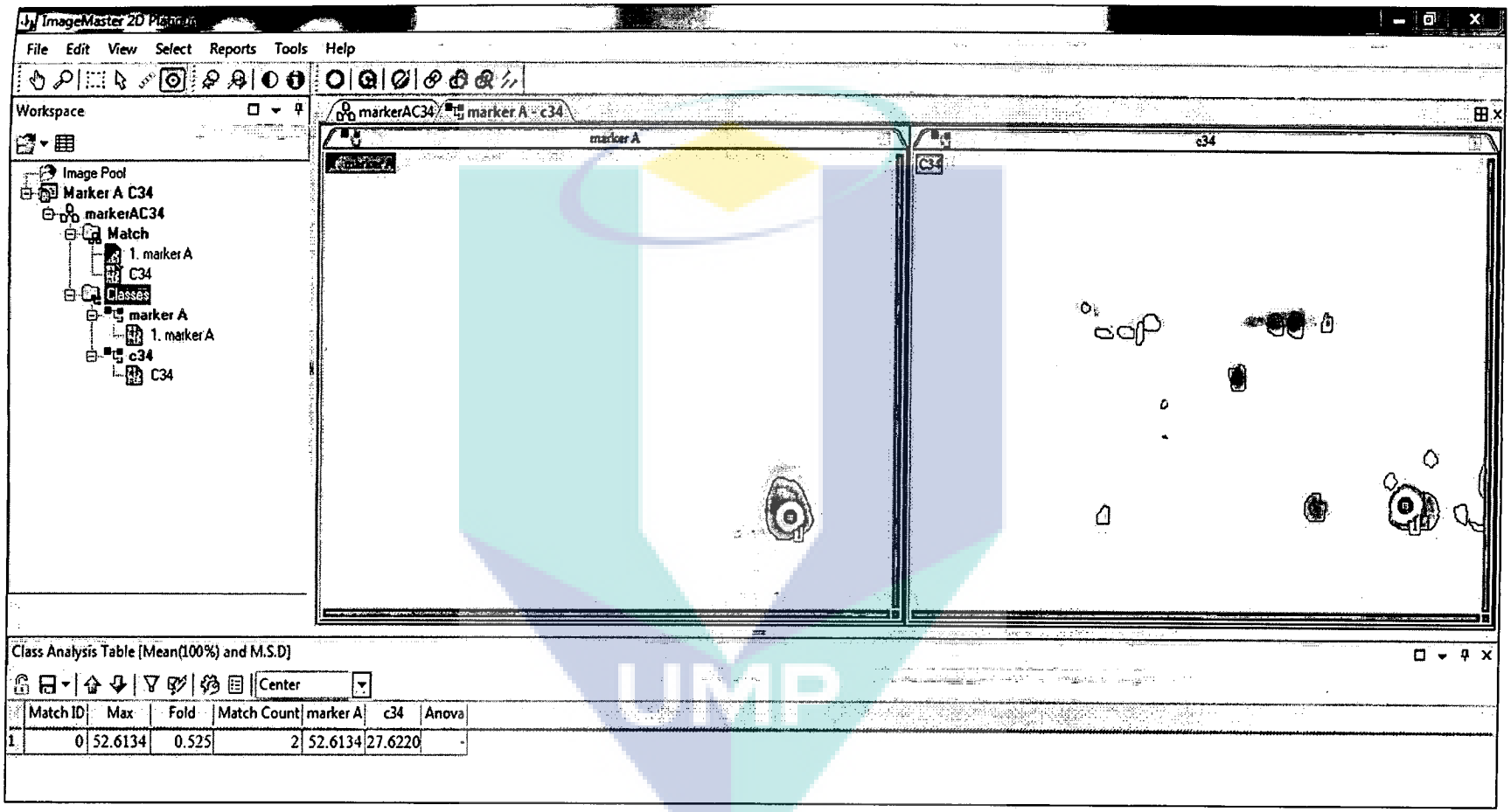

Figure 4.24: Comparison of spot intensity of purified Marker A and sample C34 using Image Master 2D Platinum 7 software. The intensity difference of spot A in the two gels was calculated to be 0.525 by the software

Table 4.10:
Marker(s) contents in samples tested

No	Sample	Eurycomanone content (% w/v)	Marker A (µg)
1	Crude Extracts	15.15	13.3
2	C7	8.48	nd
3	C28	3.82	5.7
4	C21	3.53	nd
5	C1	3.33	nd
6	C29	3.24	8.2
7	C11	2.95	5.1
8	C2	2.27	4.3
9	C41	2.12	10.1
10	C19	1.96	11.7
11	C14	1.72	8.3
12	C27	1.66	8.5
13	C26	1.03	2.4
14	C16	0.99	7.9
15	C40	0.84	10.3
16	C6	0.75	8.2
17	C34	0.55	10.5
18	C18	0.40	9.3
19	C24	0.36	2.6
20	C31	0.31	5.6
21	C17	0.26	6.1
22	C15	0.22	4.3
23	C38	0.19	7.2
24	C23	0.16	2.9
25	C4	0.08	nd
26	C8	nd	nd
27	C9	nd	nd
28	C20	nd	nd
29	C25	nd	nd
30	C3	nd	nd
31	C35	nd	nd
32	C10	nd	nd
33	C22	nd	nd
34	C30	nd	nd
35	C5	nd	nd
36	C12	nd	nd
37	C13	nd	nd

nd: not detected
samples are arranged in the order of highest containing eurycomanone to the lowest

Table 4.10 (continued):
Markers contents in samples tested

No	Sample	Eurycomanone content (% w/v)	Marker A (µg)
38	C32	nd	nd
39	C33	nd	nd
40	C39	nd	nd
41	C36	nd	nd
42	C37	nd	nd
43	C42	nd	nd
44	D1	nd	nd
45	D2	nd	nd
46	D3	nd	nd
47	D4	nd	nd
48	D5	nd	nd
49	D6	nd	nd
50	D7	nd	nd
51	D8	nd	nd
52	TA Hitam	nd	nd
53	TA Merah	nd	nd
54	Negative control	nd	nd

nd: not detected
samples are arranged in the order of highest containing eurycomanone to the lowest

In analyzing the 50 commercial *E. longifolia* products, only 20 products or 40% contained the protein marker, with at least the presence of Marker A. From eurycomanone analysis, 24 or 48% showed the presence of the chemical marker. It showed here that 4 (C1, C4, C7, C21) out of 24 products containing eurycomanone were devoid of Marker A. However, there is no product containing the protein marker but devoid of the eurycomanone. It needs to be taken into account that currently eurycomanone is considered as the 'gold standard' in determining the quality of *E. longifolia* products and these products usually are promoted based on the eurycomanone content. It could be a possibility that unscrupulous manufacturer simply spiking the product with eurycomanone compound to give the false impression that their products are high in *E. longifolia* content.

Sample C7, C21 and C1 were the first, third and fourth highest of eurycomanone among all products. However, these products showed the absence of the protein marker. Besides spiking, to enrich a product with eurycomanone, it is most likely that manufacturing process such as fractionation is carried out. For instance, fractionation process based on molecule size can lead to the separation of eurycomanone from the protein fraction. The eurycomanone fraction is put into the product while the protein fraction is omitted. C4 was the product that contained the lowest amount of eurycomanone (0.08 % w/v). Probably the amount of *E. longifolia* put into the product was too little leading to very low protein level and could not be detected by 2DE analysis. SDS PAGE analysis for these 4 products also did not show the presence of any protein bands and spots (Figure 4.25). Table 4.8 indicates that these products have low protein level.

For the 50 products, SDS PAGE and 2DE analysis delivered similar results for each product. A product that showed the presence of *E. longifolia* protein band in SDS PAGE would also have the protein spots in 2DE analysis. However, comparison between those analyses and eurycomanone revealed different observation. This is because both SDS PAGE and 2DE analyze similar constituent which is the protein, whereas eurycomanone is a secondary metabolite, a small compound. Some products that contained eurycomanone did not contain the protein marker. Water extraction in laboratory in this study revealed that the process can extract the protein together with eurycomanone as proven by HPLC and electrophoresis analysis of the freeze dried extracts. Water extraction has been the norm in *E. longifolia* products preparation, thus all of the products containing *E. longifolia* should show the presence of both protein and eurycomanone. As explained above, some of the products were rich in eurycomanone but devoid of the protein marker is probably because of extra manufacturing step such as fractionation after water extraction. A product with lowest amount of eurycomanone was devoid of the protein marker probably because since the *E. longifolia* amount itself is too low, the protein could not be detected by electrophoresis analysis.

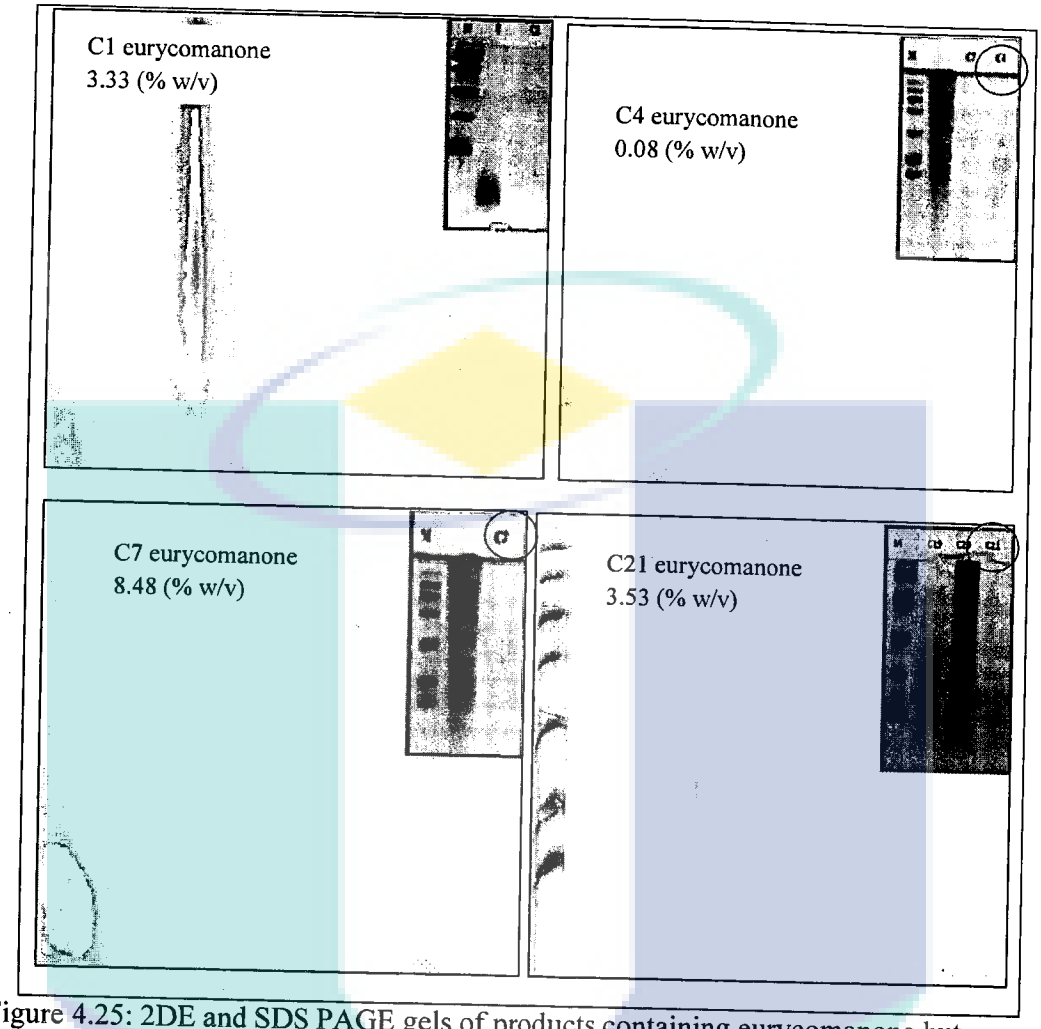

Figure 4.25: 2DE and SDS PAGE gels of products containing eurycomanone but absence of protein marker. No protein bands and spots can be observed in the SDS PAGE and 2DE gels

Statistical analysis by Student's t test (Appendix E) revealed that the amount of eurycomanone and Marker A was statistically different at significant level of $P \leq 0.05$. Figure 4.26 shows that there is no definite pattern or relationship between eurycomanone and Marker A content. This can be explained by the fact that protein and eurycomanone are different compound and the synthesis or the production of these two compounds are independent of each other. Chua et al. (2011) also discovered quassinoid and protein levels in *E. longifolia* collected from Pahang and Perak were different. They claimed geographical factors such as soil quality, terrains, rainfall, and growing condition of the plant might have caused this situation. In this study, the products were sampled from various sources and the quality of the raw *E. longifolia* itself might differ. Moreover, manufacturing conditions such as extraction temperature, fractionation and pre

concentration might lead to differences in the amount of eurycomanone and protein marker.

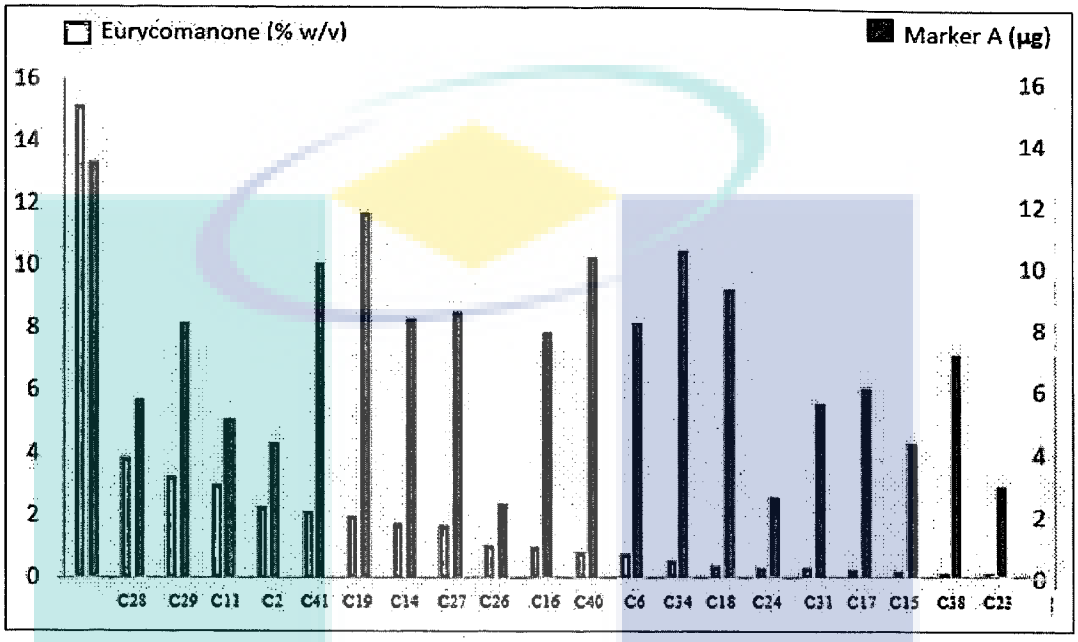

Figure 4.26: Pattern of eurycomanone and Marker A content. Samples in the chart are arranged in the order of decreasing eurycomanone content.

Eurycomanone, being a small molecule has been useful for detection of *E. longifolia* incorporated products, while Marker A, a macromolecule, has been comparable to the former with minor exceptions of some products. Marker A, being a protein, if studied further, can be developed into antibody and incorporated into portable device such as biosensor and will have the advantage of speed in analysis, ease of use and portability.

4.7 Protein Characterization

4.7.1 MALDI TOF MS

Previous researches such as Cazares et al. (2008); Flaudrops et al. (2015); Gopalakrishnan et al, (2015) have used variation of Mass Spectrometer (MS) techniques such as Surface Enhanced Laser Desorption Ionization (SELDI) and Matrix Assisted Laser Desorption Ionization (MALDI) as protein profiling and to detect biomarker

protein. Asiah et al. (2007) used SELDI MS to detect biomarker protein in ethanolic extracts of *E. longifolia* and three other plants that are famous for aphrodisiac activity; *Smilax myosotiflora, Rafflesia* sp and *Labisia pumila*. They found distinctive 4.3 kDa protein peak in the plants. Protein was also detected in bioactive fraction of *E. longifolia* as found and patented by Sambandan et al. (2006). The discovery intrigued this study to analyse specific pattern of mass signals of *E. longifolia* freeze dried water extracts using MALDI MS.

MALDI MS was performed in linear positive ion mode and protein mass profiles acquired are presented in Figure 4.27. For the extracts (a), a prominent mass of approximately 4.3 kDa was observed. Protein spectras (b, c) of *E. longifolia* that was incorporated with products excipients were also monitored. The mass spectrum of the b (excipient: maltodextrin), c (excipient: cyclodextrin) showed similar spectrum with mass signal approximately 4.3 kDa as well. It was noticed in Figure 4.28, upon overlapping the three spectras, there are similarities where they have common peak at approximately 4.3 kDa.

Asiah et al. (2007) reported 4.3 kDa mass signal in *E. longifolia* and several other plants utilizing SELDI MS. This study found this signal as well but using MALDI MS instead. SELDI MS can be considered as an extension of MALDI MS method. In both cases, protein to be analyzed are co crystalized with UV absorbing compounds and vaporised by a pulsed – UV laser beam (Vonderwulbecke, 2005).

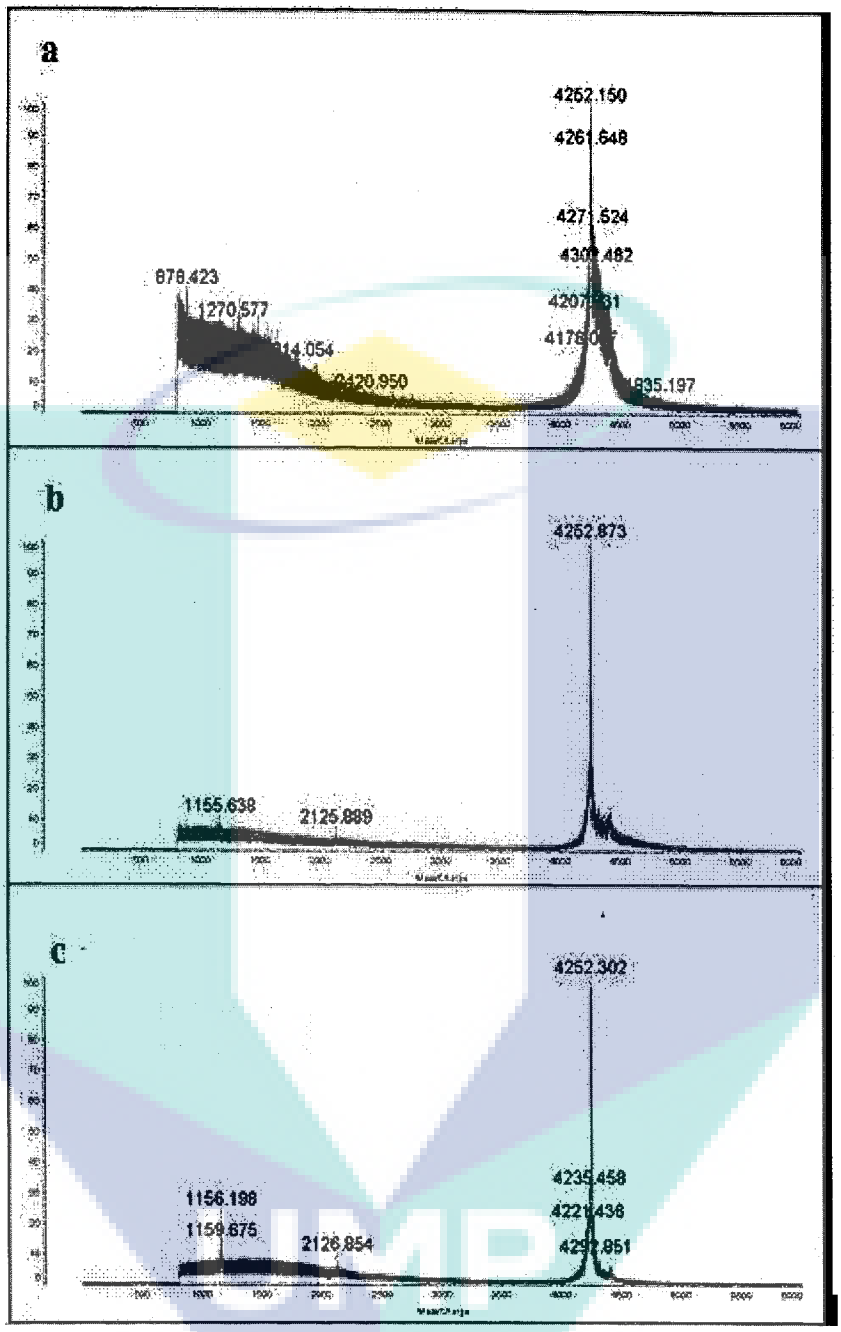

Figure 4.27: MALDI MS profiles of (a) *E. longifolia* extracts, (b) *E. longifolia* extracts incorporated with maltodextrin (c) *E. longifolia* extracts incorporated with cyclodextrin. All the profiles show the presence of 4.3 kDa protein mass.

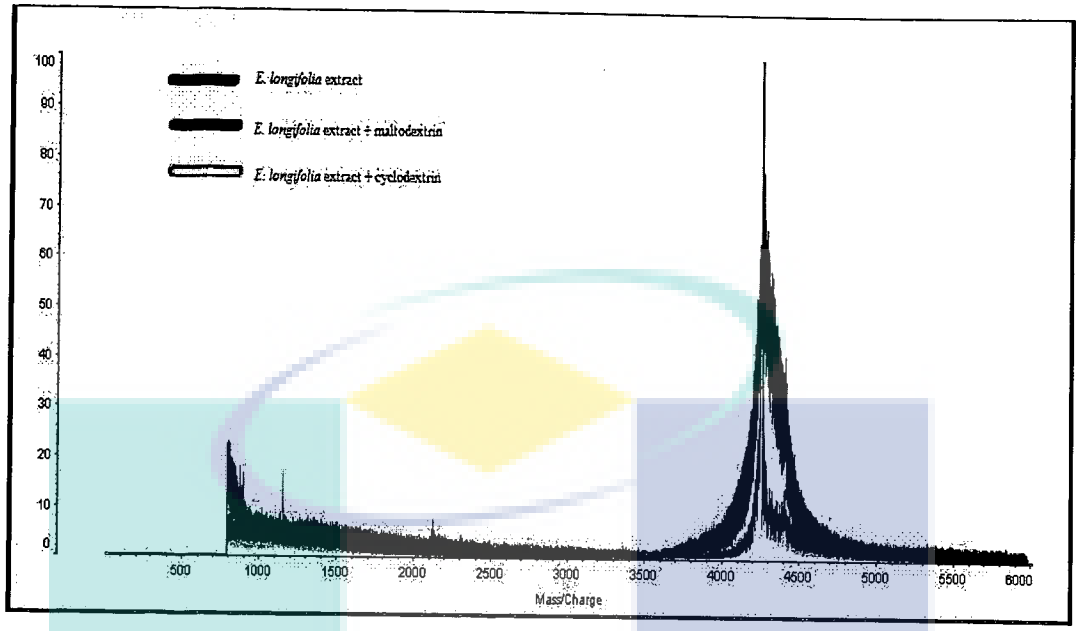

Figure 4.28: Superimposed MALDI MS profiles of *E. longifolia* freeze dried water extracts and excipients (maltodextrin and cyclodextrin). All the three profiles have common peak at 4.3 kDa.

4.7.2 Peptide Mass Finger Printing of the Protein Marker

To identify the protein marker found in the 2DE gel, the proteins were subjected to peptide mass finger printing analysis, utilizing MALDI TOF MS. The four spots (A, B, C, D) were excised by surgical blade and subjected to in-gel digestion with trypsin. Mass spectra were monitored using a reflectron MALDI TOF MS. Protein masses generated were analyzed and matched using Swissprot database form Mascot search engine program (http://www.matrixscience.com) to identify protein in each spot.

The mass spectra obtained for spot A is shown in Figure 4.29 and the protein masses are 804.028, 805.024, 806.031, 806.987, 826.995, 841.992, 858.003, 885.557, 1935.427, 1937.506, 2040.485, 2131.420, 2167.439, 2169.468, 2184.386, 2259.350, 2261.330, 2274.418, 2276.376. The mono isotopic protein masses were matched with the database of Swissprot (Appendix F2). Attempt was made to match against green plant (Viridiplantae), however, library provided no significant hit. With top score of 37, it was discovered that the proteins matched to BCP_PEA, Blue copper protein OS=*Pisum sativum* PE=2 SV=1, nominal blast of 19306 (Mr) and calculated pI value of 6.25. The sequence coverage was 32% and the matched proteins are shown in **BOLD.**

1	MAFSNALVLC	FLLAIINMAL	PSLATVYTVG	DTSGWVIGGD	YSTWASDKTF
51	AVGDSLVFNY	GAGAHTVDEV	KESDYKSCTS	GNSISTDSTG	**ATTIPLKK**AG
101	KHYFICGVPG	HSTGGMKLSI	KVKASSGSSA	APSATPSSSG	KGSPSSDDTP
151	AATTTTTTPT	KQNESSATSL	SPIVALFFTV	SWICSYVLV	

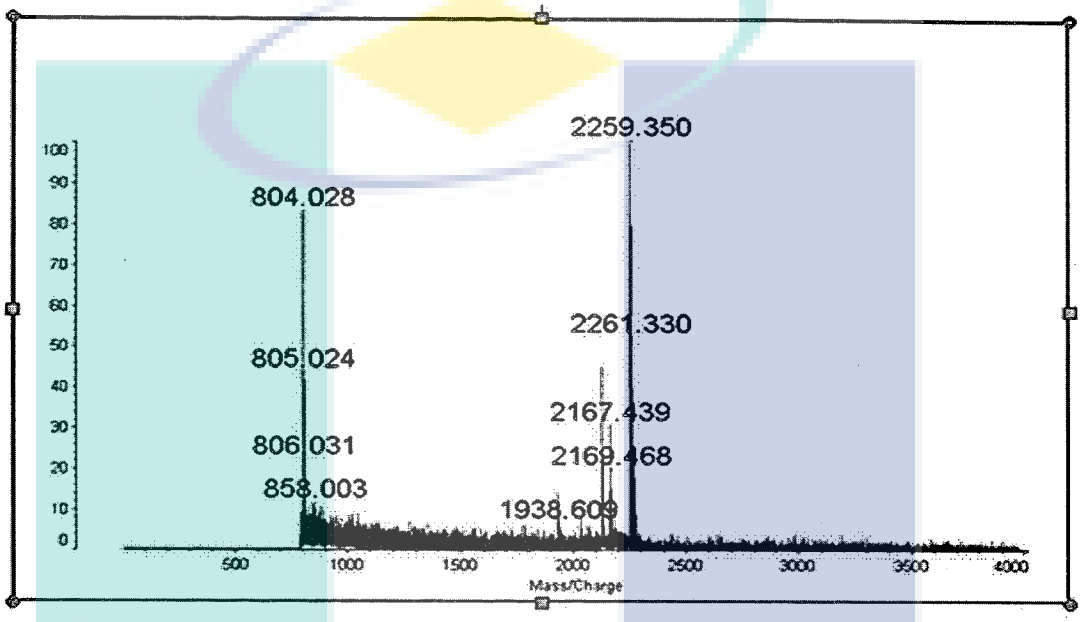

Figure 4.29: Mass spectra of Spot A from MALDI TOF MS analysis

For Spot B, the mass spectrum is in Figure 4.30. The protein masses are 802.923, 803.931, 804.930, 2039.136, 2040.619, 2042.597, 2074.879, 2131.467, 2135.237, 2147.298, 2161.959, 2168.645, 2192.305, 2202.265, 2258.273, 2260.069, 2287.621, 2317.696, 3153.901. Similarity searching using Mascot databases resulted in no significant matches. For Spot C and D, there was no good spectra could be obtained as the spot itself in the 2DE gel was too faint, indicating very low amount to be detectable.

4.7.3 *De Novo* Sequencing

One of popular method for protein identification involves submitting mass spectra of the protein in protein databases. The mass spectra will be matched againts existing protein sequence in the databases and protein with highest matching scores will be selected. However, the important point in database searching is whether the data in the spectrum allows for an unambiguous protein identification (Wattenberg et al. 2002). Thus, one of the drawbacks of database search is, it fails to recognize novel protein since it can only match to existence sequence in the database. Moreover, data based sequencing is usually predicted based on similarities with other species, and the species range in most databases is broad. If the protein of interest are from species that is absent in the databases, species-specific sequences may not overlap sufficiently with known proteins for accurate prediction and optimal prediction (Ma et al., 2005; Tannu and Hemby, 2007; Hennrich et al., 2010).

Most protein identification studies involve unknown and novel protein and results from peptide mass finger printing in Section 4.7.2 also indicated that protein signals from Marker A has no significant matches to any *E. longifolia* protein in the library. Database search scheme is also hampered by unanticipated or unknown post-translational modifications in the protein (Campbell and Vestal, 2002; Wang and Wilson 2013) and the result from 2DE analysis in Section 4.4.3 suggested that the protein in Marker A could be glycoprotein.

For any protein mass fingerprinting that failed to generate protein identification due to the protein of interest are novel or unknown, alternatively *de novo* sequencing can instead be important (Tannu and Hemby, 2007). *De novo* sequencing method involves deriving protein sequence from tandemn MS spectrum without the help of protein database. This approach identifies protein directly from MS/MS spectrum peaks, the sequence is built based on the spectrum data itself instead of relying and prediction from databases (Wang and Wilson 2013).

In LC MS/MS based *de novo* sequencing, micro capillary reversed phase liquid chromatography interfaced to a tandem mass spectrometer. Proteins are separated by nanoflow liquid chromatography, then selected by first mass analyzer (MS) according to mass to charge ratio. Each proteins then fragmented along the protein backbone, the

fragment ions are detected by second mass analyzer (MS), producing MS/MS spectrum (Hennrich et al., 2010). There are many types of fragment ions that can be formed, eg a, b, c, x, y and z ions, depending on the sites of fragmentation. It is difficult to characterize the fragment ion manually, therefore, an algorithm such as PEAKS software is used to assist in the sequencing. PEAKS also measures a 'positional confidence' for each amino acid in 'top-scoring' proteins (Tannu and Hemby, 2007). Current study utilised *de novo* sequencing as the protein in Marker A was expected to be novel. Proteins from the gel plug of Marker A spot and purified Marker A were both sequenced using this method.

In the sequencing of protein of Marker A in 2DE gel, as done by Proteomic International (full results are attached in Appendix H), the spot was excised from the gel, the protein was extracted and digested by tripsin. The proteins sequences were determined predominantly using b and y ions in the MS/MS spectra, and analyzed using PEAKS Studio Version 4.5 SP2 (Bioinformatics Solutions) and manual interpretation. Fragment ions that were assigned as b ions were 1066.41, 1317.53, 1521.68. Fragment ions that were assigned as y ions were 175.15, 262.19, 361.22, 432.29, 489.31, 755.38, 812.39, 959.45, 1073.46, 1210.53, 1357.60, 1414.62, 1577.67, 1809.70. The mass difference between adjacent b and y ions was calculated and it is unique for each amino acid residue. Based on the interpretation, it was proposed that protein in marker A consisted of 20 amino acid residue, with projected sequence of TFMSMTYGFHNFGYCGAVSR, with probability score of 94%.

In the sequencing of protein of purified Marker A, fragment ions that were assigned as b ions were 233.11, 212.14, 397.27, 511.32, 1006.5, 1192.5, 1706.7. Fragment ions that were assigned as y ions were 1750.7, 1471.7, 1116.5, 1017.4, 916.39, 845.32, 657.28, 560.23, 503.27, 390.2, 261.15, 2014.13, 147.11. Based on the interpretation, it was proposed that protein in Marker A consisted of 22 amino acid residue, with projected sequence of CELLHAAMHSVTAMGPGLEGGK, with probability score of 87%.

The results from the sequencing of the gel plug and purified Marker A showed that the amino acid sequence for analysis is ambiguous even though they are from the same protein. Many factors that can cause difficulties and inaccuracy in interpreting MS/MS data. The factors can be incorrect assignment of y n b ions, some fragments ion are missing, noise peaks that come form internal cleavages and contaminants (Liu et al

2012), similar mass residue such as leucine and isoleucine, and post translational modifications (Lim et al 2003). These factors can cause *de novo* sequencing to figure out only partially correct sequence from the spectrum (Campbell and Vestal, 2002).

Current studies used two soft ionization techniques to sequence Marker A. First approach was MALDI TOF MS where peptide masses of proteolytic protein digests are determined. It is also known as peptide mass fingerprinting. The second method was electrospray ionization (ESI) mass spectrometry where fragment ion masses of proteolytic peptides. It is also known as fragment ion mass searches.

Previous studies (Medzihradszky et al. 1997; Bienvenut et al. 2002; Wattenberg et al. 2002; Irungu, et al. 2008) also found that results from MALDI MS and ESI did not correspond to each other.

This different could be due to how both methods operate. They differ both in how the molecules are ionized and how the mass separation is performed. Peptide fragment ions obtained from doubly charged precursors produced by ESI are mainly y-type ions with some b-ions in the lower mass range. In contrast, peptide fragment ions produced from the singly charged ions originating from the MALDI source are a mixture of y-, b- and a-ions accompanied by ions resulting from neutral loss of ammonia or water (Wattenberg et al. 2002). IN ESI, a minor amount of salts and detergents can weaken protein ion intensities and degrade spectra qualities, thus additional sample preparation step is necessary, while MALDI is known to be more tolerant towards common impurities (Irungu, et al. 2008). Both methods have drawbacks where in MALDI MS, results obtained are not always unambiguous while ESI which based on fragment ion-based searches are comparably slow (Bienvenut et al. 2002).

To get reliable sequence from *de novo* sequencing, many studies have conducted chemical derivatization towards the proteins. Hennrich et al (2010) introduced metalloendopeptidase Lys-N to protein, which resulted in single charged ion and simplified spectrum. Samgina et al. (2010) discovered acetylation and sulfobenzoylation of N-amino group provided better spectrum than intact protein. Evaristo et al. (2015) conducted reduction and alkylation towards their protein of interest prior *de novo* sequencing.

In this study, there were no chemical modification and derivatization carried out prior *de novo* sequencing, thus resulting in partially complete sequence. However, in the

future it will be interesting to try this method and probably complementary with other sequencing method such as Edman degradation.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

This study focused on screening and isolating novel protein marker for authentication of *E. longifolia* commercial products. Products were sampled from the markets, screened for the presence of eurycomanone and protein marker. The identified protein marker was isolated and characterized.

Among all samples, *E. longifolia* freeze dried extracts contained the highest amount of eurycomanone (15.15%). A total of 24 products (10 Malaysian Registered Products, 10 Malaysian Unregistered Products, 4 International Products) or 48% contained eurycomanone while 26 products or 52% did not. Products with single formulation generally showed higher content of eurycomanone. Even though some products were not registered, they showed high presence of eurycomanone. It is probably due to the facts that *E. longifolia* extracts can be obtained easily with little processing.

In electrophoretic profiles analysis, *E. longifolia* extracts showed the presence of distinctive single band in SDS PAGE at 10 kDa molecular weight, and four spots, with the same isoelectric point in 2DE analysis. The four spots were denoted as Marker A, B, C and D. 2DE analysis showed similar results with SDS PAGE analysis where 20 products that showed the presence of the distinctive single band in SDS PAGE would have the visibility of one or more of the protein marker spots in 2DE analysis. Marker A was chosen as the ultimate marker.

For the analysis of Tongkat Ali Hitam and Tongkat Ali Merah no eurycomanone and protein marker were detected in both samples. It can be concluded that the two plants share the same root morphology and local names, however, they are from different constituents. Beverage products, which consisted of premixed coffee and canned drinks were devoid of eurycomanone and protein marker. It can be generally said that, consumers ingesting these products are not getting the benefits that they intend to get from this plant as probably no *E. longifolia* was added.

Eurycomanone and protein profile analysis in SDS PAGE and 2DE showed similar results with minor exceptions. Four products which showed the presence of eurycomanone marker, were devoid of the protein marker and statistical analysis revealed the content of the eurycomanone marker was statistically different. The possible explanation is, specific manufacturing process caused this situation, where the products were enriched and standardized to a defined eurycomanone content.

Marker A was isolated after size exclusion chromatography followed by anion exchange chromatography. Successful isolation of Marker A was proven by the presence of single protein spot which has the same electrophoretic profile with Marker A in *E. longifolia* freeze dried extracts.

Protein mass finger printing in Mascot Database search resulted in no matches. This was expected as the protein in Marker A is expected to be novel and the available database for *E. longifolia* was only 11 proteins which were related to photosynthetic mechanism. This total number of protein of *E. longifolia* protein in the database can be considered as very little in comparison with other species such as *Arabidopsis thaliana* and *Oryza sativa* where 3846080 and 883300 of protein respectively from the species were available in the protein database (search date; 15 December 2015). Due to insufficient library information, purified Marker A was subjected to *de novo* sequencing. Based on the partial sequencing method and interpretation, it is proposed that protein in marker A consisted of 22 amino acid residue, with projected sequence of CELLHAAMHSVTAMGPGLEGGK, with probability score of 87%. The protein sequence will be useful for primer design for molecular works. Consequently, the full sequence can only be obtained by performing N Terminal sequencing of Marker A.

One of the most noteworthy contributions in this study is the isolation of Marker A. As far as publications and literatures are concerned, there is no successful protein isolation of *E. longifolia* root extracts has been reported so far. Thus it can be concluded here that this protein is a new protein. In conclusion, this study discovered the potential of a protein to be made as marker.

5.2 Recommendation

The isolated protein, though isolated in laboratory scale, may pave the way and provide insights for future research. From this research, eurycomanone was found to be useful for *E. longifolia* authentication. However, it is expected that *E. longifolia* product development in Malaysia can be improved by applying eurycomanone and the protein marker as complimentary marker for products standardization and quality control.

Eurycomanone is the analytical marker to authenticate *E. longifolia*. However, this compound is not yet to be proven to contribute to the aphrodisiac effects, which *E. longifolia* is most famous for. Thus, other research that could be ventured is testing the isolated protein marker in sexual enhancing effects assays such as Leydig cells assay and animal test. If this isolated protein possesses the aphrodisiac effects, it will have the advantage to be made as efficacy marker.

As 2DE results showed that the protein marker is probably glycosylated, further studies such as metabolic labelling, affinity enrichment, mass tagging and affinity tagging, should be carried out to confirm the possibility of post translational modification of this protein.

Marker A has been partially sequenced in this study. From the amino acid sequence, further research into the area may include creating the antibody of Marker A. The developed antibody can be incorporated and developed into antibody based biosensor. In the future, the marker A should be completely sequenced using method such as Edman degradation, or *de novo* sequencing with chemical derivatization.

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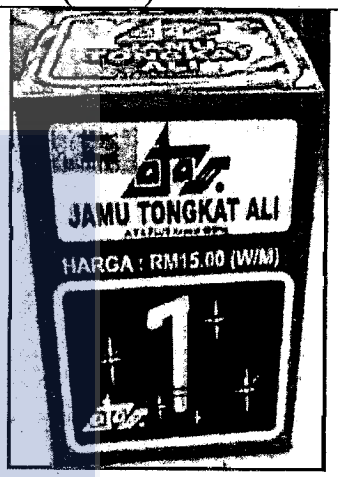
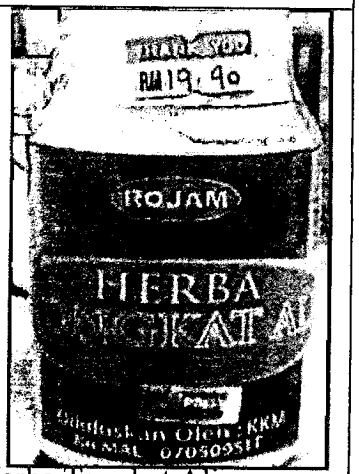
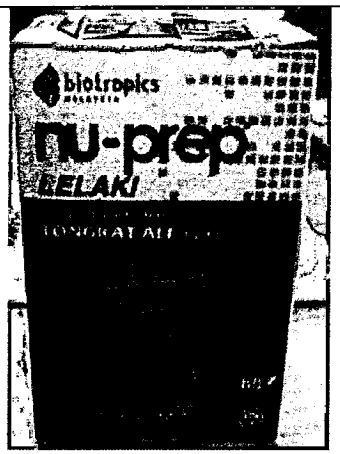
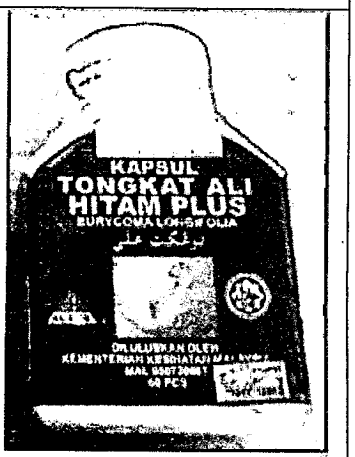
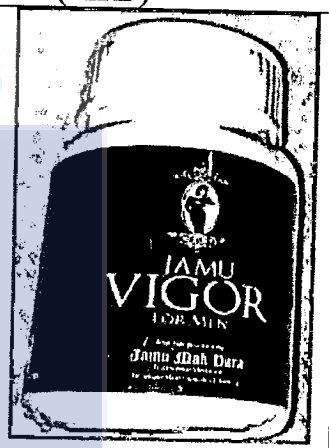
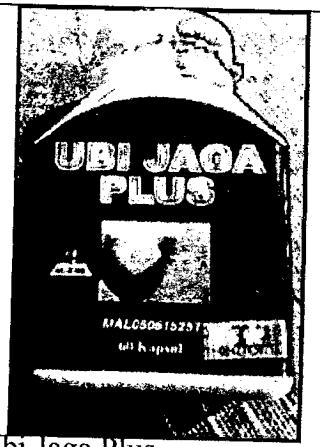
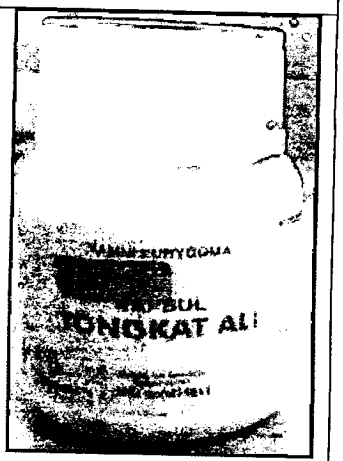
Appendix A1 – Pictures of commercial products

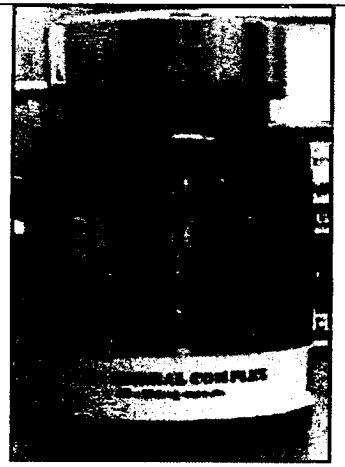
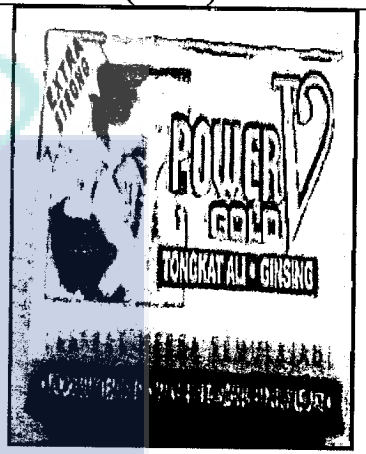
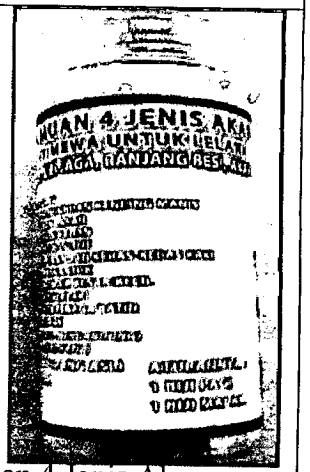
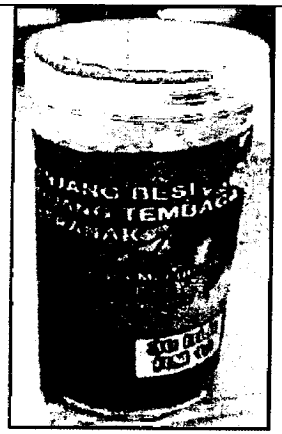
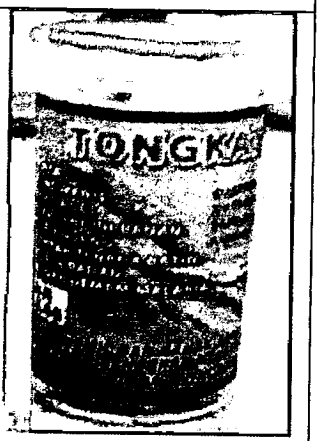
Malaysian Registered Products (MRP)			
C1  Tongkat Ali Plus	C2  Tongkat Ali Capsule	C3  Jamu Tongkat Ali	C6  Herba Tongkat Ali
C7  Nu Prep	C8  Tongkat Ali Maca Plus	C9  Hurix Plus	C20  Tongkat Ali Hitam Plus

Appendix A1 – Pictures of commercial products

Malaysian Registered Products (MRP)

C21  Fenugreek Tongkat Ali Plus	C24  Ubi Jaga Tongkat Ali	C25  Jamu Vigor	C26  Ubi Jaga Plus
C27  Formulation For Men	C28  Ubi Jaga Tongkat Ali	C35  Al Jabbar 101	C38  Kapsul Tongkat Ali

Appendix A1- Pictures of commercial products

Malaysian Unregistered Products (MUP)			
C4  Black Jack	C10  Tanduk Rusa	C11  Power Gold	C15  Ramuan 4 Jenis Akar
C16  Tongkat Ali dan Ubi Jaga	C17  Ranjang Besi dan Tongkat Ali	C18  Ramuan Akar Kayu	C19  Tongkat Ali

Appendix A1 – pictures of commercial products

Malaysian Unregistered Products (MUP)

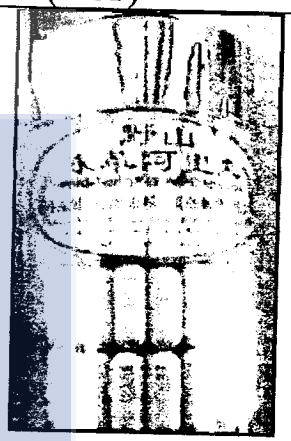
C22

Kapsul Tongkat Ali Hitam

C23

Orang Kampong Tongkat Ali

C29



Wild Tongkat Ali

C30

Urat Madu

C31

Jamu Sakti

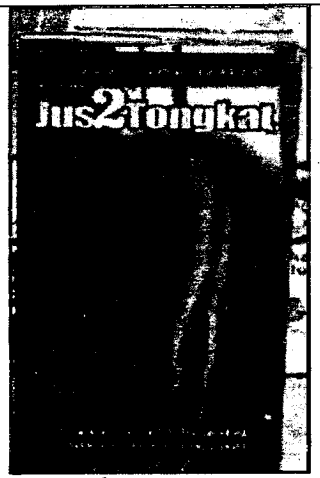
C42

Maajun Pak Tani

Appendix A1- Pictures of commercial products

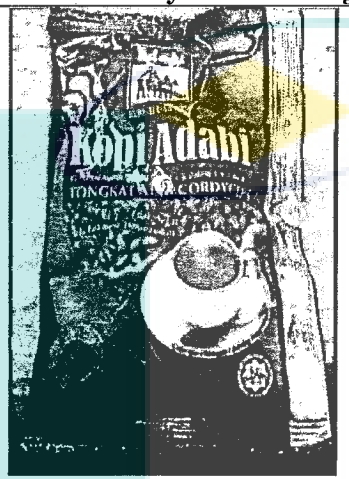
Malaysian Beverages Products (MBP)

D7



Jus 2 Tongkat

D6

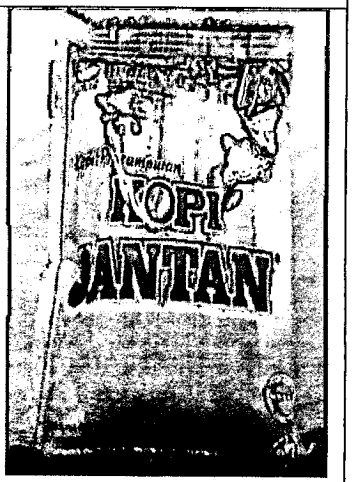


Kopi Adabi

D3

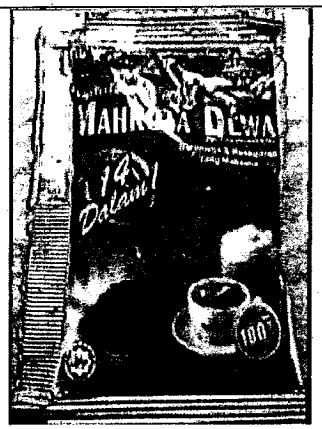
Capuccino Tongkat Ali

D2



Kopi Jantan

D4



Tongkat Ali Mahkota Dewa

D8

Healthy Men Coffee

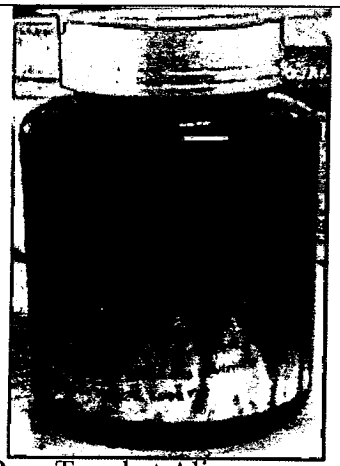
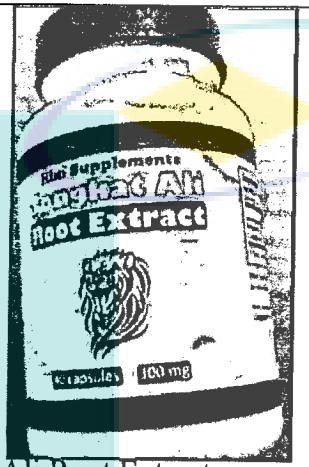
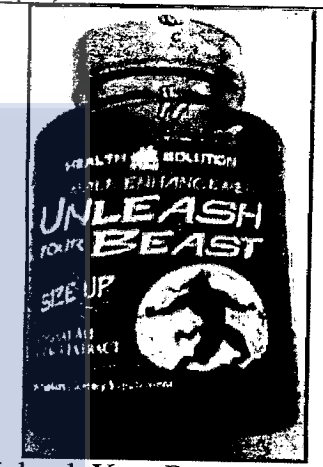
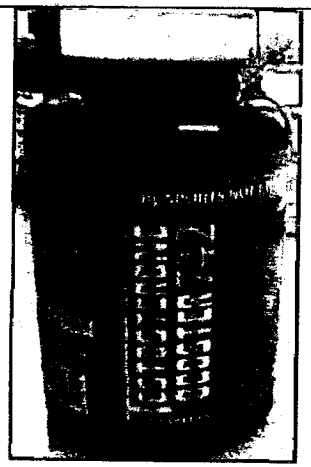
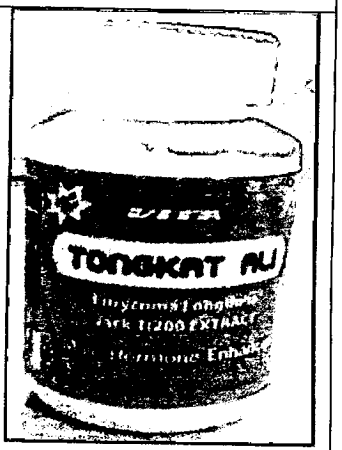
D5

Tongkat Ali n Ginseng Coffee

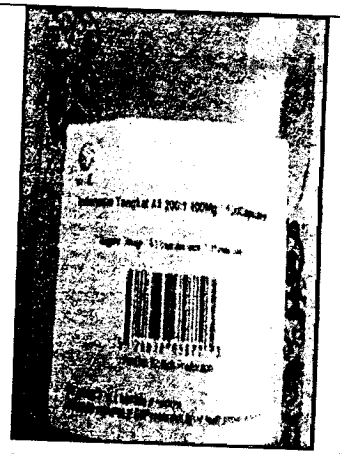
D1

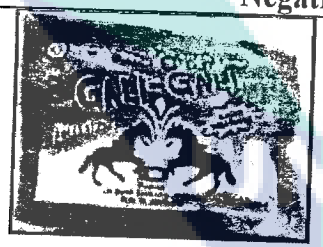
Alicafe

Appendix A1- Pictures of commercial products

International Products (IP)			
C5  Pure Tongkat Ali	C12  Tongkat Ali Root Extract	C13  Unleash Your Beast	C14  Pure D
C32  Testosterone Booster	C33  Longjack	C34  Tongkat Ali Doctor Formulated	C36  Tongkat Ali

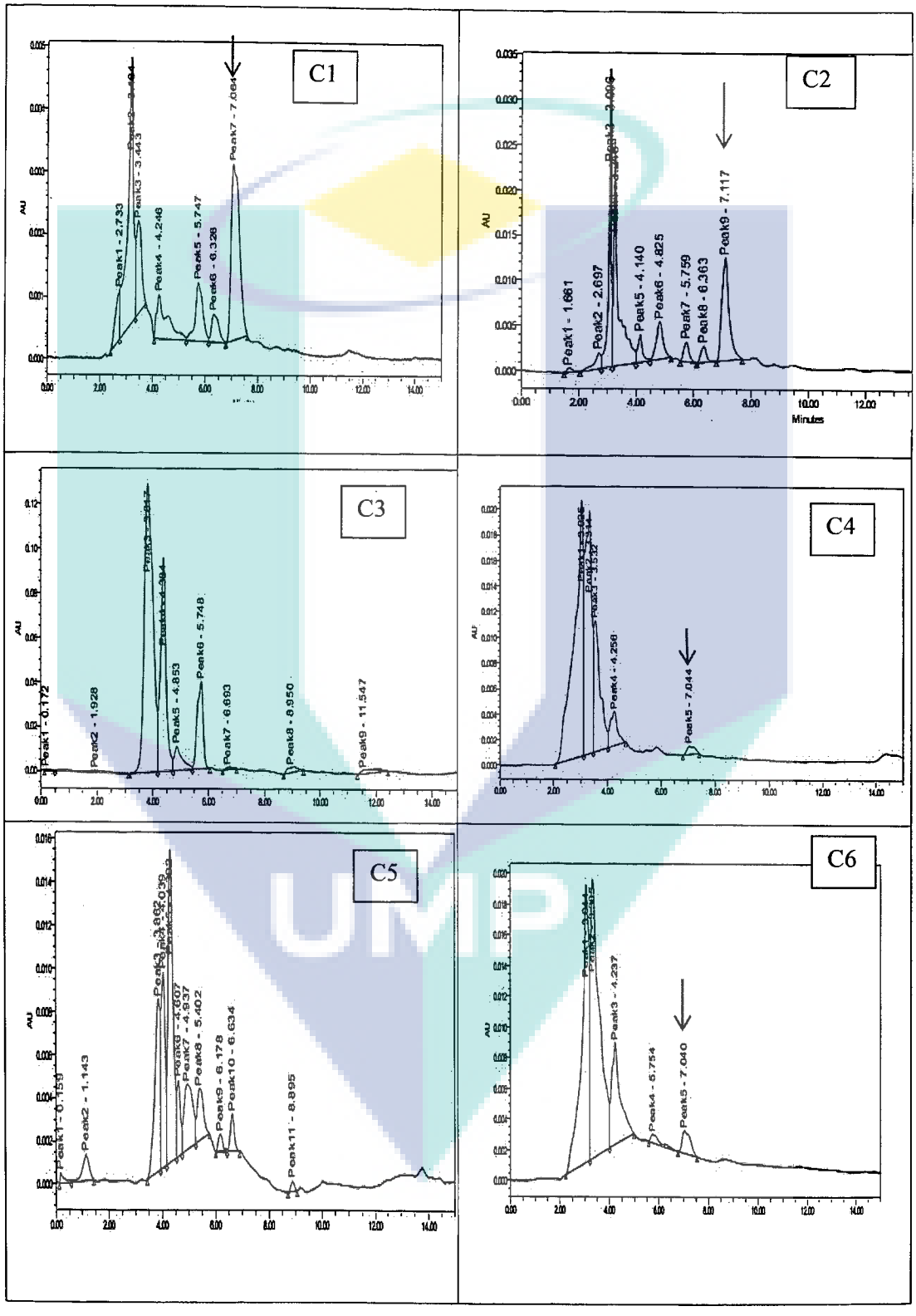
Appendix A1 – Pictures of commercial products

International Products (IP)			
C37  Make My Pepper Big	C39  Unleash Power	C40  Herb Natural Tongkat ALi	C41  Indonesia Tongkat Ali

Negative Control (NC)			
	 NC1 Gali Gali	NC2  Ubi Jaga	

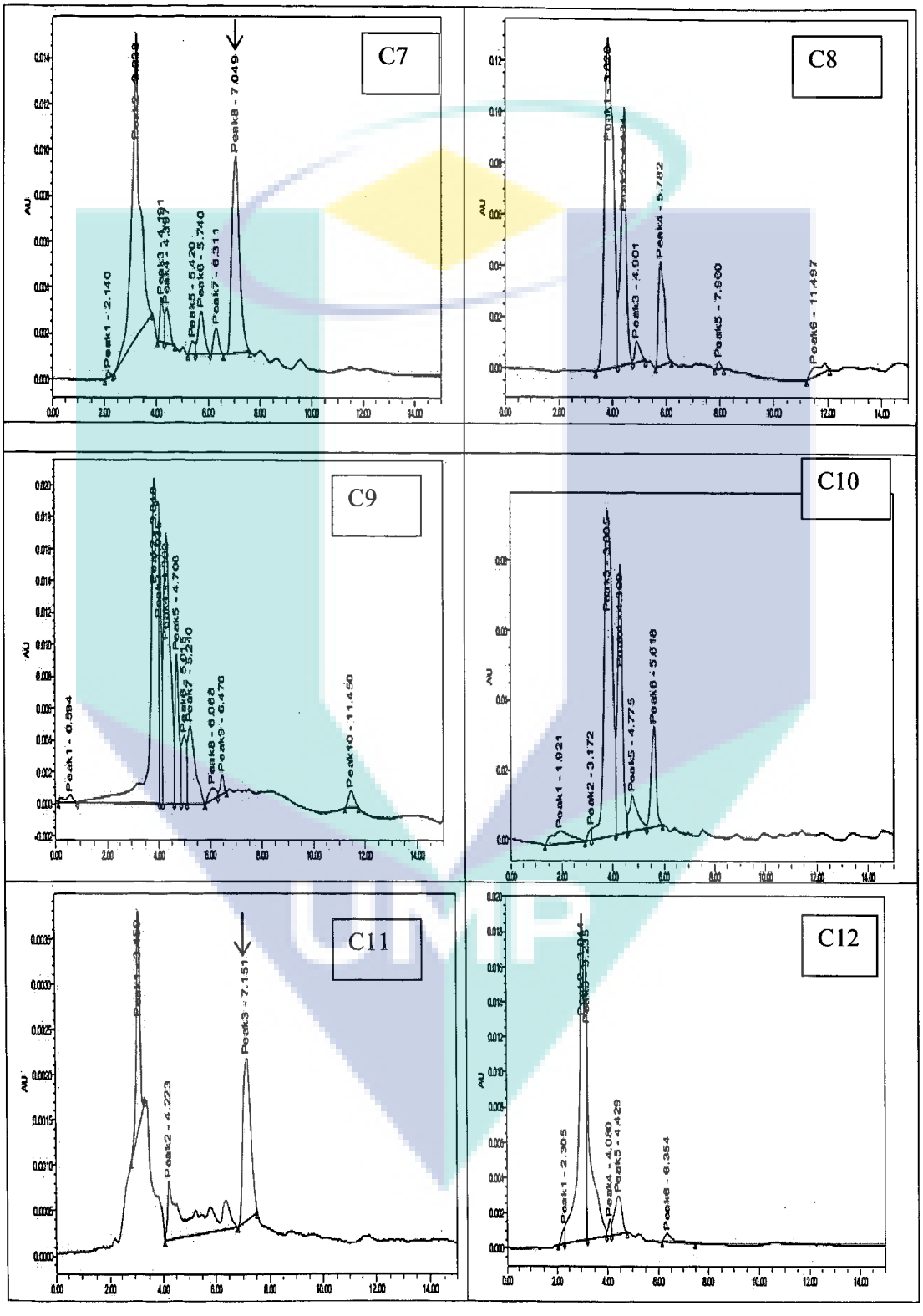
APPENDIX B

HPLC chromatograms of samples



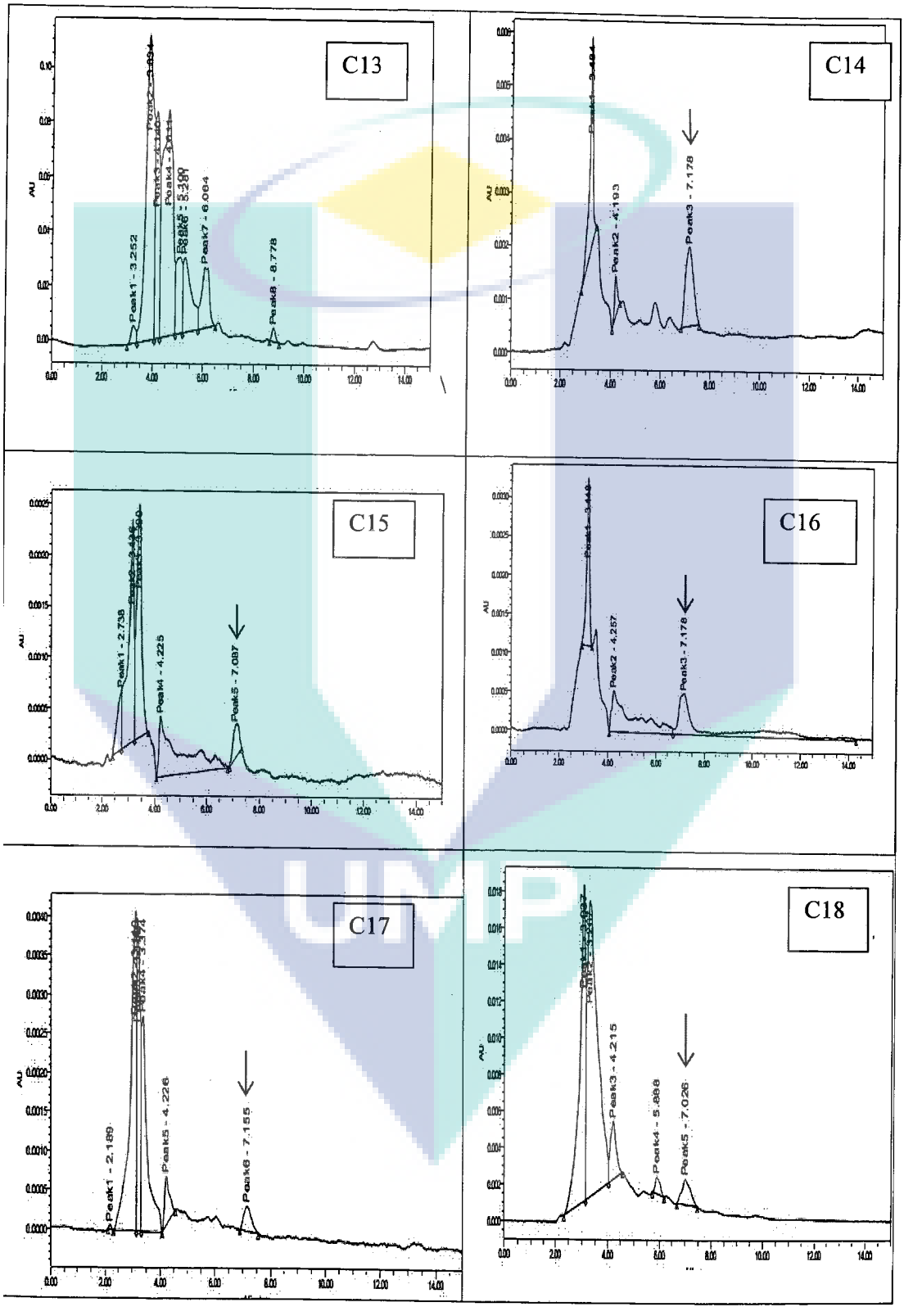
APPENDIX B

HPLC chromatograms of samples



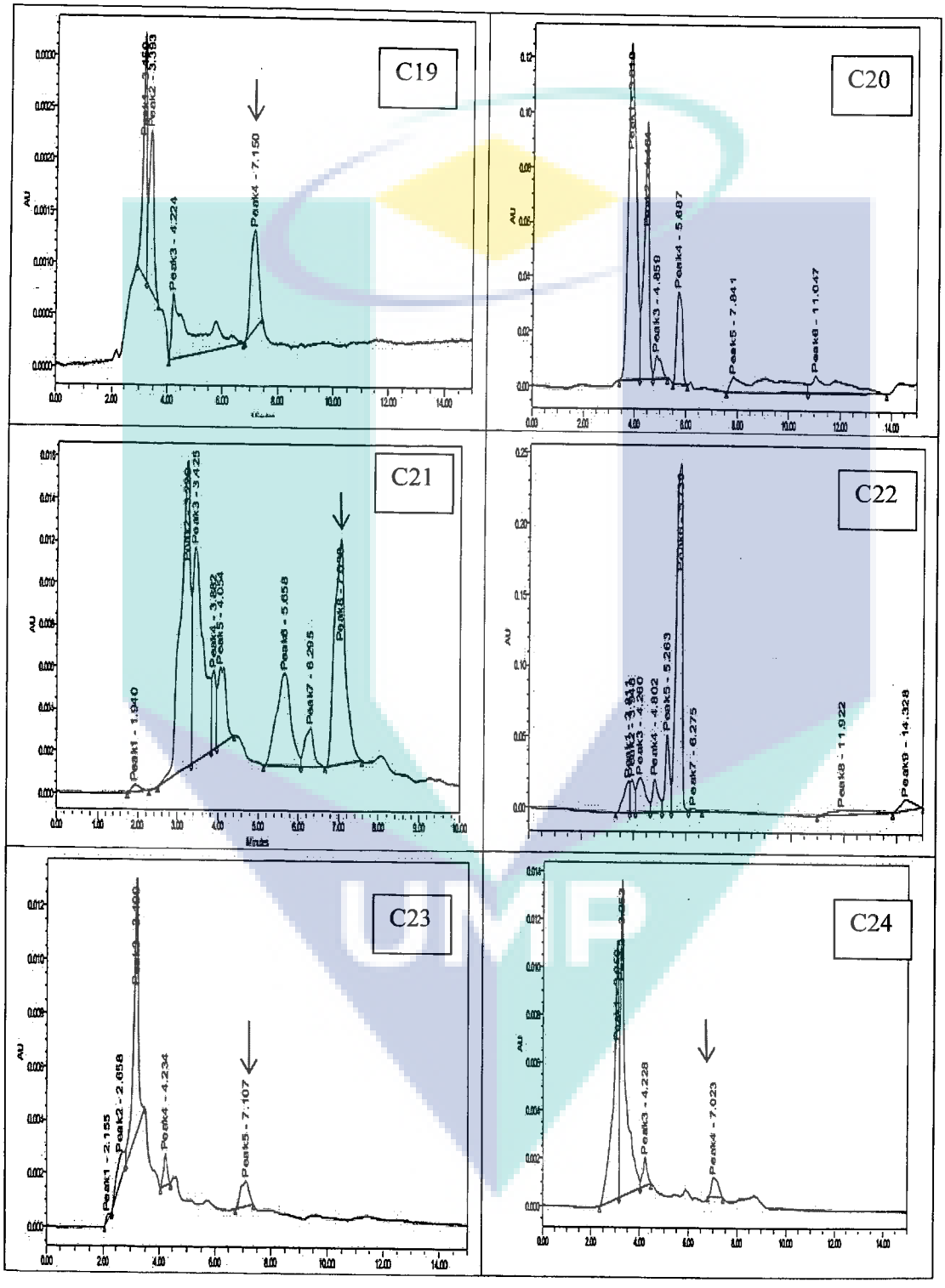
APPENDIX B

HPLC chromatograms of samples



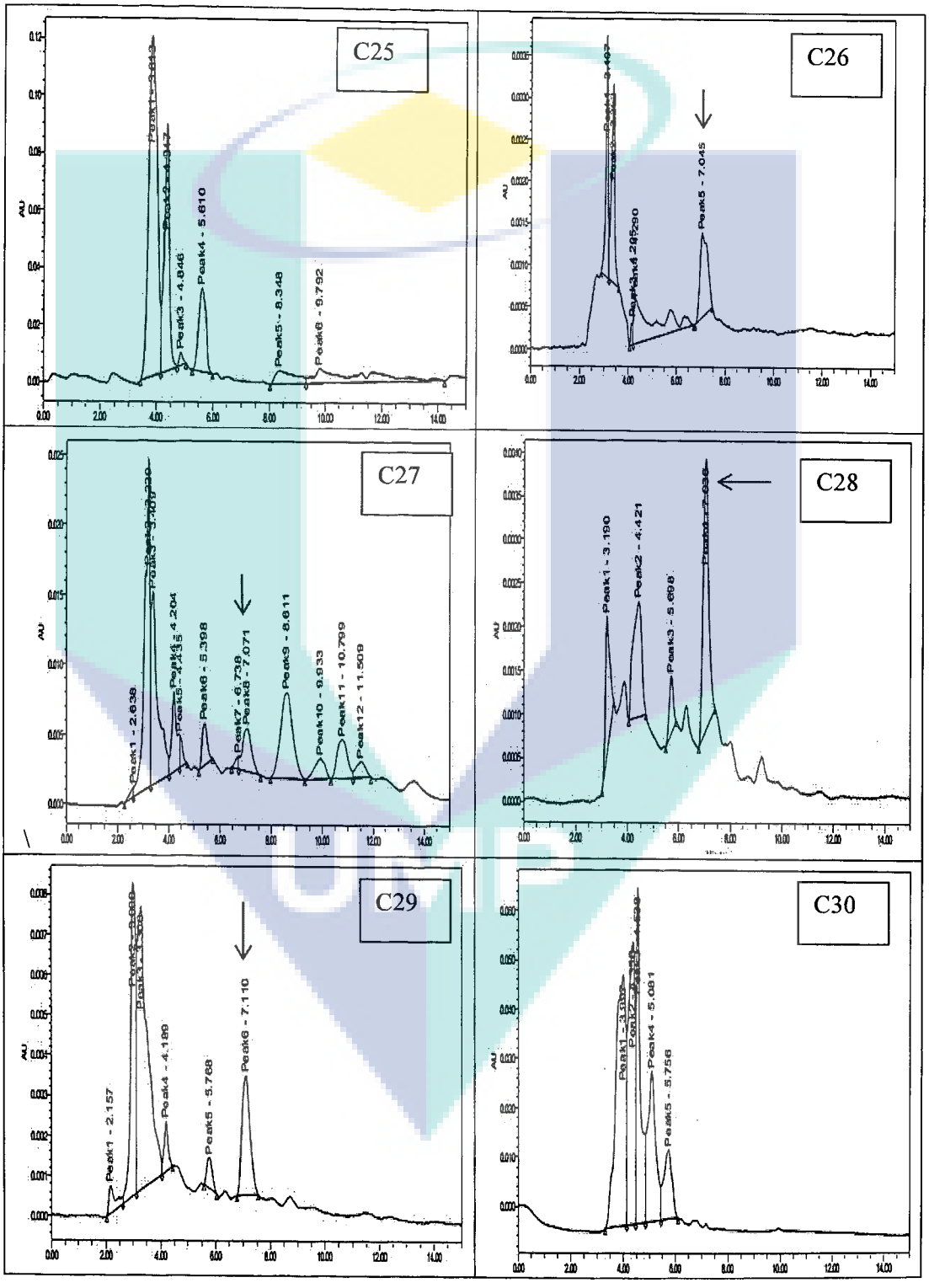
APPENDIX B

HPLC chromatograms of samples



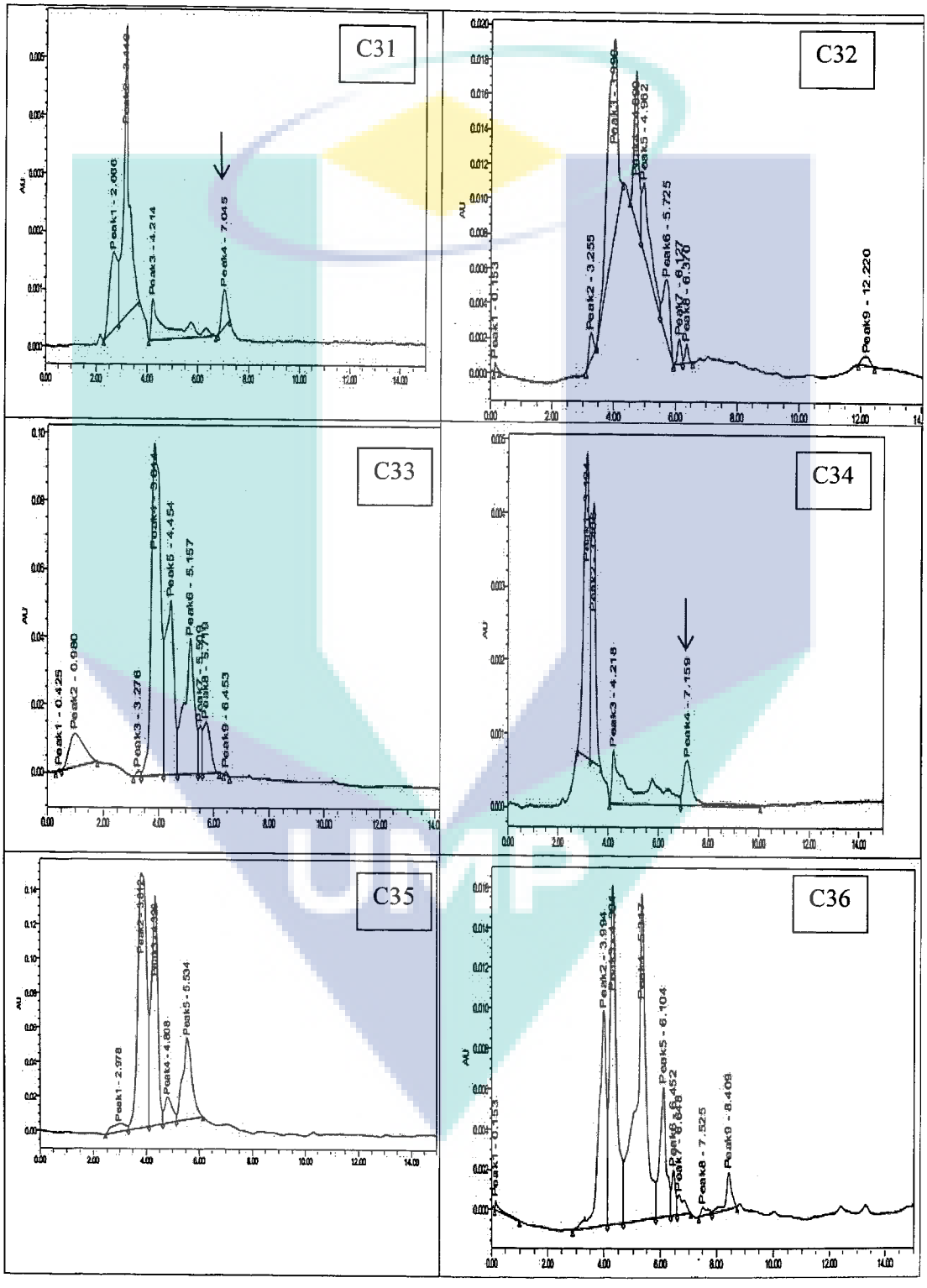
APPENDIX B

HPLC chromatograms of samples



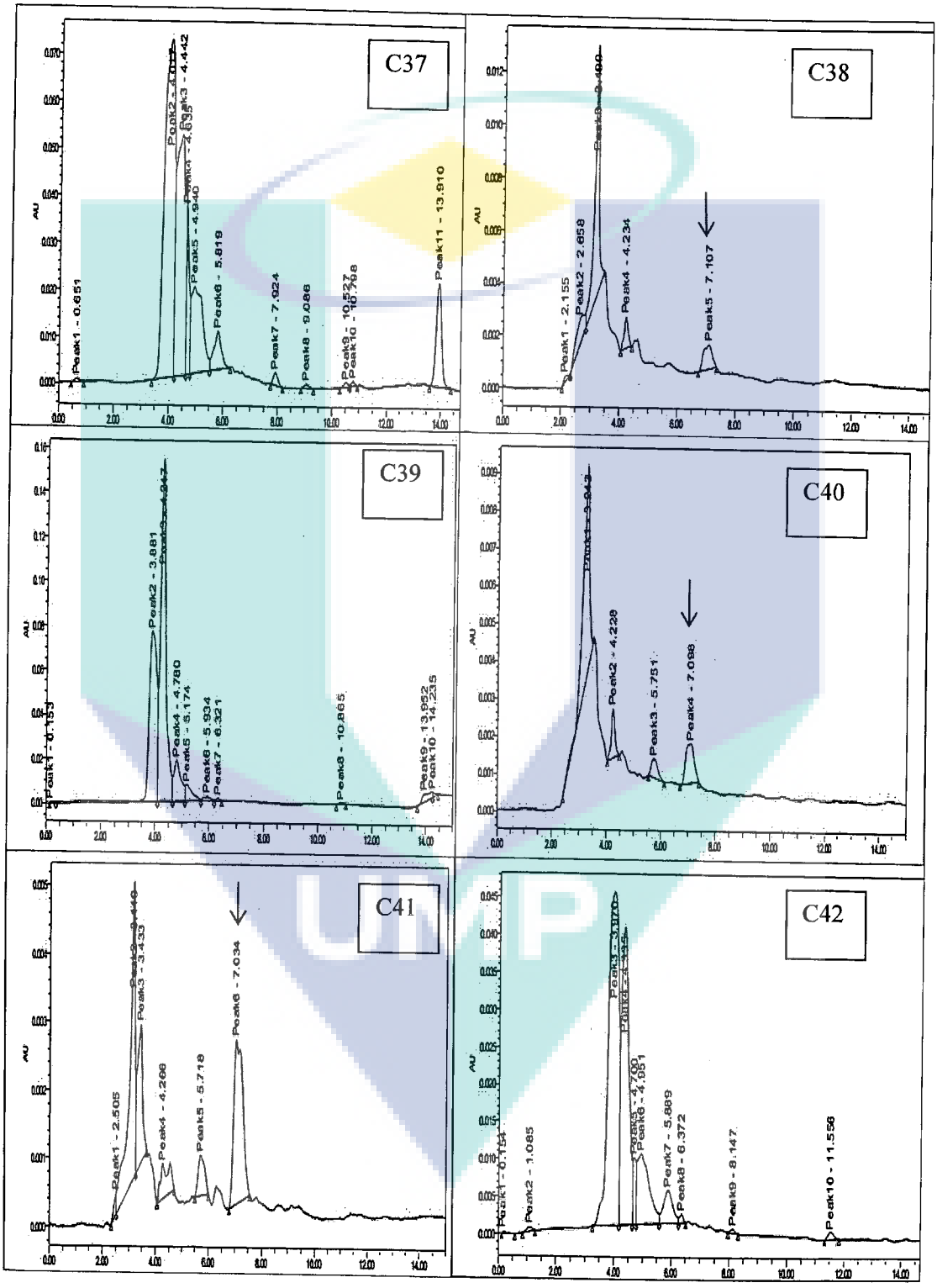
APPENDIX B

HPLC chromatograms of samples



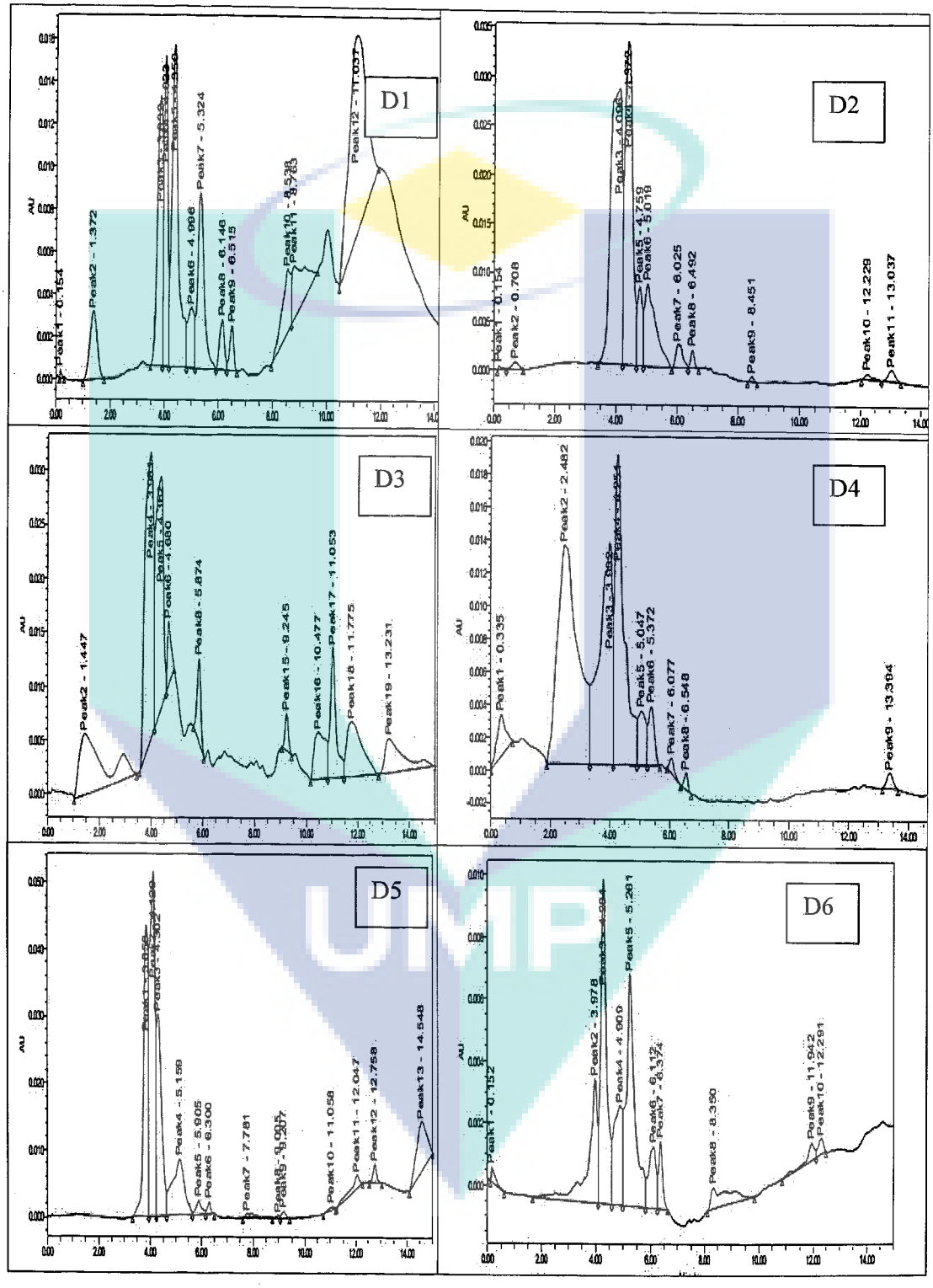
APPENDIX B

HPLC chromatograms of samples



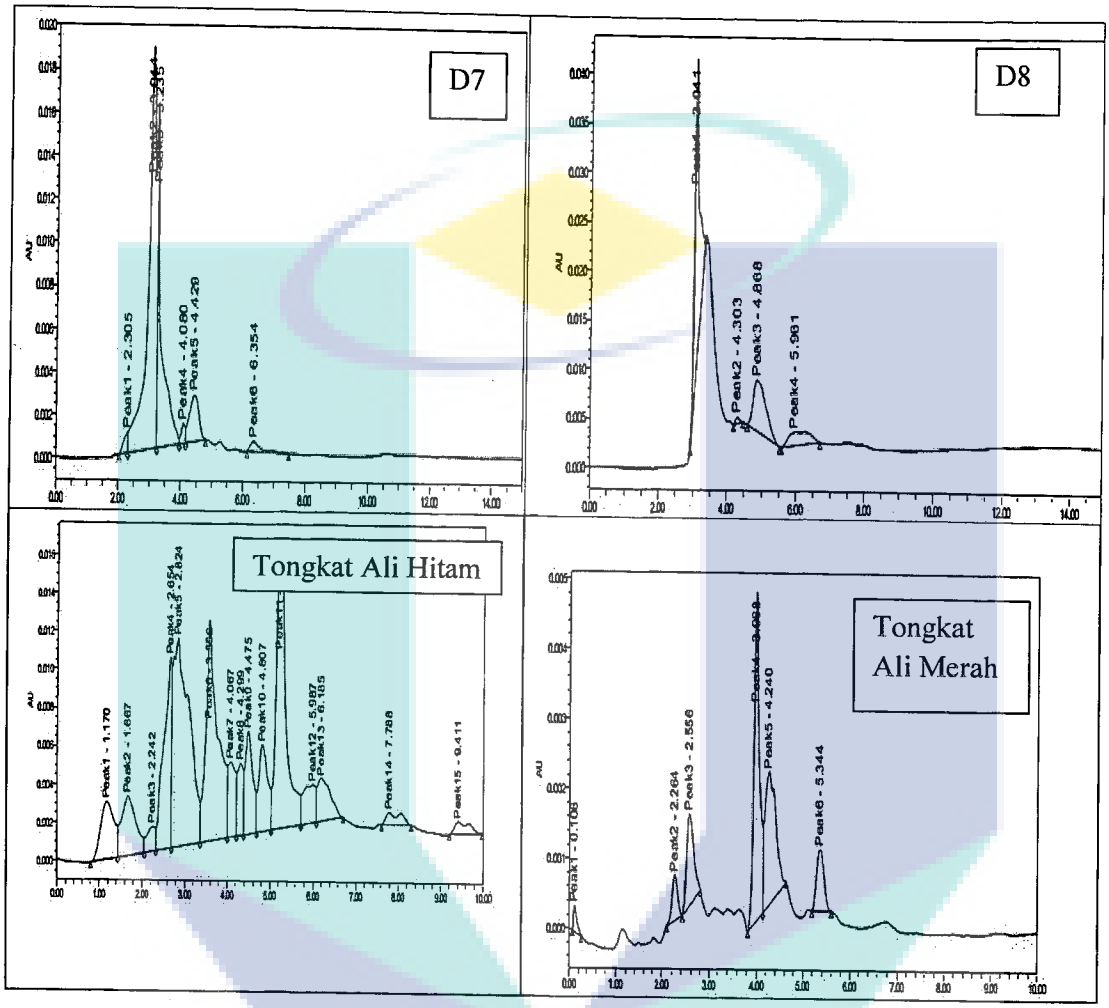
APPENDIX B

HPLC chromatograms of samples



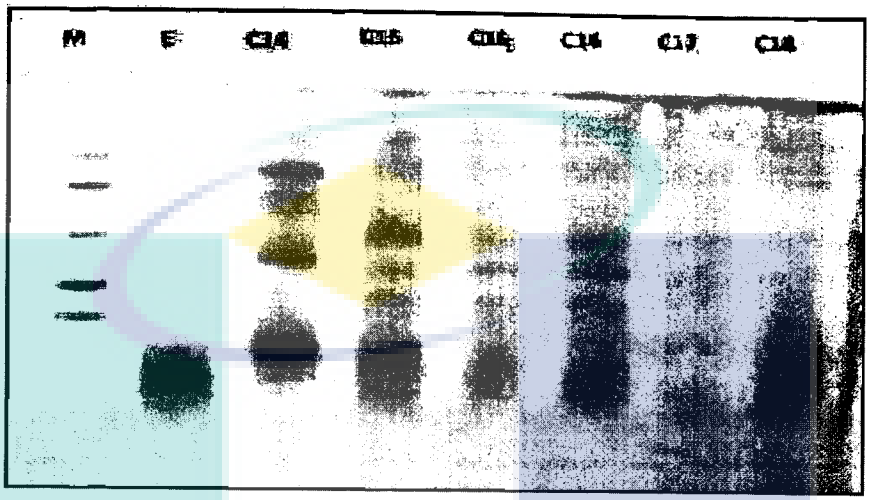
APPENDIX B

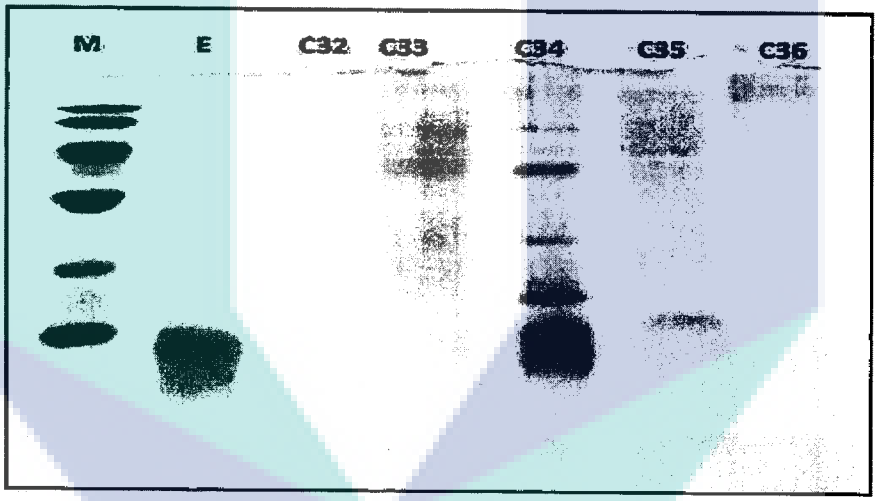
HPLC chromatograms of samples

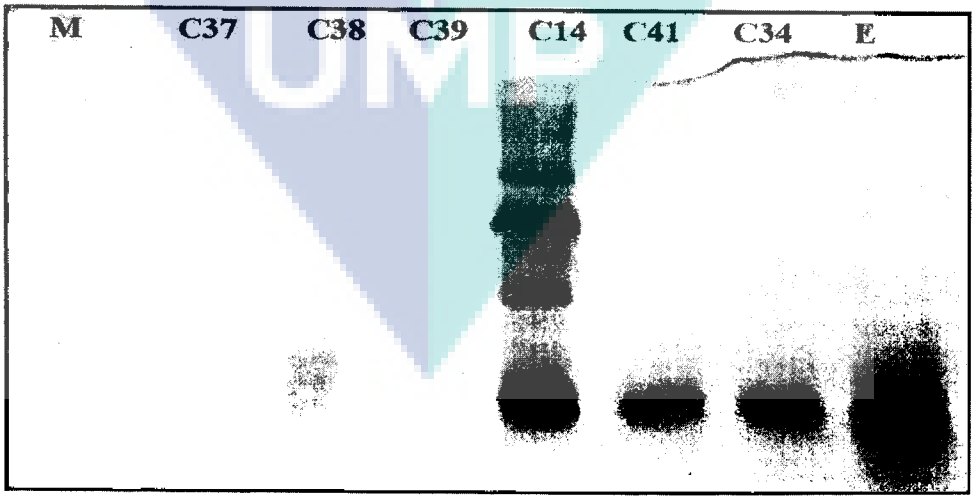


APPENDIX C

SDS PAGE gels of samples

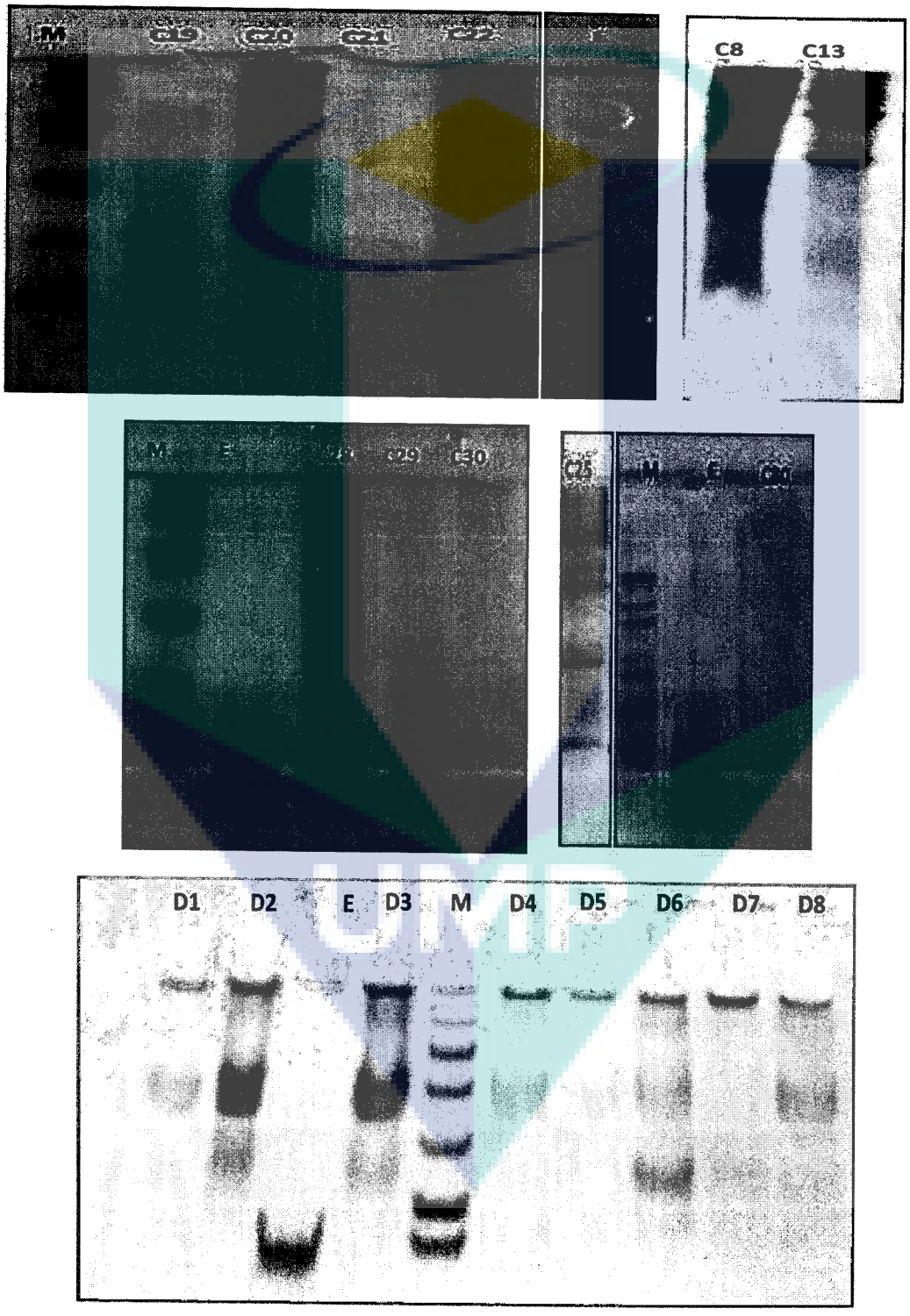






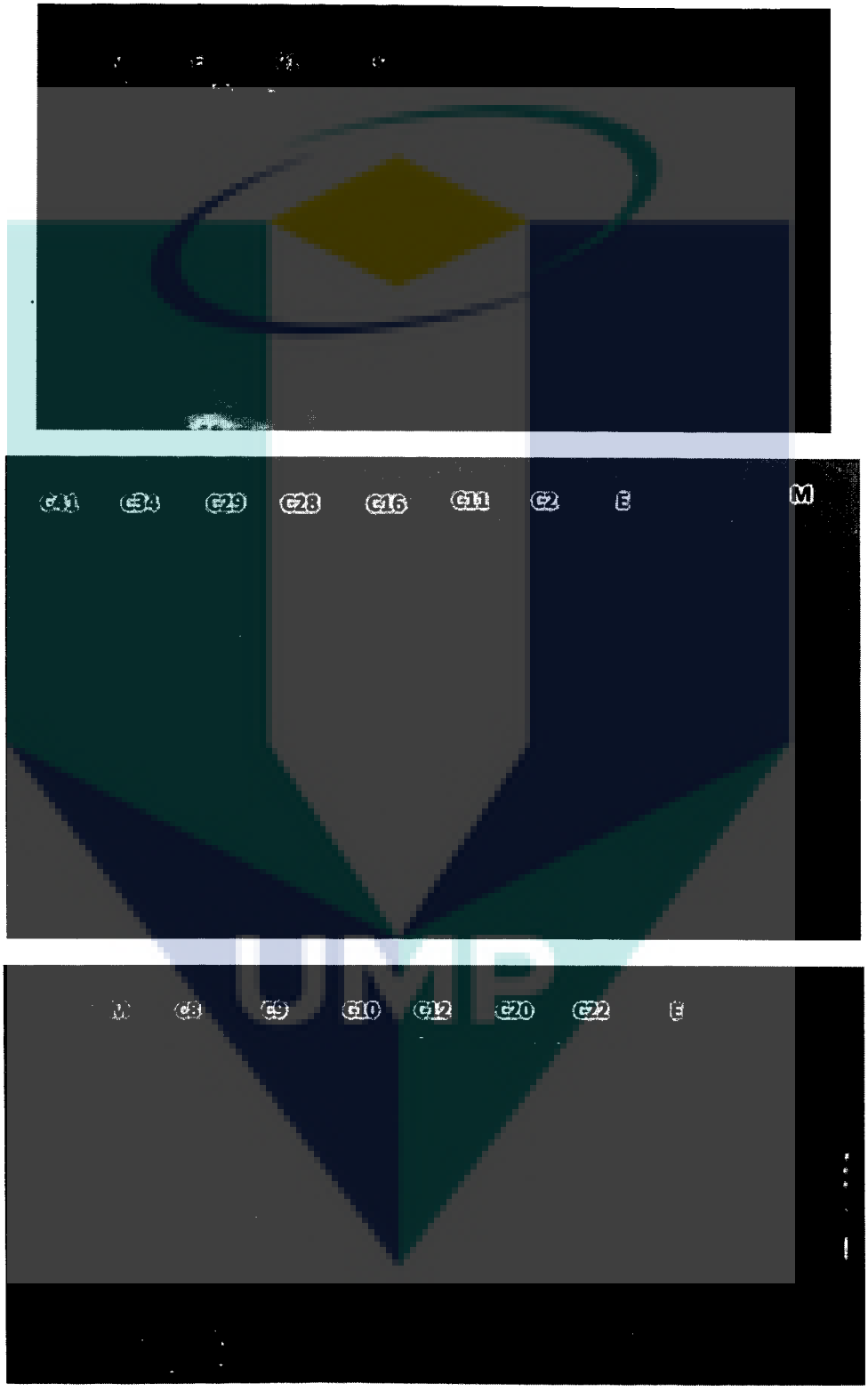
APPENDIX C

SDS PAGE gels of samples



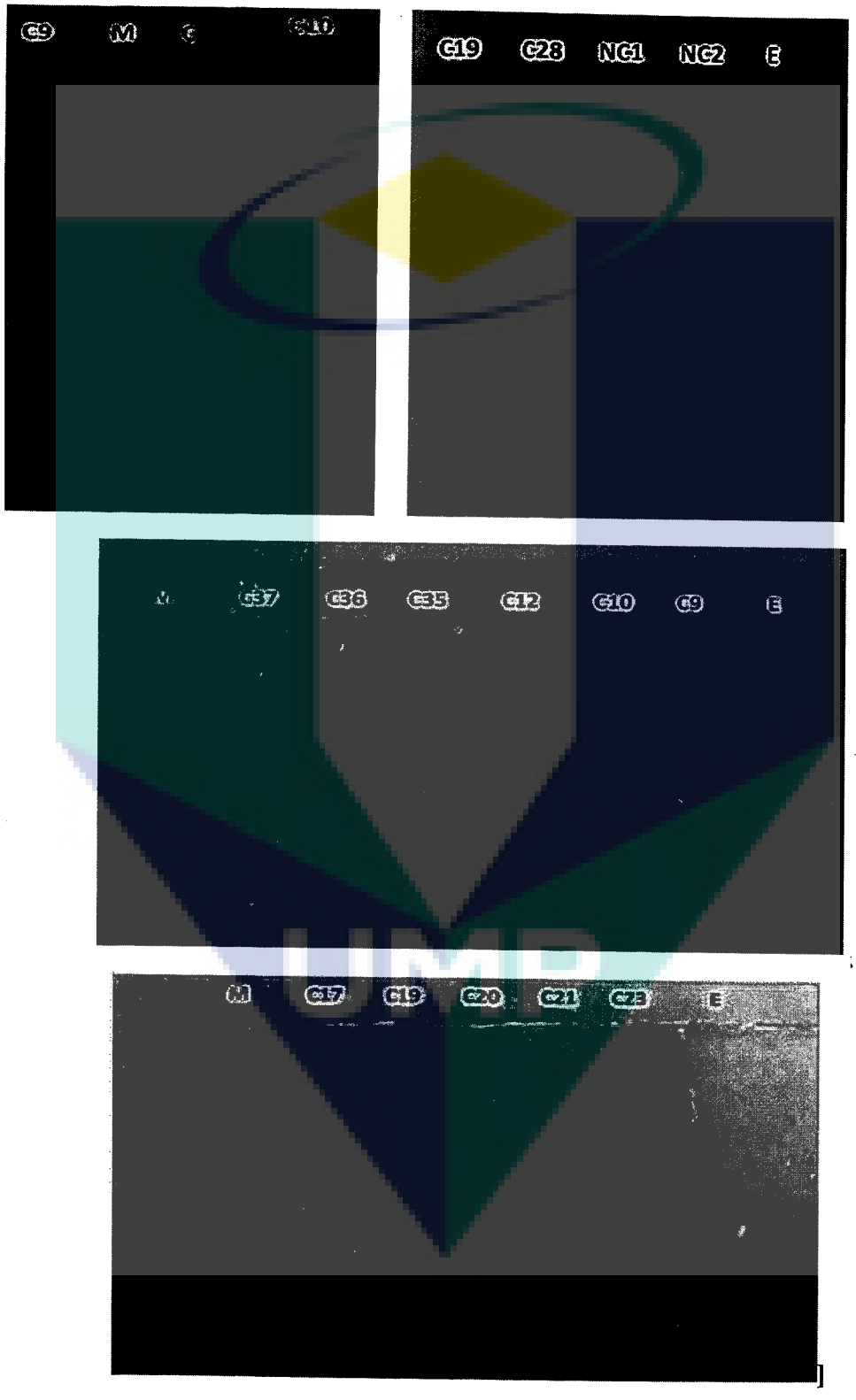
APPENDIX C

SDS PAGE gels of samples



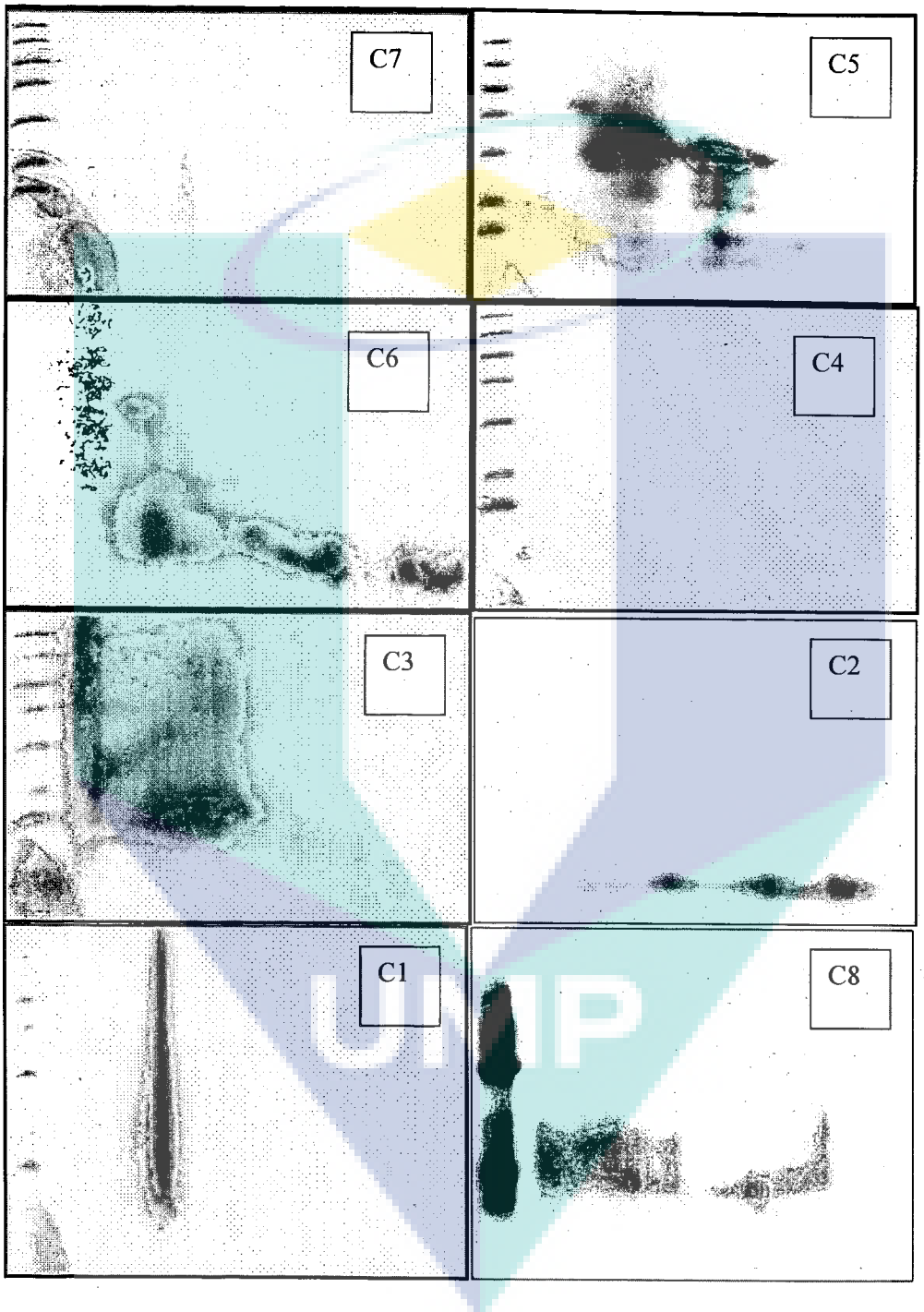
APPENDIX C

SDS PAGE gels of samples



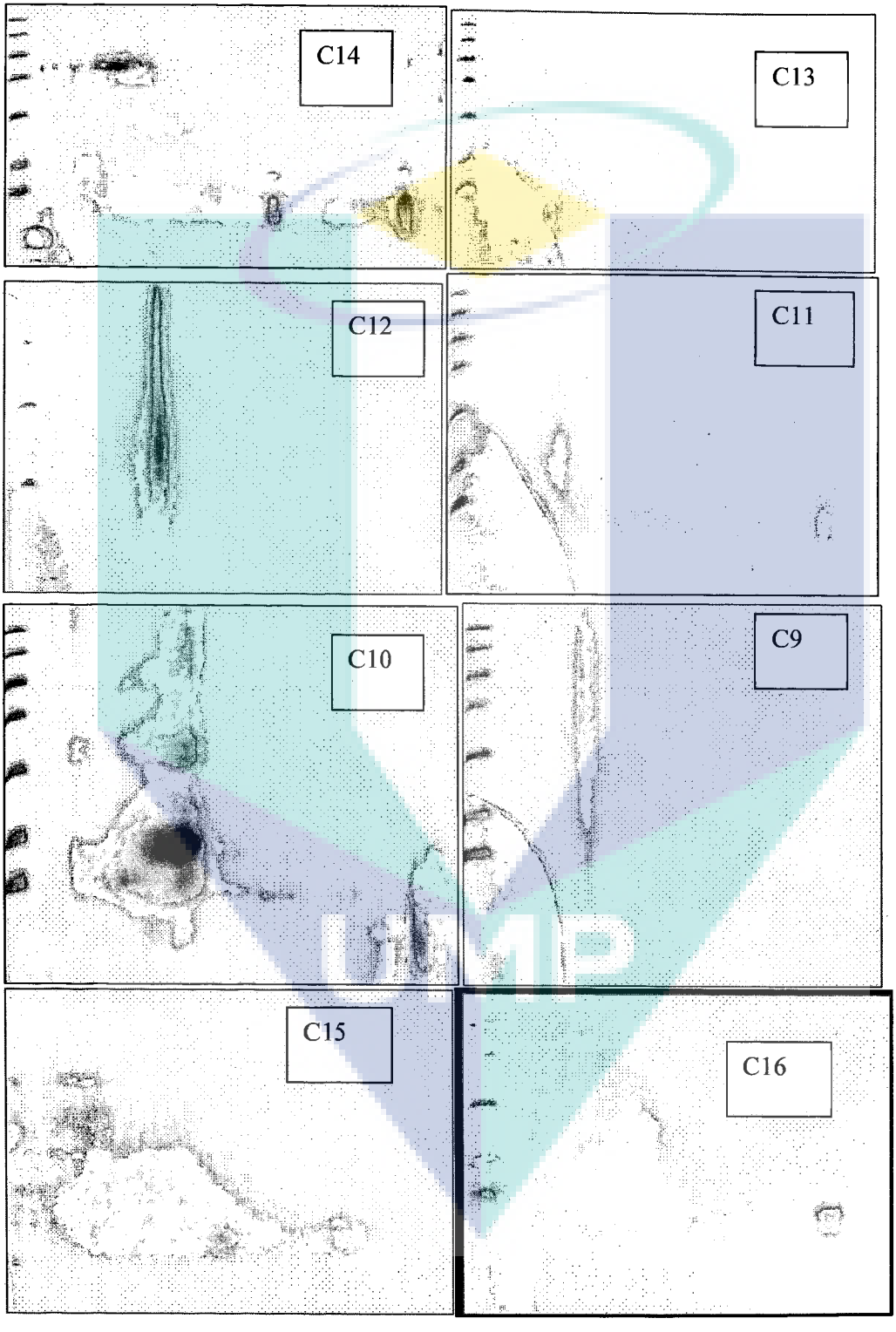
APPENDIX D

2DE gels of samples



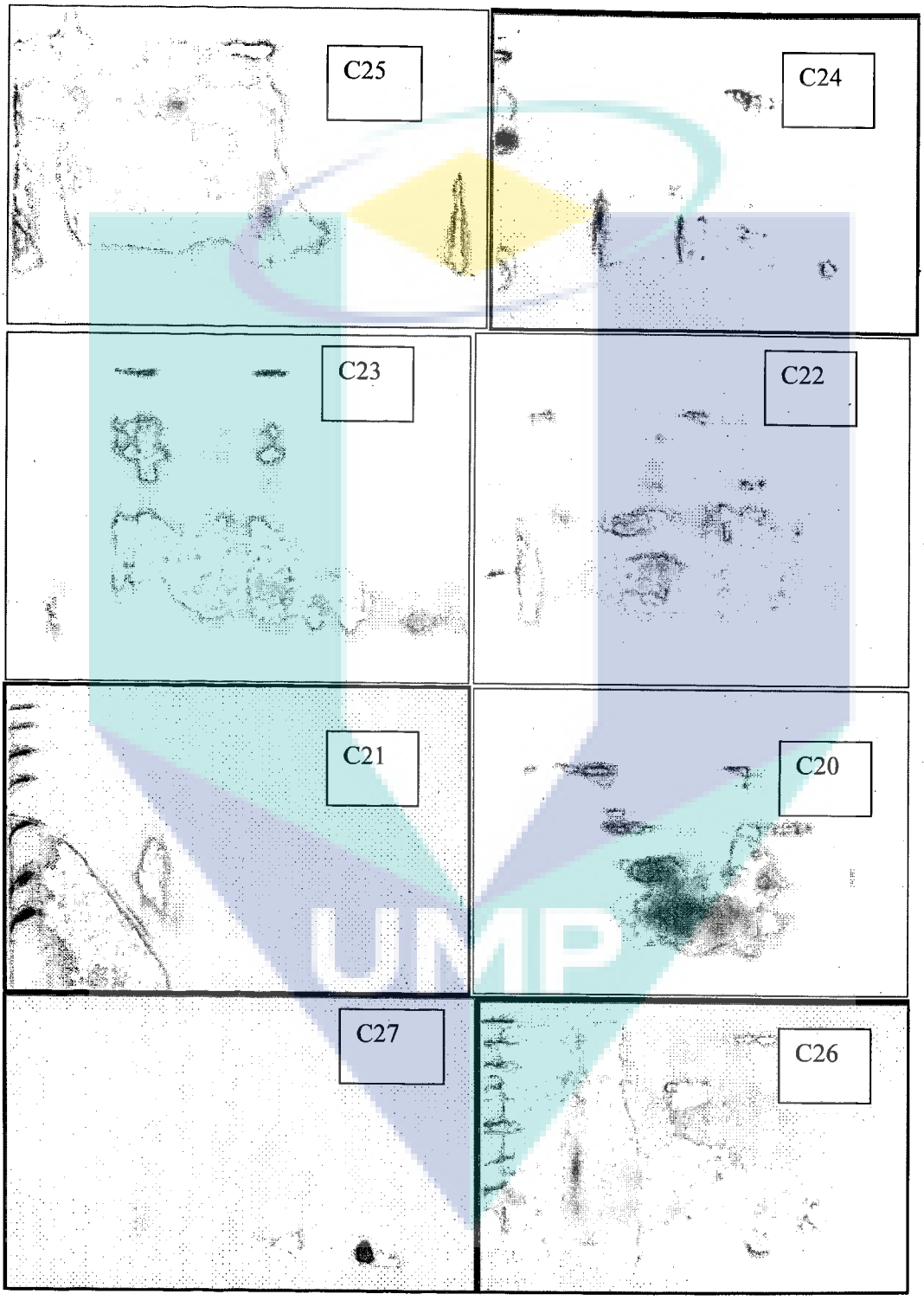
APPENDIX D

2DE gels of samples



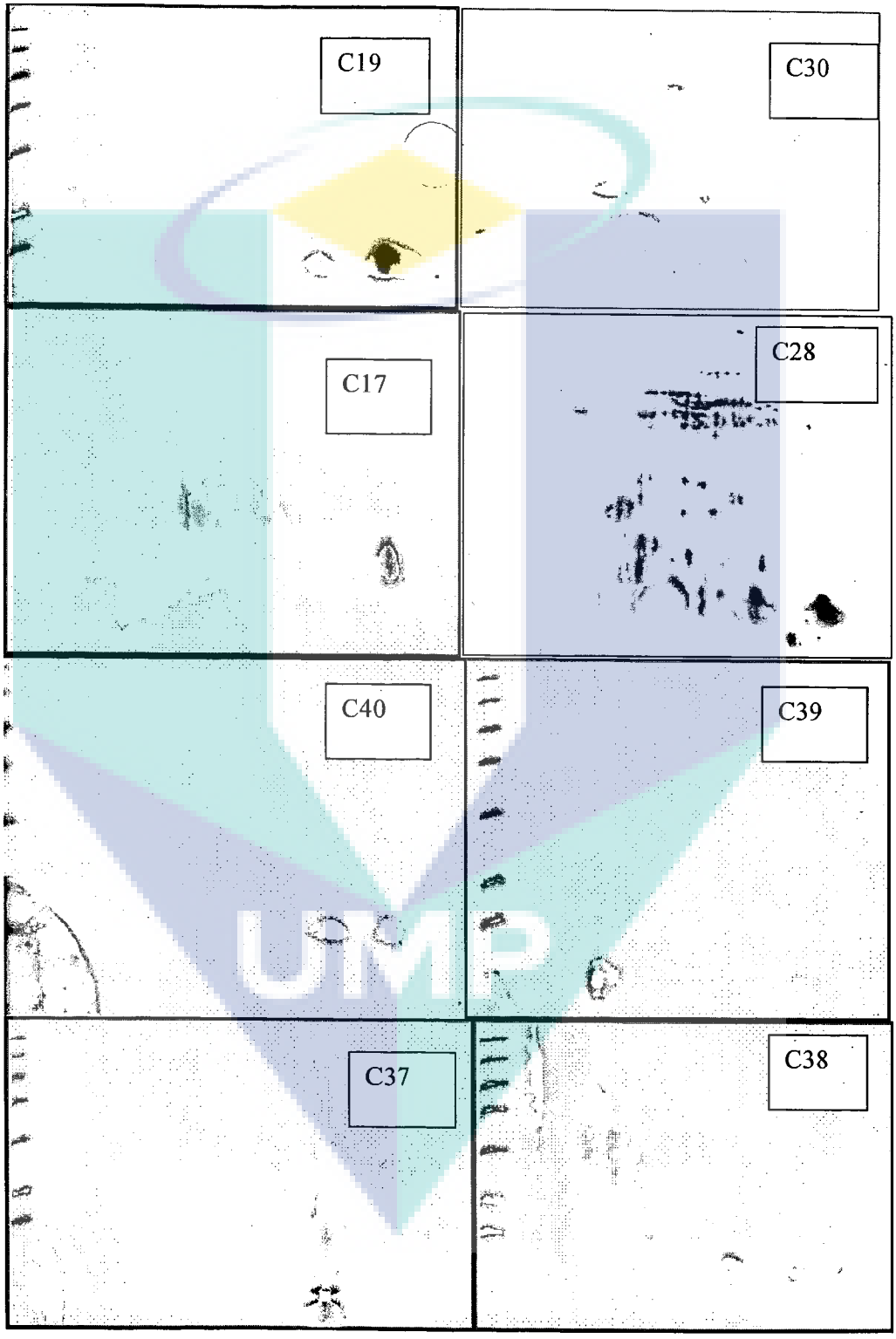
APPENDIX D

2DE gels of samples



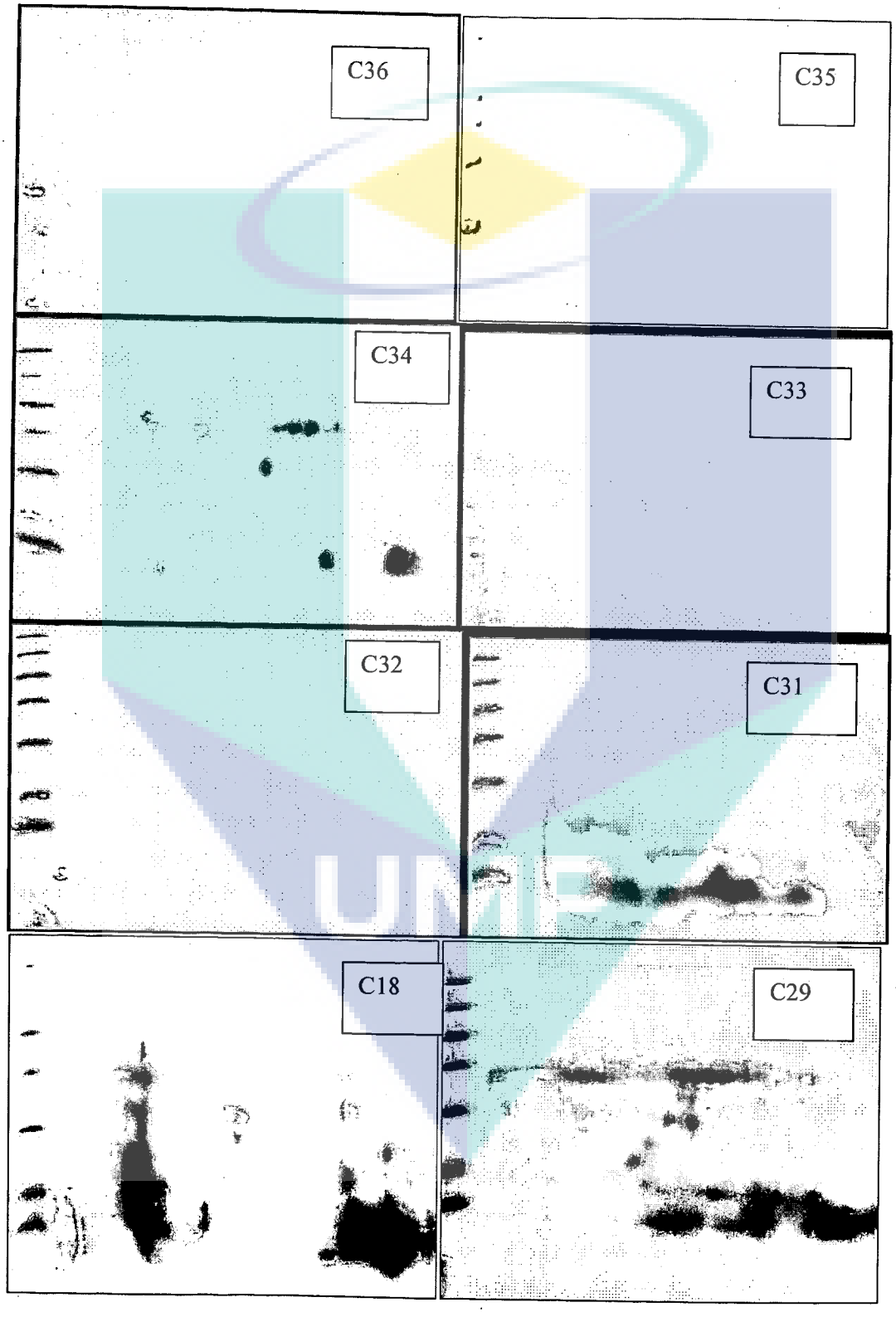
APPENDIX D

2DE gels of samples



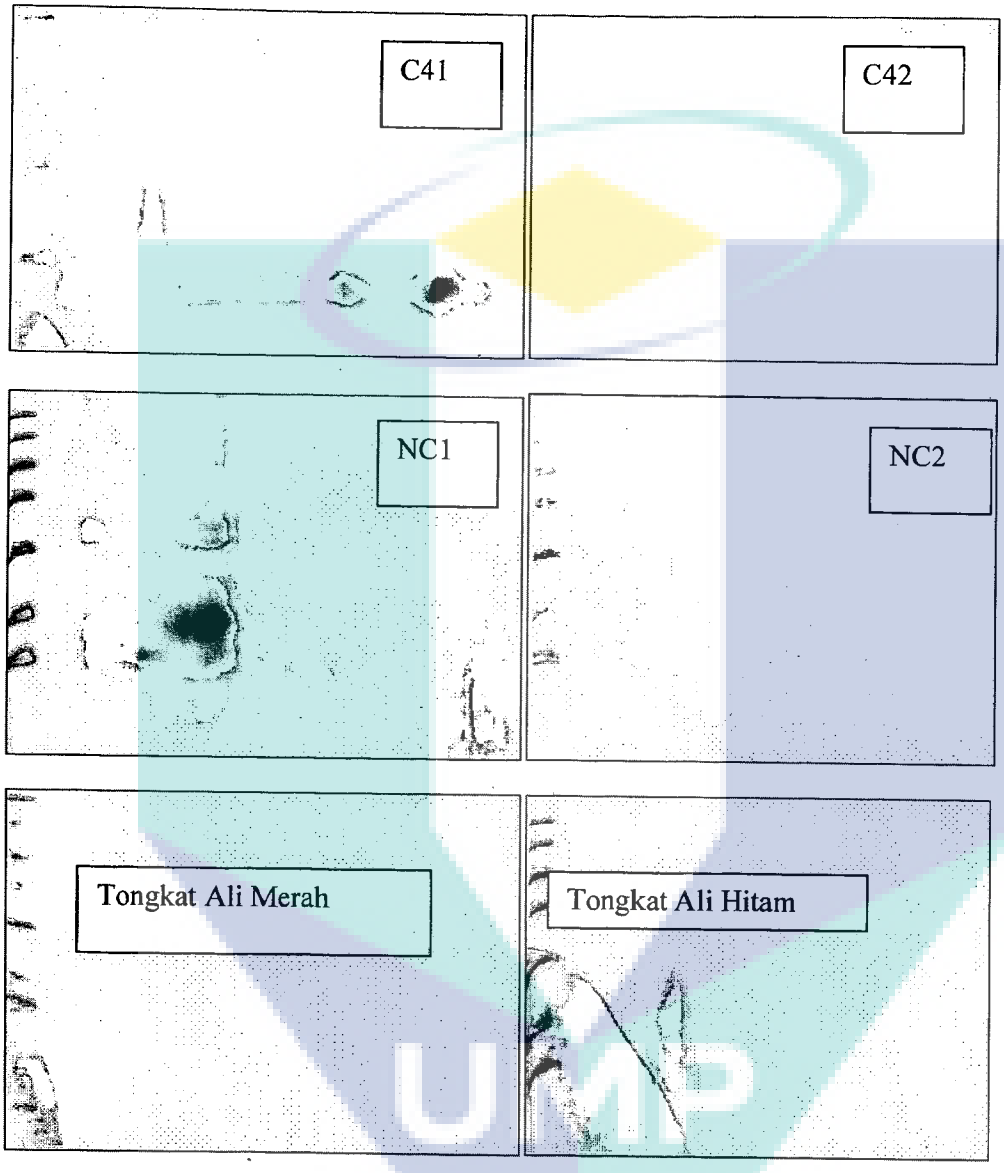
APPENDIX D

2DE gels of samples



APPENDIX D

2DE gels of samples



APPENDIX E

Student's t test analysis of eurycomanone and Marker A

	Eurycomanone	Marker A
Crude extract	15.15	13.3
C28	3.82	5.7
C29	3.24	8.2
C11	2.95	5.1
C2	2.27	4.3
C41	2.12	10.1
C19	1.96	11.7
C14	1.72	8.3
C27	1.66	8.5
C26	1.03	2.4
C16	0.99	7.9
C40	0.84	10.3
C6	0.75	8.2
C34	0.55	10.5
C18	0.4	9.3
C24	0.36	2.6
C31	0.31	5.6
C17	0.26	6.1
C15	0.22	4.3
C38	0.19	7.2
C23	0.16	2.9

t-Test: Paired Two Sample for Means

	Eurycomanone	*Marker A*
Mean	1.95	7.261904762
Variance	10.34202	9.30147619
Observations	21	21
Pearson Correlation	0.467090061	
Hypothesized Mean D	0	
df	20	
t Stat	-7.518939215	
P(T<=t) one-tail	1.49873E-07	
t Critical one-tail	2.527977003	
P(T<=t) two-tail	2.99747E-07	
t Critical two-tail	2.84533971	

2t critical > t Stat = statistically not significant

APPENDIX F

Eurycoma longifolia protein in NCBI database

Summary 20 per page Sort by Default order

Items: 11

1. maturase K, partial (chloroplast) [Eurycoma longifolia]
   269 aa protein
   Accession: BAO71833.1 GI: 619328442

2. ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) [Eurycoma longifolia]
   177 aa protein
   Accession: BAO72441.1 GI: 619326975

3. ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) [Eurycoma longifolia]
   177 aa protein
   Accession: BAO72400.1 GI: 619326893

4. maturase K, partial (chloroplast) [Eurycoma longifolia]
   243 aa protein
   Accession: AIK19444.1 GI: 672917582

5. ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) [Eurycoma longifolia]
   155 aa protein
   Accession: AIG54763.1 GI: 667480300

6. ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) [Eurycoma longifolia]
   172 aa protein
   Accession: AIG54762.1 GI: 667480298

APPENDIX F

Eurycoma longifolia protein in NCBI database

7. AtpB, partial (chloroplast) [Eurycoma longifolia]
   498 aa protein
   Accession: ABU75161.1 GI: 156530129

8. ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) [Eurycoma longifolia]
   474 aa protein
   Accession: ABS87159.1 GI: 154814215

9. PhyC, partial [Eurycoma longifolia]
   315 aa protein
   Accession: ABS87091.1 GI: 154814078

10. maturase K (chloroplast) [Eurycoma longifolia]
    505 aa protein

http://www.ncbi.nlm.nih.gov/protein/?term=eurycoma+longifolia

---

Accession: ABS87023.1 GI: 154813941

11. maturase K, partial (chloroplast) [Eurycoma longifolia]
    260 aa protein
    Accession: BAO71792.1 GI: 619328360

APPENDIX F2

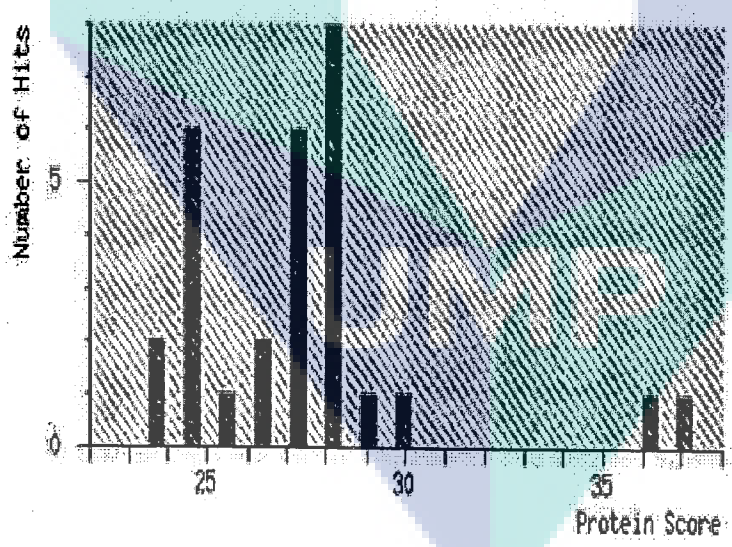
Mascot search results of Marker A

MATRIX SCIENCE **Mascot Search Results**

User : aini
Email : aininorhidayah@gmail.com
Search title :
Database : SwissProt 2015_12 (550116 sequences; 196219159 residues)
Taxonomy : Viridiplantae (Green Plants) (37193 sequences)
Timestamp : 15 Jan 2016 at 17:28:30 GMT
Top Score : 37 for BCP_PEA, Blue copper protein OS=Pisum sativum PE=2 SV=1

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 58 are significant (p<0.05).



Concise Protein Summary Report

APPENDIX F2

Mascot search results of Marker A

Concise Protein Summary Report

Format As: Concise Protein Summary ▼ Help

Significance threshold p< 0.05 Max. number of hits AUTO

Preferred taxonomy All entries ▼

Re-Search All | Search Unmatched

1. BCP_PEA Mass: 19306 Score: 37 Expect: 7.3 Matches: 4
Blue copper protein OS=Pisum sativum PE=2 SV=1

Search Parameters

```
Type of search        : Peptide Mass Fingerprint
Enzyme                : Trypsin
Mass values           : Monoisotopic
Protein Mass          : Unrestricted
Peptide Mass Tolerance : ± 1.2 Da
Peptide Charge State  : 1+
Max Missed Cleavages  : 1
Number of queries     : 19
```

Mascot: http://www.matrixscience.com/

http://www.matrixscience.com/cgi/master_results.pl?file=../data/20160115/FFTTpSrYtS.dat&sessionID=guest_guestsession

APPENDIX F2

Mascot search results of BCP_PEA

MATRIX SCIENCE **MASCOT Search Results**

Protein View: BCP_PEA

Blue copper protein OS=Pisum sativum PE=2 SV=1

Database:	SwissProt
Score:	37
Expect:	7.3
Nominal mass (M_r):	19306
Calculated pI:	6.25
Taxonomy:	Pisum sativum

Sequence similarity is available as an NCBI BLAST search of BCP_PEA against nr.

Search parameters

Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Mass values searched:	19
Mass values matched:	4

Protein sequence coverage: 32%

Matched peptides shown in ***bold red.***

```
  1 MAFSNALVLC FLLAIINMAL PSLATVYTVG DTSGWVIGGD YSTWASDKTF
 51 AVGDSLVFNY GAGAHTVDEV KESDYKSCTS GNSISTDSTG ATTIPLKKAG
101 KHYFICGVPG HSTGGMKLSI KVKASSGSSA APSATPSSSG KGSPSSDDTP
151 AATTTTTTPT KQNESSATSL SPIVALFFTV SWICSYVLV
```

Unformatted sequence string: 189 residues (for pasting into other applications).

APPENDIX F2

Mascot search results of BCP_PEA

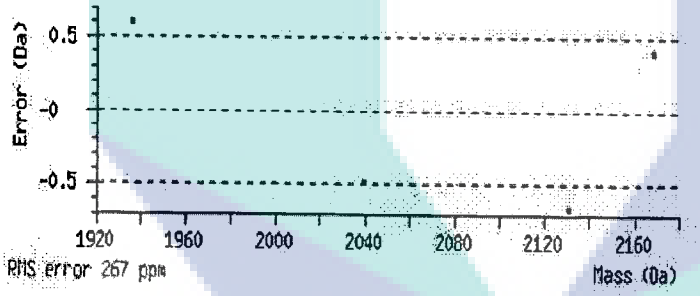
Unformatted sequence string: **189 residues** (for pasting into other applications).

Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

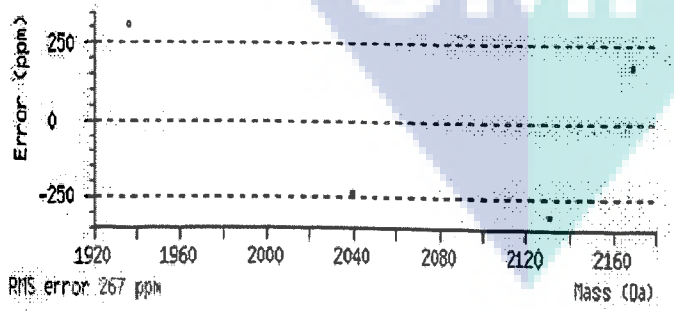
Show predicted peptides also

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	M	Peptide
77 - 97	2040.4850	2039.4777	2039.9681	-0.4904	0	K.SCTSGNSISTDSTGATTIPLK.K
77 - 98	2169.4680	2168.4607	2168.0631	0.3976	1	K.SCTSGNSISTDSTGATTIPLKK.A
102 - 121	2131.4200	2130.4127	2131.0707	-0.6580	1	K.HYFICGVPGHSTGGMKLSIK.V
142 - 161	1937.5060	1936.4987	1935.8909	0.6078	0	K.GSPSSDDTPAATTTTTTPTK.Q

No match to: 804.0280, 805.0240, 806.0310, 806.9870, 826.9950, 841.9920, 858.0030, 885.5570, 1935.4270, 2167.4390, 2184.3860, 2259.3500, 2261.3300, 2274.4180, 2276.3760



ttp://www.matrixscience.com/cgi/protein_view.pl?file=..%2Fdata%2F20160115%2FFTTpSrYtS.dat&hit=1&db_idx=1



APPENDIX F3

Mascot search results of BCP_PEA

```
AC   Q41001;
DT   01-NOV-1997, integrated into UniProtKB/Swiss-Prot.
DT   01-NOV-1996, sequence version 1.
DT   16-SEP-2015, entry version 63.
DE   RecName: Full=Blue copper protein;
DE   Flags: Precursor;
OS   Pisum sativum (Garden pea).
OC   Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
OC   Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae;
OC   Pentapetalae; rosids; fabids; Fabales; Fabaceae; Papilionoideae;
OC   Fabeae; Pisum.
OX   NCBI_TaxID=3888;
RN   [1]
RP   NUCLEOTIDE SEQUENCE [MRNA].
RC   STRAIN=cv. Line 59; TISSUE=Pod;
RA   Drew J.E.;

RL   Thesis (1994), Durham University, United Kingdom.
CC   -!- SIMILARITY: Contains 1 phytocyanin domain. {ECO:0000255|PROSITE-
CC       ProRule:PRU00818}.
DR   EMBL; Z25471; CAA80963.1; -; mRNA.
DR   PIR; T06555; T06555.
DR   ProteinModelPortal; Q41001; -.
DR   GO; GO:0009055; F:electron carrier activity; IEA:InterPro.
DR   GO; GO:0046872; F:metal ion binding; IEA:UniProtKB-KW.
DR   GO; GO:0055114; P:oxidation-reduction process; IEA:UniProtKB-KW.
DR   Gene3D; 2.60.40.420; -; 1.
DR   InterPro; IPR028871; BlueCu_1_BS.
DR   InterPro; IPR008972; Cupredoxin.
DR   InterPro; IPR003245; Phytocyanin_dom.
DR   Pfam; PF02298; Cu_bind_like; 1.
DR   ProDom; PD003122; Plcyanin-like; 1.
DR   SUPFAM; SSF49503; SSF49503; 1.
DR   PROSITE; PS00196; COPPER_BLUE; 1.

DR   PROSITE; PS51485; PHYTOCYANIN; 1.
PE   2: Evidence at transcript level;
KW   Copper; Disulfide bond; Electron transport; Glycoprotein;
KW   Metal-binding; Signal; Transport.
FT   SIGNAL        1     24       {ECO:0000255}.
FT   CHAIN        25    189       Blue copper protein.
FT                                 /FTId=PRO_0000002870.
FT   DOMAIN       25    124       Phytocyanin. {ECO:0000255|PROSITE-
FT                                 ProRule:PRU00818}.
FT   METAL        65     65       Copper. {ECO:0000255|PROSITE-
FT                                 ProRule:PRU00818}.
FT   METAL       106    106       Copper. {ECO:0000255|PROSITE-
FT                                 ProRule:PRU00818}.
FT   METAL       111    111       Copper. {ECO:0000255|PROSITE-
FT                                 ProRule:PRU00818}.
FT   CARBOHYD    163    163       N-linked (GlcNAc...). {ECO:0000255}.
FT   DISULFID     78    106       {ECO:0000255|PROSITE-ProRule:PRU00818}.
SQ   SEQUENCE   189 AA;  19319 MW;  8B6EB652C7145098 CRC64;
     MAFSNALVLC FLLAIINMAL PSLATVYTVG DTSGWVIGGD YSTWASDKTF AVGDSLVFNY
     GAGAHTVDEV KESDYKSCTS GNSISTDSTG ATTIPLKKAG KHYFICGVPG HSTGGMKLSI
     KVKASSGSSA APSATPSSSG KGSPSSDDTP AATTTTTTPT KQNESSATSL SPIVALFFTV
```

http://www.matrixscience.com/cgi/protein_view.pl?file=..%2Fdata%2F20160115%2FFTTpSrYtS.dat&hit=1&db_idx=1

APPENDIX G

Rf of protein standard

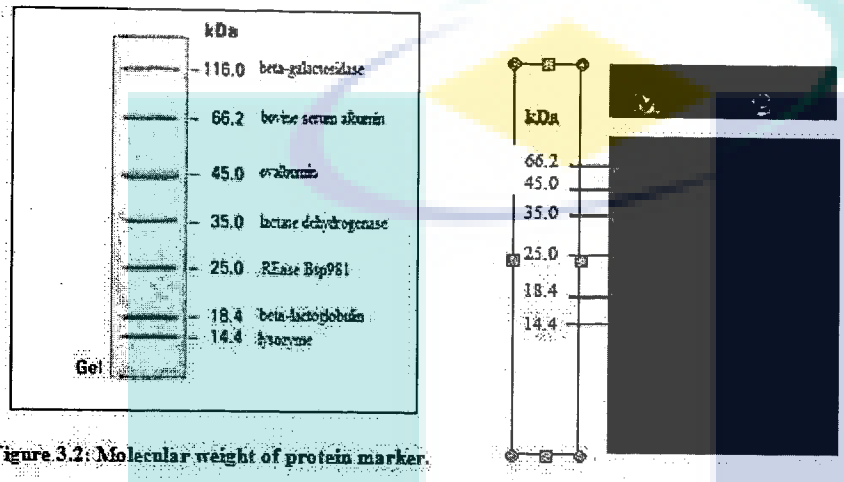


Figure 3.2: Molecular weight of protein marker.

Mw	Migration rf	log mw
116	0.133	2.06445
66.2	0.216	1.8208
45.0	0.2833	1.65321
35.0	0.366	1.544
25.0	0.5	1.3979
18.4	0.66	1.2648
14.4	0.733	1.15836
x	**0.833**	**1**

APPENDIX H

De novo sequencing of Marker A- gel plug

Table 1: Sequencing results of sample PI-3485

m/z	No.	Peptide	Score (%)	Rank
569.76	1	TFMSMTYGFHNFGYCGAVSR	94	0
	2	HQNSMTYGFHNFGYCGAVSR	2	1
	3	HVETMTYGFHNFGYCGAVSR	1	2
	4	EEAHMTYGFHNFGYCGAVSR	1	3
	5	TEVHMTYGFHNFGYCGAVSR	<1	4

Table 2: Sequencing result for peptide m/z 569.76 (PI-3485).
Sequence: TFMSMTYGFHNFGYCGAVSR

#	b	sequence	y	#
1		T		20
2		F		19
3		M		18
4		S		17
5		M	1809.70	16
6		T		15
7		Y	1577.67	14
8		G	1414.62	13
9	1066.41	F	1357.60	12
10		H	1210.53	11
11	1317.53	N	1073.46	10
12		F	959.45	9
13	1521.68	G	812.39	8
14		Y	755.38	7
15		C		6
16		G	489.31	5
17		A	432.29	4
18		V	361.22	3
19		S	262.19	2
20		R	175.15	1

= detected y and b ions

APPENDIX H

De novo sequencing of Marker A- gel plug

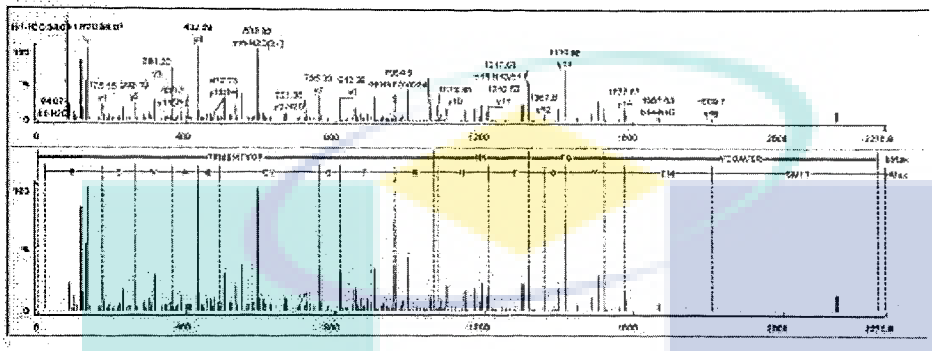


Figure 1: *De novo* spectra for peptide m/z 569.76 (PI-3485).
Reference: 141218.

De novo sequencing of purified Marker A

Table 1: Sequencing results of sample PI-3617

	No.	m/z	Peptide	Score (%)	Rank
PI-3617	1	736.99	CELLHAAMHSVTAMGPGLEGGK	87	0
			ECLLHAAMHSVTAMGPGLEGGK	7	1
			CELLAAHMHSVTAMGPGLEGGK	2	2
			ECLLAAHMHSVTAMGPGLEGGK	1	3
			CELLAHAMHSVTAMGPGLEGGK	1	4

APPENDIX H

Table 1a: Sequencing result for peptide m/z 736.99 (PI-3617). Sequence: CELLHAAMHSVTAMGPGLEGGK

#	b	sequence	y	#
1		C		22
2	233.11	E		21
3	212.14	L		20
4	397.27	L		19
5		H	1750.7	18
6	511.32	A		17
7		A		16
8		M	147.17	15
9	1006.5	H		14
10		S		13
11	1192.5	V	1116.5	12
12		T	1017.4	11
13		A	916.39	10
14		M	845.32	9
15		G		8
16		P	657.28	7
17	1706.7	G	560.23	6
18		L	503.27	5
19		E	390.2	4
20		G	261.15	3
21		G	204.13	2
22		K	147.11	1

= detected y and b ions

APPENDIX H

De novo sequencing of purified Marker A

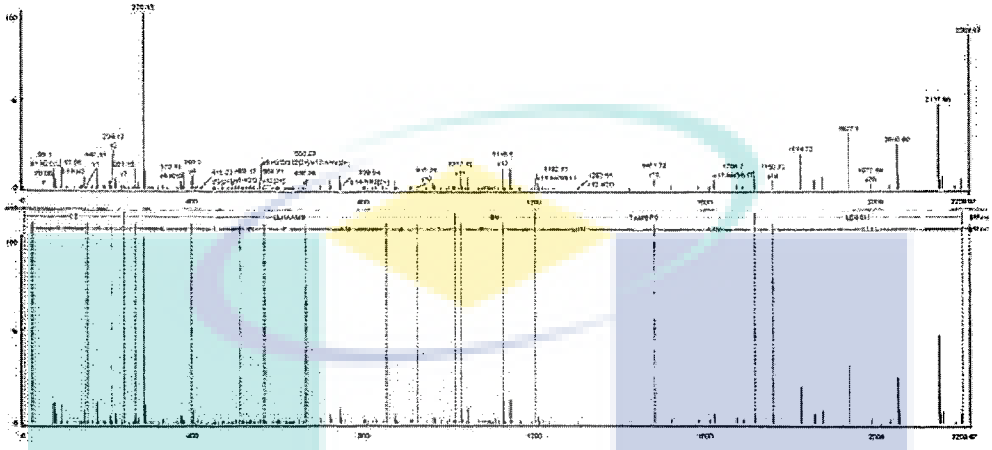
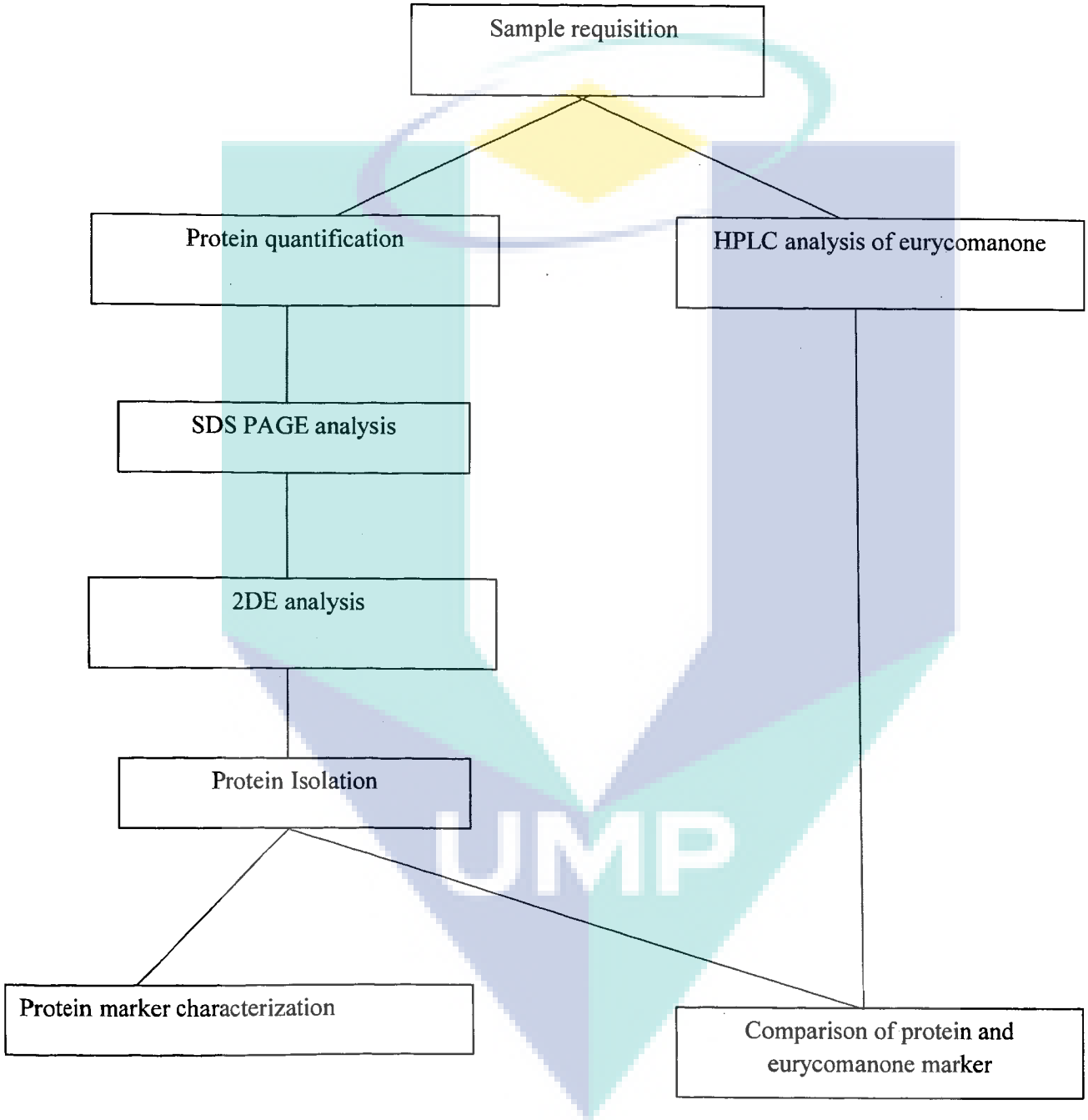


Figure 1: *De novo* spectra for peptide m/z 736.99 (PI-3617).
Reference: 150730

APPENDIX I

Method Flow Chart



APPENDIX J

Buffer preparation

Bradford Reagent Preparation

100 mg Coomasie was dissolved in 50 mL ethanol. 100 mL phosphoric acid was added into the solution. The solution volume was brought to 1L with deionized water. After overnight stirring, the solution was filtered with Whatman filter paper.

10% SDS

10 g SDS was dissolved in 9 mL water with gentle stirring. The solution volume then was brought to 100 mL.

Acrylamide Stock Solution (30% T, 2.67% C)

87.6 g acrylamide and bis (N,N methylenebisacrylmide) were dissolved in deionized water, the solution volume then was brought to 300 mL.

Electrophoresis Buffer

3 g tris, 14.4 g glycine, 1g SDS were dissolved in water and the solution was brought to 1L.

Tris HCl pH 6.8

6 g tris was dissolved in 60 mL deionized water. pH was adjusted to 6.8 with 6 N HCl. The volume then was brought to 100 mL.

APPENDIX J

Buffer preparation

Tris HCl pH 8.8

90.75 g tris was dissolved in 250 mL deionized water. pH was adjusted to 8.8 with 6 N HCl. The volume then was brought to 500 mL.

10% APS

100 mg APS was dissolved in 1 mL deionized water. This solution was prepared fresh daily.

20 mM Tris HCl pH 8.7

2.422 g tris was dissolved in 900 mL deionized water. The pH then was titrated to 8.7 using HCl. The solution volume then was brought to 1L.

0.50 M NaCl Solution in 20 mM Tris HCl, pH 8.7

2.422 g tris and 28.329 g NaCl were dissolved in 900 mL deionized water. The pH then was titrated to 8.7 using HCl. The solution volume then was brought to 1L.

APPENDIX K

Publication and conferences

Aini Norhidayah, Jaya Vejayan and Mashitah Mohd Yusoff. (2015). Review on Eurycoma longifoliaPharmacological and Phytochemical Properties. *Journal of Applied Sciences.* **15** (6): 831-844

Aini Norhidayah, Jaya Vejayan and Mashitah Mohd Yusoff. (2015). Detection and Quantification of Eurycomanone Levels in Tongkat Ali Herbal Products. *Journal of Applied Sciences.* **15** (7): 999-1005

Jaya Vejayan, Aini Norhidayah Mohamed, Mashitah Mohd Yusoff. Novel Protein Marker to Authenticate Tongkat Ali Herbal Products in the Market. CITREX UMP. Silver Awards. 8 MAC 2015

Title: Proteomics applications in authenticating *Eurycoma longifolia* contained herbal aphrodisiac products. Presented at the National Conference on Industry-Academia Initiatives in Biotechnology 2013 held from 5th-7th Dec 2013. Organised by Faculty of Industrial Sciences & Technology, University Malaysia Pahang (UMP) at Equatorial Cameron Highlands.

Title: Proteomics applications in studying Eurycoma longifolia extracts and its herbal aphrodisiac products. Presented at the 7th AOHUPO Congress and 9th International Symposium of the Protein Society of Thailand. Venue: Bangkok, Thailand held from August 6-8, 2014.

Title: Issues and Challenges in Authenticating *Eurycoma longifolia* Contained Herbal Aphrodisiac Products. Presentation: Presented at the Faculty of Industrial Sciences and Technology (FIST) Colloquium held on 3rd Jan 2014. Organised by Faculty of Industrial Sciences & Technology, University Malaysia Pahang (UMP).