A NEW SERVICE FOR CENTRAL LABORATORY, UMP: DEVELOPING EURYCOMANONE AS A CHEMICAL MARKER FOR TESTING AND STANDARDIZING TONGKAT ALI PRODUCTS

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(UJIAN BAHARU BAGI MAKMAL PUSAT, UMP: PEMBANGUNAN EURYCOMANONE SEBAGAI PENANDA KIMIA UJIAN KENALPASTI PRODUK TONGKAT ALI YANG ASLI)

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PREFACE

This grant has been awarded by University Malaysia Pahang as an internal funding with the intention to develop a new service for Central Laboratory of Universiti Malaysia Pahang. The main objective is to utilize the existing marker i.e. Eurycomanone as a service to authenticate and quantify herbal products been incorporated with Tongkat Ali. The work already been accomplished in this study whereby a standard operating procedure document been prepared describing in detail the required parameters for the analytical HPLC equipment. In addition, to reduce the cost in the purchase of the expensive Eurycomanone standard periodically, a suggested method also been given in this report on the manner possible to obtain high yield of pure Eurycomanone using a preparative HPLC. The analytical HPLC technique suggested also been well defined using some of the general HPLC method validation procedures. Consequently, the technique of detecting Eurycomanone was used in the authentication of a number of selected Tongkat Ali incorporated products in the market and found to provide useful information.

ACKNOWLEDGEMENT

The principal investigator would like to acknowledge the contributions of these individuals as well as organizations in the successful completion of this project. Firstly, to University Malaysia Pahang for providing the internal grant coded as RDU1405305. The fund provided been very helpful in initiating the work on Tongkat Ali in UMP. Tongkat Ali being an important plant in Malaysia has been traded worldwide and the need to establish the test in Central Laboratory, UMP is without a doubt a necessity. The support provided by the collaborator i.e. Prof Dr Mashitah Mohd binti Yusoff been astounding and dearly appreciated. Similarly, Central Laboratory being the beneficiary of this test also contributed towards the final product i.e. a standard operating procedure in conducting the test using HPLC. The SOP is now ready to be utilized for the purpose of testing by any small or medium sized entrepreneur manufacturing Tongkat Ali incorporated products. The labour and groundwork done by the sole postgraduate student channelled out of this project i.e. Aini Hidayah is also acknowledged here. This was followed by further refining done by a final year student, Cik Amira Alia Zulkifly.

ABSTRACT

Eurycoma longifolia is widely known for their aphrodisiac properties. Due to this, abundant of the *E. longifolia* based products are sold in the market without any specific regulation to control the quality and authentication of *E. longifolia* product. The only marker can be used to detect the presence of *E. longifolia* is eurycomanone. This study is to develop a method for the Central Laboratory of Universiti Malaysia Pahang to determine the presence and quantity of eurycomanone content in herbal products manufactured with Tongkat Ali. The analysis was carried out using RP-HPLC at 245nm with isocratic mobile phase that comprised of acetonitrile and water (86:14) with a flow rate is 0.8ml/min. The separation was done using Zorbex SB-C18 column (5µm, 250mm X 4.6mm) and injection volume is 10µl. Validation tests of the method is performed to indicate the linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ). The result indicated that the linearity was in the range of 0.01325-0.5 mg/ml (R^2=0.9997). The precision of retention time and peak area for intraday analysis of eurycomanone standard in relative standard deviation is <0.15 and <0.50 %; the precision of retention time and peak area for interday is <0.55 and <6.60%, respectively. The accuracy as the percent recovery of eurycomanone was in the range of 94.60-104.67%, while LOD and LOQ were 0.0196 and 0.0593, respectively. The concentration of eurycomanone in *E. longifolia* freeze dried crude water extract is 0.82±0.05w/w% and herbal products is around 0.06±0.00-0.30±0.00w/w%. The proposed method shows a good linearity, accurate, precise, reproducibility and short elution time. This method can be an affordable approach for the quality control of *E. longifolia* crude extract and its herbal products for any small and medium sized entrepreneurs of Tongkat Ali having no QC laboratory of their own.

ABSTRAK

Eurycoma longifolia sangat terkenal dengan ciri-ciri afrodisiak. Oleh itu, banyak produk yang berasaskan herba ini dijual di pasaran tanpa ujian dan peraturan tertentu untuk mengawal kualiti dan ketulenan produk berasaskan *E. longifolia*. Satu- satunya cara untuk mengesan kehadiran *E. longifolia* adalah mengesan kehadiran eurycomanone. Kajian ini bertujuan membangunkan kaedah untuk menentukan kualiti and kuantiti petanda eurycomanone bagi dijadikan satu ujian perkhidmatan di Makmal Berpusat, Universiti Malaysia Pahang. Petanda ini boleh diguna bagi menentukur produk Tongkat Ali yang asli. Analisis dijalankan dengan menggunakan RP-HPLC pada 245nm dengan fasa bergerak isocratic yang terdiri daripada asetonitril dan air (86:14) dengan kadar aliran 0.8ml/min. Pemisahan dilakukan dengan menggunakan ruangan Zorbex SB-C18 (5µm, 250mm X 4.6mm) dan jumlah suntikan 10µl. Ujian pengesahan kaedah yang dilakukan untuk menunjukkan kelinearan, ketepatan, kejituan, had pengesanan dan had kuantifikasi. Hasilnya menunjukkan kelinearan dalam lingkungan 0.01325-0.5 mg/ml (R^2 = 0.9997). Ketepatan masa tahanan dan kawasan puncak untuk analisis eurycomanone dalam hari yang sama dalam sisihan piawai relatif ialah <0.15 dan <0.50%; ketepatan masa tahanan dan kawasan puncak bagi hari berlainan adalah <0.50 dan <6.60%. Ketepatan peratus pemulihan eurycomanone adalah 94.60-104.67% manakala had pengesanan dan had kuantifikasi adalah 0.0196 dan 0.0593. Kepekatan eurycomanone dalam ekstrak mentah *E. longifolia* dan produk herbanya adalah 0.82 ± 0.05w/w%. Eurycomanone dikesan dalam 6 daripada 23 produk berasaskan *E. longifolia* dan kandungan eurycomanone adalah sekitar 0.06 ± 0.00-0.30 ± 0.00 w/w%. Kaedah ini menunjukkan kelinearan, kejituan, ketepatan dan kebolehulangan yang baik. Kaedah ini boleh menjadi pendekatan yang berpatutan untuk kawalan kualiti ekstrak mentah *E. longifolia* dan produk herbanya oleh mana-mana industri kecil dan sederhana yang tidak berkemampuan mempunyai makmal "QC" tersendiri.

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%RSD	Relative standard deviation
µL	Microliter
^{0}C	Degree Celcius
Cf	Found concentration
Cs	Sample concentration
ICH	International Conference on Harmonization
LOD	Limit of Detection
LOQ	Limit of Quantification
mAU	Miliabsorbance units
mg	Miligram
min	Minute
mL	Milliliter
mm	Milimeter
MS	Malaysian standard
nm	Nanometer
P	P-value statistical hyphothesis
R^2	Regression coefficient
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
S	Slope of calibration curve
UPLC	Ultra Pressure Liquid Chromatography
UV-Vis	Ultraviolet visible
w/v%	Percentage weight per volume
w/w%	Percentage weight per weight
α	Alpha

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Eurycoma longifolia is tropical herbal plants from Simaroubaceae family, which popular in South-East Asian countries such as Malaysia, Indonesia, Cambodia and Vietnam. This herbal plant also widely known as Tongkat Ali in Malaysia and Pasak Bumi in Indonesia. *E. longifolia* is an evergreen plant that can attain the maximum height, which is fifteen to eighteen meters long (Bhat and Karim, 2010).

In Malaysia, *E. longifolia* relatively common tree let of the sub-canopy layer. This plant characteristics are tall with slender shrubby tree. The leaves are pinnate, long, spirally arranged with ten to thirty slender leaflets. The fruits are green and contain a pulpy layer which will turn to dark after ripening. The flower grows in fairly large panicles with reddish and hairy petals (Bhat and Karim, 2010).

E. longifolia is widely known for its aphrodisiac properties that is used as an adaptogen for energy and vitality; and also for testosterone enhancer. Traditionally, almost all part this medicinal plant extract has been used for anti-malarial, ulcers, antimicrobial, prevent gum disease, anti-diabetic, anti-pyretic activities (Bhat and Karim, 2010) and anti-aging (Talbott et al, 2013) which have been proved scientifically. The roots of this plant which popularly known as 'Malaysian ginseng' are an invaluable components which is used as a treatment for malaria, persistent fever, aches, glandular swelling, dysentery and sexual

insufficiency (Bhat and Karim, 2010). Nowadays, the scientist has been studied that this medical herb has potential in other medical properties such as anti-cancer and anti-parasitic (Jiwajinda et al., 2002).

The healing factor that exists in *E. longifolia* especially the effect on male sexual health, has led the traders and pharmaceutical company to commercialize *E. longifolia* extract into popular products. Abundance of *E. longifolia* products available in market such as 'maajun', water extract, energy drink, tablet, pill, capsule, powder, premix coffee and as food supplement. The *E. longifolia* based product not only being produced in South-East Asia such as Malaysia and Indonesia but companies from western countries such as America and Europe which sold easily on online websites.

Eurycomanone is a one of the major quassinoids found in the *E. longifolia* root being a major bioactive compound exhibiting the antimalarial and antineoplastic activities (Wong et al., 2012). In addition, eurycomanone has shown to boost anti-proliferative effect on several cancer cell lines (promote proliferation cell death and inflammation), anti-inflammatory and enhanced testosterone level. As a major constituent that was found in *E. longifolia*, eurycomanone has been used as a reference and a marker to check the present of the *E. longifolia* extract in its derived product.

This quality control procedure is to determine the concentration of the *E. longifolia* in the various product forms. The qualitative and quantitative analysis is important to detect the presence and concentration of *E. longifolia* active ingredient in its commercialized product. The analysis of the eurycomanone in *E. longifolia* derived product can be done by using reverse phase High Performance Liquid Chromatograpy (HPLC) (Khari et al., 2014; FRIM, 2014) or Ultra Pressure Liquid Chromatography (UPLC) (Benslman, 2008; FRIM, 2014) by using eurycomanone as a marker.

High Performance Liquid Chromatograpy (HPLC) is a separation technique in analytical chemistry used to separate the component in the mixture, estimate the purity and determine the content of the substances. It is the form of chromatography column that pumps an analyte or sample mixture in a solvent (mobile phase) under a high pressure through the column with the solid adsorbent material (stationary phase). HPLC is basically an improved form of the column chromatography and each component in the mixture will interact differently with adsorbent material that caused the different in flow rate for different component, this will lead to the component separation as they flow out from the column.

1.2 PROBLEM STATEMENT

E. longifolia root extract had gained wide recognition for their aphrodisiac activities and had been consumed traditionally especially from the water extract. Nowadays, *E. longifolia* extract has been commercialized into many types of product such as 'maajun', tablet, pill, food supplement, capsule and pre-mix coffee.

Over the years, the condition that allows production and sale of the health supplement and beauty product is the product need to be free from the presence of heavy metals, steroid and microbial contamination. The toxicity and the safety evaluation have been done with the modern herbal medicine such as *E. longifolia* product in regard of consumer safety, but for the presence and concentration of the *E. longifolia* extract in the commercialized product is completely unverifiable by the company that offering the product.

As the marketing strategy and gimmicks to sell the product, the company claim that extraction ratios of the potent *E. longifolia* is 100:1 and 200:1 and this is not reproducible because the extraction ratio cannot be considered as a valid method to evaluate the herbal extract for more than 20 years. In addition, *E. longifolia* only exist in South-East Asia, but there is a foreign company that

manufactures the product based on *E. longifolia* extract. There is also a case they adulterate by incorporating sildenafil (Viagra), steroid or other plant extract that also may have the aphrodisiac properties, but as a marketing strategy they claim that their product contain *E. longifolia* extract instead.

The only verifiable method to evaluate the presence and concentration of *E. longifolia* extract by using the chemical marker which is eurycomanone, but most of US product manufacturers claim that they used marker of glycosaponins, eurypeptides and polysaccharides to evaluate E. *longifolia* extract. The only chemical marker that can be used to detect the presence and the concentration of *E. longifolia* is eurycomanone. Eurycomanone is the main active compound in *E. longifolia* that responsible for the pharmaceutical effect and also unique fingerprint that presence in the root of *E. longifolia*. The test of the eurycomanone detection in *E. longifolia* can be done using Reverse-phase High Performance Liquid Chromatography (HPLC) (Khari, 2014).

1.3 RESEARCH OBJECTIVES

1. To extract eurycomanone from trustworthy *E. longifolia* root source.
2. To establish a eurycomanone standard curve for eurycomanone detection using reverse-phase HPLC.
3. To detect the presence and concentration of eurycomanone in the *E. longifolia* crude extract and other selected commercialized products using reverse-phase High Performance Liquid Chromatography (HPLC).
4. To isolate eurycomanone compound from *E. longifolia* crude extract.
5. To develop a Standard Operating Procedure (SOP)

1.4 SCOPE OF STUDY

The extraction of water extract of *E. longifolia* root can be achieved by heating the root powder under reflux process. The eurycomanone standard is used to construct the eurycomanone standard curve by diluting the known concentration of eurycomanone and injecting into HPLC column to get the peak area of various concentrations of eurycomanone. The Limid of Detection (LOD) and Limit of Quantification (LOQ) for eurycomanone standard can be calculated from eurycomanone standard calibration curve in Microsoft Excel using the specific formula. The method validation was validated according to the ICH (International Conference on Harmonization) guidelines by evaluated the methods linearity, selectivity, precision, accuracy and the LOD and LOQ. The presence and concentration of eurycomanone in *E. longifolia* extract and selected 21 commercial products of *E. longifolia* is conducted by injecting the sample into HPLC system and peak of the sample compound were detected by UV/Vis wavelength.

The negative control in this studies is a known aphrodisiac tablet that does not contain the *E. longifolia* extract. The eurycomanone compound is isolated from the water extract of *E. longifolia* by spiking the sample solution with the eurycomanone standard and next the peak signifying eurycomanone will be collected. The compound collected was injected back to HPLC and the single peak at the specified retention time of eurycomanone indicated that the isolated eurycomanone compound.

CHAPTER 2

LITERATURE REVIEW

2.1 TAXONOMY OF *EURYCOMA LONGIFOLIA*

Eurycoma Longifolia or locally known as Tongkat Ali in Malaysia is a tropical herbal plants from Simaroubaceae family. The name Tongkat Ali which is popularly called in Malaysia means 'walking stick' which is assigned because of the presence of long twisted root in the herbal plant. This plant is popular in South-East Asian country not only in Malaysia but also popular in Indonesia, Vietnam, Myanmar, and Thailand, it also found in a certain region in Cambodia. In Indonesia, this plant is known as Pasak Bumi (Bhat and Karim, 2010).

The other popular name of this plant is include Malaysian Ginseng, Long Jack, natural Viagra, Setunjang Bumi, Pokok Jelas, Cay ba binh (tree that cures hundreds of diseases), Ian-don, Payung Ali, Bedara Pahit, Tongkat Baginda, Pokok Syurga, Jelaih, Penawar pahit (Bhat and Karim, 2010), Petala Bumi, Bedara Putih, Bedara Merah, Hempedu Pahit, Akar Jangat Semang,Tongkat Rasul and Muntah Bumi.

Generally, the complete maturation for Tongkat Ali is 25 years but for the commercial application, the root are harvested after 4 years of cultivation. It can grow 15-18 meters long and bearing a fruit after 2-3 years. As an evergreen herbal plant, it has a pinnate leaves which can reach one meter in length, spirally arranged, long and with ten to thirty leaflets. Each leaflet can reach about 5-20 cm long and 1.5-6 cm wide (Bhat and Karim, 2010).

The flower are male and hermaphrodite (having both male and female reproductive system) on different plant, consist of small petals and has a very

long axillary pellicles. The fruit color are green with 2-3 cm long and it will turn to dark after ripening.

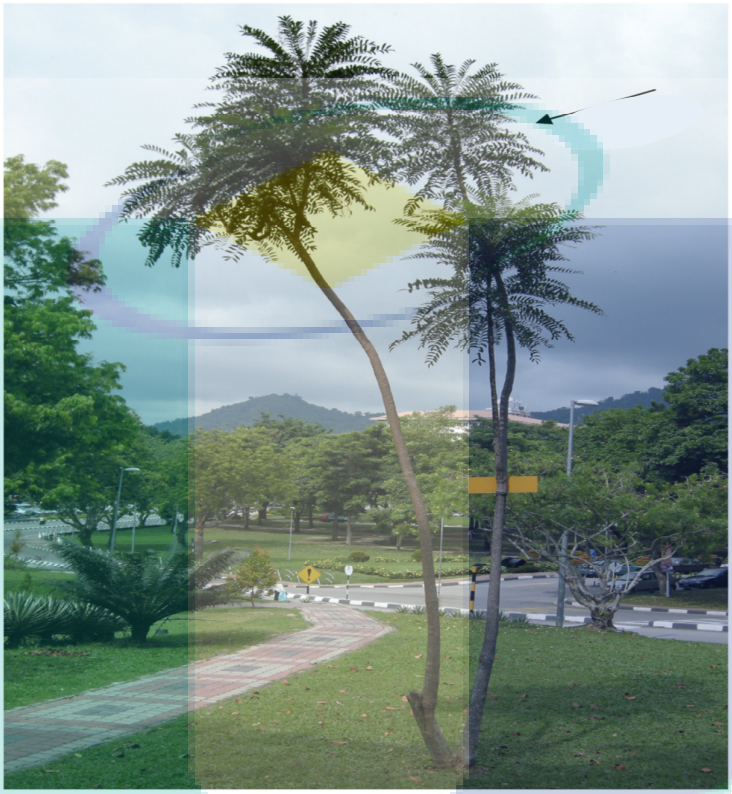


Figure 2.1: *Eurycoma longifolia* at the Universiti Sains Malaysia Campus, Malaysia.

Adapted from Bhat and Karim (2010)

Figure 2.2: *Eurycoma longifolia* root.

Adapted from http://tongkatali.sg/

2.2 CHEMICAL COMPOSITION IN *EURYCOMA LONGIFOLIA*.

Eurycoma longifolia have broad range of chemical composition that already been isolated and identified by using the scientific approach. This plant is rich in quassinoids, squalene derivatives, biphenylneolignans triterpenes, canthin-6-ones and β-carboline alkaloids (Hajjouli et al., 2014).

The chemical composition from every part of this herb plant has been isolated included root, bark, wood and leaves. Thoai et al. (1970) has investigated the chemical constituent in *E. longifolia* leaves. As a result, two steroid were isolated which is the bitter principle called eurycomalactone, 2, 6-dimethoxybenzoquinone, p-sitosterol and campesterol. Eurycomalactone is closely related to the quassinoid that encountered in *Simaroubaceae* family and it is the first compound with a keto group at C-6 and a position of r-lactone group is between 14 and 7.

Jiwajinda et al. (2001) has isolated seven quassionoid compound from *E.longifolia* leaves including a new compound which 12-epi-11-dehydroklaineanone and the other six compound is lonilactone, 6-dehydrolonilactone, 11-dehydroklaineanone, 15β-hydroxyklaineanone, 14,15 β-dihydroxyklaineanone and 15- β -Oacetyl-14-hydroxyklaineanone. This quassinoid were isolated to test as the plant growth inhibitor on the cucumber seedling and the result shows that 14,15 β-dihydroxyklaineanone has the strongest activity against the cucumber seedling growth.

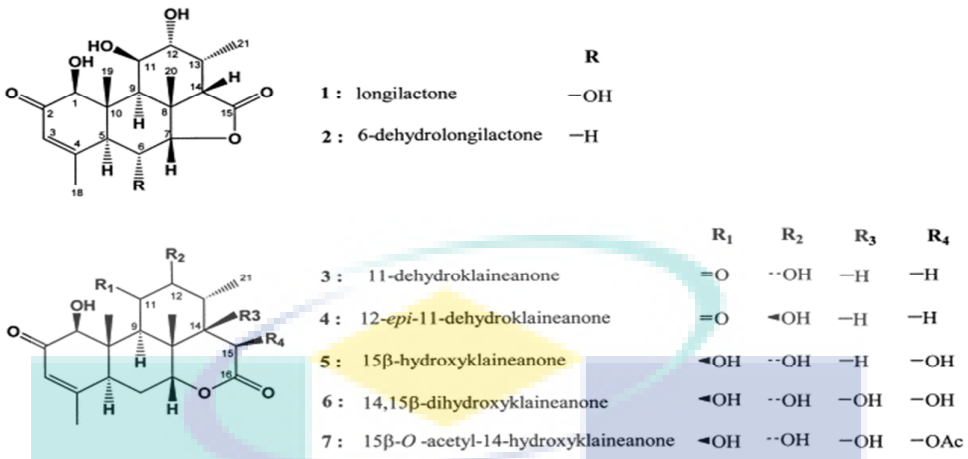


Figure 2.3: Structures of quassinoid isolated from *E. longifolia.*

Adapted from Jiwajinda et al. (2011)

Mitsunaga et al. (1994) has investigated the chemical constituents in *E. longifolia* bark and root, five new canthin-6-one alkaloids and two known β-carboline alkaloids were isolated. The canthin-6-one alkaloids is 10-hydroxy-9-methoxycanthin-6-one, 9, 10-dimethoxycanthin-6-one, 11-hydroxy-10-methoxycanthin-6-one, 9-methoxy-3-methylcanthin-5,6-dione and 5,9-dimethoxycanthin-6-one. The structure of this constituent were determine from spectroscopic data. In addition, two novel biphenyls and two novel isomeric 2,2′-dimethoxy-4-(3-hydroxy-1-propenyl)-4′-(1,2,3-trihydroxypropyl) diphenyl ethers have been isolated from *E. longifolia* wood and the structures is characterized by spectroscopic means (Morita et al., 1992).

E. longifolia root is the most valuable part in this plant and it is rich with active constituent that has been proven has a pharmaceutical properties. Kuo et al. (2004) has isolated sixty five compound in from *E. longifolia* roots. These compound were characterize by comprehensive analyses of 1D and 2D NMR and mass spectral data. For the first time, four quassinoid diterpenoids which is eurycomalide A, eurycomalide B, 13β, 21-dihydroxyeurycomanol, and 5α, 14β, 15β trihydroxyklaineanone were isolated from natural sources.

Yusuf et al. (2013) identified several compounds in *E. Longifolia* root and they are quassinoids, canthin-6-one alkaloids, tirucallane-type triterpenes, beta-carbolines, biphenylneolignans and squalene derivatives. Hajjauli et al. (2014) also state that majority of these component were found in roots of *E. longifolia*.

Furthermore, Ang et al. (2002) isolates eurycolactone D, eurycolactone E and eurycolactone F which is three types of quassinoids, and this structure were elucidated using spectroscopic method and was further confirm by X-ray crystallography. Using the same method, two other known quassinoid, laurycolactone B and eurycomalactone also were identified.

Recently, four new quassinoid was identified by Meng et al. (2014) from *E. Longifolia* root and three of them are diastereomers for each other. By performing systematic phytochemical investigation, new isolated quassinoid were discover including three known compound eurycomanone, eurycomanol and 13β,21-dihydroxyeurycomanone. The new four quassinoid is eurycomadilactone , $\Delta^{4,5}$,14-hydroxyglaucarubol, 5-iso-eurycomadilactone and 13-epi-eurycomadilactone, these compound were tested in cytotoxic activities against cancer lines. Furthermore, in recent studies, five new quassionoid has been isolated by Park et al. (2014) which is eurylactone E, eurylactone F, eurylactone G, eurycomalide D, and eurycomalide E, along with ten known quassinoids from *E. longifolia* root. The extensive spectroscopic methods were used to determine their structures inclusing 1D and 2D NMR, and MS spectra data. From this studies, 13β-methyl,21-dihydroeurycomanone (6) been reported as synthetic derivative but for the first time it was isolated from the natural product.

2.3 EURYCOMANONE (MAJOR QUASSINOID COMPOUND)

Eurycomanone is one of the major component in quassinoid. Quassinoid is isolated as s bitter principles from the Simaroubaceae family plant which is responsible for the bitter taste of *E. longifolia* extract and has attracted attention

because of wide range of their biological properties (Guo et al., 2005). It also known as a major bioactive group in *E. Longifolia* plant especially in roots.

Eurycomanone show the highest concentrated quassinoid in the root extract other quassinoid compound. Because of that, eurycomanone is the only chemical marker and unique fingerprint to confirm the correct *E. longifolia* plant. In addition, eurycomanone is the main bioactive constituent in *E. longifolia* that responsible for its pharmacological effects. Over the years, studies has been conducted to test the pharmacological potential of the plant extract of *E. longifolia*. Through the research many pharmacological potential has been found and verified scientifically, including the potential which traditionally known which verified based on scientific research and new properties that has been found to treat the disease.

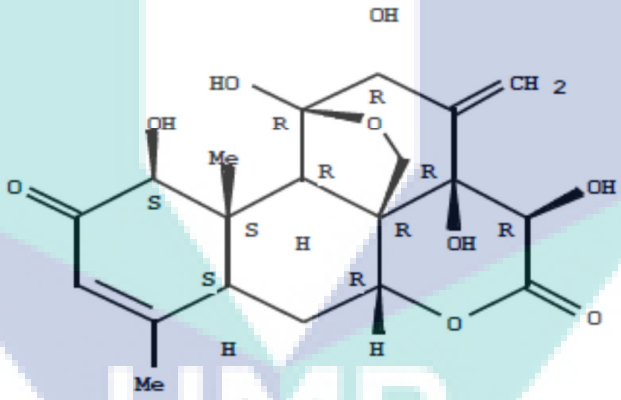


Figure 2.4: Chemical structure of eurycomanone ($C_{20}H_{24}O_9$).
Adapted from Mohamad et al. (2013)

2.4 PHARMACEUTICAL PROPERTIES

2.4.1 Aphrodisiac Properties

E. longifolia is popular for their aphrodisiac properties and the water extract was traditionally used to enhance the testosterone level in man. There are

many literature review available for aphrodisiac properties in *E. longifolia*. One of the experiment was conducted by Ann and Lee (2002) to studies the various fractions effects of *E. longifolia* on the orientation activities of the 9 months old, inbred, adult, middle-aged Sprague–Dawley rats and retired breeders for ten days towards the receptive females, the environment, themselves and mobility after treating these subjects twice daily.

The result shows that when the subject was treated with 800 mg/kg of *E. longifolia,* there is an increased towards the receptive females in orientation activities, genital grooming toward themselves and restricted movements to a particular area of the cage compared with the control during the investigation period. This experimental results give confirmations that different fractions of *E. longifolia* modified the orientation activities of the male rat.

As Ang and Sim (1997) carried out their study of the effects different fractions of *E. longifolia* on the libido of sexually experienced male rats after dosing them twice daily of for ten days. The result shows that with 400 mg/kg of methanol, water, chloroform, and butanol fractions resulting in mounting frequencies of 5.3 ± 1.2, 4.9 ± 0.7, 4.8 ± 0.7 and 5.2 ± 0.1. produced a dose-dependent increase of the treated animals, and further increased to 5.4 ± 0.8, 5.4 ± 0.8, 5.2 ± 0.6 and 5.3 ± 0.2 respectively with 800 mg/kg but there were no erections, ejaculations, intromissions or seminal emissions during the 20-min of observation period that allowed for the measurement of sexual arousal reflected by mounting frequency uninfluenced by other behavioral components. This provide that, in the absence of feedback from genital sensation, *E. longifolia* is a strong stimulator of sexual arousal in sexually vigorous male rats.

In addition, Ang et al. (2003) studies the effect of *E. longifolia* on sexual qualities in middle aged male rats. For twelve weeks, a middle aged male rats are dosing with 0.5 g/kg of various fractions of *E. longifolia* and the control group

received 3 ml/kg of normal saline daily. As a results, by decreasing their hesitation time as compared to controls with various fractions of *E. longifolia,* it enhanced the sexual qualities of the middle aged male rats. This experiment shows that *E. longifolia* enhanced the sexual activity and support the traditional use of *E. longifolia* as an aphrodisiac.

2.4.2 Cytotoxic to Cancer Cell

Cancer is a disease that caused by uncontrolled division of cell which mainly caused by the environmental factors which mutate the genes that encode for the critical cell-regulatory protein. World Cancer accounting for 8.2 million death in 2012 and leading the cause of death worldwide. Lung cancer (1.59 million deaths) is the most causes of death followed by liver cancer and stomach cancer. (World Health Organisation, 2014).

As bioactive compound in *E. longifolia*, eurycomanone have been shown to stimulate anti-proliferative effect on various cancer cell lines. The effect of eurycomanone on the expression of selected genes of the A549 lung cancer cells has been examined. In this studies, at the concentration ranging from five to twenty µg/ml, A549 lung cancer cell proliferation was inhibited by eurycomanone in a dose-dependent manner. In addition, eurycomanone treatment also suppress the expression of prohibitin, annexin and resulted in significant down-regulation of endoplasmic reticulum protein 28 (ERp28) which is precursor of endoplasmic reticulum protein 29 (ERp29). ERp29 is soluble protein that involved in secretary protein product. The conclusion in this finding is eurycomanone exhibit significant anti-clonogenic and anti-proliferative cell growth effects on A549 lung cancer cells, suppression of the lung cancer cell tumor markers and several known cancer cell growth-associated genes (Wong et al., 2012).

In the past studies, p53 role as an apoptotic trigger has been demonstrates by both *in-vitro* and *in- vivo*. Zakaria et al. (2009) investigated the cytotoxicity of eurycomanone against human hepato carcinoma cell (HepG2) and Mahfudh and Pihie (2008) in Human Cervical Carcinoma Cell (Hela cell) through the Up-Regulation of p53.

Zakaria et al. (2009) studied the cytotoxicity of eurycomanone against human hepato carcinoma cell *in-vitro* and the toxicity was evaluated using MTT assay and Hoechst 33258 nuclear staining to detect the mode of cell death. Mahfudh and Pihie (2008) investigated the Human Cervical carcinoma cell using the methylene blue staining to assay to evaluated the cytotoxicity of eurycomanone and Hoechst 33258 nuclear staining, TUNEL assay and flow cytometry with Annexin-V/propidium iodide double staining detect the mode of the cell death. Western blotting was used to study the protein expression of p53, E6, E6-AP, Bax and Bcl-2 and Immunostaining assay was used to confirm the up-regulation of p53 and Bax in cancer cells. In both studies, eurycomanone triggered the apoptotic process of p53 tumor suppressor protein that involved in the up-regulation. The up-regulation of p53 followed by increasing the pro-apoptotic Bax, it also increase the cytochrome C levels in cytosol by apoptosis induction. The apoptosis characteristics that found by eurycomanone treatment including DNA fragmentation, chromatin condensation and apoptotic bodies (Zakaria et al., 2009; Mahfudh and Pihie, 2008). The result shows that eurycomanone is cytotoxic towards HepG2 cells by inducing the apoptosis through the down-regulation of Bcl-2 and up-regulation of p53 and Bax (Zakaria et al., 2009); and cytotoxic towards HeLa cells by induce the apoptosis through the down-regulation of Bcl-2, up-regulation of p53 and Bax, independently of functional E6 and E6-AP activity (Mahfudh and Pihie, 2008). A conclusion in both findings is eurycomanone was cytotoxic on cancerous cells such as HeLa, HepG2, CaOv-3, MCF-7 and HM3KO but less toxic on normal cells such as MDBK, Vero, Chang's liver and WLR-68.

Three alkaloid from roots of *E. longifolia* [5-hydroxymethyl-9-methoxycanthin-6-one, *n*-pentyl *â*-carboline-1-propionate and 1-hydroxy-9-methoxycanthin-6-one] and 19 known *â*-carboline alkaloids were screened for cytotoxic properties *in-vitro* shows significant cytotoxicity against human breast cancer (MCF-7) and lung cancer (A-549) cell lines (Kuo et al., 2003).

Recently, two quassinoid from E. longifolia root, eurycomanone and eurycomanol were test on the K562 human leukemia cell models and Jurkat cell and compared to peripheral blood mononuclear cells from a healthy donors. Without affecting healthy cells, both quasssinoid component inhibit K562 and Jurkat proliferation and cell viability. The studies also found that only eurycomanone inhibit NF-κB signaling through the inhibition of IκBα phosphorylation and upstream mitogen activated protein kinase (MAPK) signaling which conclude that both quassinoid compound present different toxicity towards leukemia cells. Eurycomanone prevent the induction of NF-κB and MAPK by TNFα without affecting the healthy cell viability (Hajjouli et al., 2014).

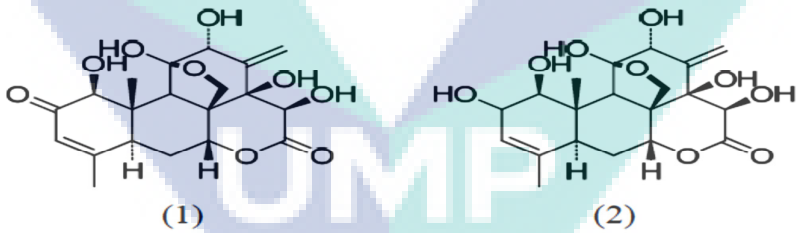


Figure 2.5: The major quassinoid, eurycomanone (**1**) and its derivative, eurycomanol (**2**) which found most in the collected root samples.

Adapted from Hajjouli et al. (2014)

In addition, there is another constituent in *E. longifolia* other than eurycomanone that has cytotoxic properties against cancer cell lines. Kardono et al. (1991) investigated the effect of chemical constituent from roots to test on

various cancer cell lines, four canthin-6-one alkaloids and one quassinoid which is eurycomanone.

These compounds was evaluated against various of cell lines consist of a number of human cancer cell types that is breast, colon, lung, melanoma, fibrosarcoma, KB, KB-V1 and murine lymphocytic leukemia (P-388). The result from this studies shows that canthin-6-ones 1-4 were active with all cell lines tested except KB-V1 cell line and eurycomanone was significantly active when tested against all human cell lines except against murine lymphocytic leukemia (P-388). These constituent found to be cytotoxic principles.

2.4.3 Antimalarial Constituent

Malaria is the one of the killer infectious disease that cause by a parasitic protozoans belongs to *Plasmodium falciparum*. This disease is spread and transmitted by the infected female Anopheles mosquito. The problem of multidrug-resistant Plasmodium falciparum has been aggravating particularly in Southeast Asia and development of new potential antimalarial drugs is urgently required. (Thiengsusuk et al., 2013). Since the effective vaccine for malarial prevention is absence and antimalarial drug resistance give the effective treatment of the disease, antimalarial drug discovery and development of potential antimalarial designates from natural product become one of the important approach.

Active constituent in *E. longifolia* has potential in antimalarial and *E. longifolia* extract has been used as traditional remedy to treat malaria disease. Recently, several studies has been reported to provide a scientific base for traditionally *E. longifolia* extracts against malaria or the intermittent fever (Bhat and Karim, 2010).

Studies has proven that eurycomanone and pasakbumi B exhibited marginal antimalarial activity against both the W2 and D6 P. falciparum clones *in-vitro* (Kuo et al., 2003). In addition, same studies by Kuo et al. (2003) which

screened these three isolated compound [*n*-pentyl *â*-carboline-1-propionate, 5-hydroxymethyl-9-methoxycanthin-6-one, and 1-hydroxy-9-methoxycanthin-6-one] and 19 known *â*-carboline alkaloids that isolated from root has display antimalarial activity against the resistant *Plasmodium falciparum in vitro* (Kuo et al., 2003).

In addition, Kardono et al. (1991) and Chan et al. (2004) also studies the antimalarial properties in *E. longifolia* extract. Kardono et al. (1991) characterized five chemical constituent which isolated from root of *E. longifolia* collected in Kalimantan, Indonesia. Two chemical component shows significant antimalarial activities against cultured P. falciparum strains which is 7-methoxy-P-carboline-1-propionic acid and eurycomanone.

Chan et al. (2004) tested four quassinoid and an alkaloid to determine their effect on the lactate dehydrogenase activity of chloroquine-resistant Gombak A isolate and chloroquine-sensitive D10 strain of *Plasmodium falciparum* parasitesin vitro. The activity of antiplasmodium were compared with their known cytotoxicity against KB cells *in vitro*. Four quassinoid which is eurycomanone, 13α(21)-epoxyeurycomanone, 13,21-dihydroeurycomanone, eurycomalactone and an alkaloid, 9-methoxycanthin-6-one shows high antiplasmodial activity against Gombak A isolate but not active against D10 strain when compared with chloroquine. Among of these compound tested, eurycomanone and 13, 21-dihydroeurycomanone shows higher selectivity indices obtained to antiplasmodial activity.

Recently, Yusof et al. (2013) synthesizes new eurycomanone derivatives by esterification process using isolated eurycomanone and acylating agent. The eurycomanone and its derivatives were tested for antimalarial activity *in-vitro*. This test were conducted by candle jar method, eurycomanone and its derivatives were incubated with *Plasmodium falciparum* strain 3D7 in CO_2 incubator at 37^{o}C

for 72 hours. After making thin blood smear and staining it with Giemsa 10%, the antimalarial activity were assessed microscopically. The growth inhibitory of fifty percent of *P. falciparum* were determined by linear regression analysis. The result shows that eurycomanone is more active than chloroquine and its derivatives. Monoacyl eurycomanone more potent than diacyl and triacyl eurycomanone.

2.4.4 Anti-Diabetic Activity

Traditionally, it has been belief that daily consumption of *E. longifolia* roots and leaves can reduce blood sugar levels but the scientific information available about this characteristics relevant to human model is very scarce (Bhat and Karim, 2010).

Husen et al. (2004) studied the effect of aqueous extract of *E. longifolia* to determine the hyperglycaemic effect in rat model system. To determine their blood glucose lowering effect, this is conducted in normoglycaemic and Streptozotocin-induced hyperglycaemic rats. The positive result was obtained in hyperglyacaemic rats when 150 mg/kg body weight of the aqueous extract and freeze-dried material were used.

2.4.5 Other Pharmaceutical Properties

Besides cytotoxic properties, antimalarial, antidiabetic and the most popular is aphrodisiac, *E. longifolia* also possess many other pharmaceutical properties such as antimicrobial (Farouk and Benafri, 2007; Tzar et al., 2011; Khanam et al., 2015), antifungal (Tzar et al., 2011), anti-inflammatory (Varghese et al., 2013; Hajjouli et al., 2014), anti-oxidant (Varghese et al., 2013) and anti-pyretic (Bhat and Karim, 2010).

2.6 TOXICITY STUDIES OF *E. LONGIFOLIA*

The major drawback using the traditional medicines is lack of adequate scientific research that support the level of safety, toxicity and quality of *E. longifolia* product in the market. There is no available report of the safety and long term side effect use of product prepared from *E. longifolia* and the criteria of commercial standard might not meet the standard criteria regarding the concentration of bioactive constituent (Bhat and Karim, 2010).

Nowadays, many of the product formulation are non-traditional and was mixed with other herbs that may increase the chance the E. *longifolia* based-product being contaminated with heavy metals and change the cytotoxicity status due to combination of plant metabolites (Razak and Aidoo, 2011).

One studies has been done to test the toxicity of the *E. longifolia* commercial product. Razak and Aidoo (2011) studied the effect of toxicity mixing herbs in polyherbal product containing mixture of *E. longifolia* with other herbs, authenticated *E. longifolia* and a product contain only *E. longifolia* using methanol-chloroform extraction. The extract of three herbal product were tested with Hep2 human cell and viability of Hep2 cell was analyzed using the effect of the extract on Hep2 3-4,5-dimethylthiazole-2,5-diphenyl-tetrazolium bromide (MTT) assay.

The mutagenicity of the extract were tested on *Salmonella* TA98 and *Salmonella* TA100 using Ames test. The content of heavy metals such as Cu, Mn, As, Pb and Cadmium of the product were analyzed by flame atomic absorption spectrometry. The result from three products in three tests conducted shows that the IC_{50} which is the concentration of an inhibitor where the response were reduced by half. Respectively, IC_{50} of the crude extract of pure E. longifolia was 22.23 µg ml^{-1} and the product that containing only E. longifolia was 50.00 µg ml^{-1}. The IC_{50} of two polyherbal product is 15.20 µg $ml^{-1}$and 18.89 µg ml^{-1}, respectively.

All product are mutagenic except product containing the mixture of *E. longifolia* and *Cistanche deserticola* but compared to the toxicity of remedies containing solely *E. longifolia,* there is a risk of increased toxicity and mutagenicity of the extract of *E. longifolia*-based remedies. Results also shows that heavy metal are detected in a very low concentration in all products. The conclusion in this studies is the toxicity of newly formulated polyherbal products cannot be deduced from the information of the toxicity of each individual component of the polyherbal products. Other experimental studies on the toxicity of the E. longifolia extract on male rats has been done by Shuid et al. (2011) and Choudhary et al. (2012).

2.7 HERBAL DRUG STANDARDIZATION AND QUALITY CONTROL

There is increasing acceptability and awareness of the use of herbal drugs in today's medical practices, it is a known fact that more than 80% of the world population depending on herbal medicines although these applications are unorthodox. The rise in the use of the herbal product also lead to various forms of abuse and adulteration of the product leading to manufacturers and consumer disappointment and also fatal consequences (Kunle et al., 2012). To distinguish useful plants with the beneficial effects from those that were toxic, the primitive people learned by trial and error, and also which processing methods and combination had to be used to gain consistent and optimal results. Herbal plants are most widely distributed all over the world, but most abundantly in tropical countries and it has been estimated that 25% of modern medicines are directly or indirectly derived from the herbal plants.

Kunle et al. (2012) reviewed on their studies stress on the importance of herbal medicines standardization from all aspects such as from producers and consumers perspective and processes and procedures. In addition, Ong (2004)

also stated that the standardization of the extraction method, chemical standardization and herbal preparations are also important.

Higher incidence of adverse drug reaction because of overuse of synthetic drugs with higher impurities in advanced communities has motivated mankind to go back to nature for safer remedies. Therefore, quality control standards of various medicinal plants used in indigenous system of medicine are becoming more relevant today in view of commercialization of formulations based on medicinal plants. In general, the methods for quality control of herbal medicines involve sensory inspection, which is macroscopic and microscopic examinations; and the analytical inspection using instrumental techniques such as HPLC, thin layer chromatography (TLC), LC-MS, GC-MS, near infrared (NIR) and spectrophotometer.

One of the most popular methods for analysis of herbal medicines is HPLC because it is not limited by stability of the sample compound, easy to learn and use. In addition, reversed phase column (RP) column may be the most popular column used in the analytical separation of herbal medicines because it provide the good separation for some extracts of herbal medicines and its versatility for the analysis of chemical compound (Liang et al., 2004). However, since lots of chemical compounds in herbal medicines are non-chromophoric compound, the single wavelength UV detector seems to be unable to fulfill the task (Liang et al., 2004). It is a limitation that been addressed using other detectors such as RI, MS, Fluorescence and etc.

The recent approaches in herbal drug standardization is DNA fingerprinting, chromatographic fingerprinting (HPLC, TLC), gross morphology, microscopy and chemical analysis to evaluating that there is no adulterants present in the plant material (Yadav and Dixit, 2008)

2.8 HIGHLIGHTED CURRENT ISSUES ABOUT *E. LONGIFOLIA* HERBAL PRODUCTS (TV NEWS AND NEWSPAPER)

There is no scientific literature that look into the misuse and abuse of *E. longifolia* especially for the aphrodisiac properties. There has been tremendous increase in demand for this plant extract and nearly 200 *E. longifolia* commercial herbal products available in the health-food market mainly for its aphrodisiac properties. *E. longifolia* products are available in market either in the form of raw crude powder from root, capsule, pre-mix coffee, maajun etc.

Mass media often broadcasted the news related to the 'abuse' of *E. longifolia* in commercialized products. Due to overinflux of *E. longifolia* based-products in the market there has been doubts whether the products are genuine or fake one as well as if the concentration of the bioactive component of *E. longifolia* extract in the products are sufficient to provide the pharmaceutical effects.

Figure 2.6: NTV7 Report – *E. longifolia* Products in Market, Genuine or Artificial. (8 September 2013)

Adapted from https://www.youtube.com/watch?v=i_m8a2rvwKY (watched on 19 March 2015)

On 8 September 2013, Edisi 7 (NTV7 News) has broadcasted about the *E. longifolia* based-products in the market which has been alleged to contained high concentration of *E. longifolia* extract however they have been found to be fakes one. The news reported that by knowing the facts that *E. longifolia* can increase the testosterone level in man, many parties took advantage by claiming their product contain *E. longifolia* even it is actually artificial (NTV7 report, 2013).

According to Forest Research Institute of Malaysia (FRIM) studies, seven from ten of the product and drinks based on *E. longifolia* extract from popular brands contain only 0.25%- 1.6% of *E. longifolia* extract. In addition, it only contain 7.8 to 49.3 ng of *E. longifolia* active constituent and this amount is far from the level that can be beneficial to health (NTV7 report, 2013).


NST - 13/9/2011

life & times health

Beware of wonder cures

Herbal supplements are not always beneficial to health.
KASMIAH MUSTAPHA looks into the safety aspects of their use

Consumers have to ensure the herbal products are scientifically proven to be effective

Figure 2.7: Newspaper report. Adapted from Mustafa, K. 2011. Beware of wonder cures. *New Straits Times*. 13 September: 2.

News Straits Times newspaper also reported on the safety of the herbal supplements in the market. The article reported that the customer believes that

product with *E. longifolia* can increase the male libido. Cashing in on this belief, some company manufacture the product which is supposedly contain these herb for example *E. longifolia* but most of the cases, the product contain drug or other stimulant. In 2011, The Health Ministry raided a company that was producing coffee mixed with the controlled drug Tadalafil and the manufacture claim that the coffee contain herbal and organic extract which can boost the man's sexual function (Mustafa, 2011).

In this article, Dr. Shaiful said that patient need to check the scientific background of the product to ensure the active ingredient is not combine with other potentially harmful ingredient especially in *E. longifolia* product. He also added that some product maybe contain probably only 0.001 percent of the herbal extract and the rest are the drugs which can cause the health problems (Mustafa, 2011).

CHAPTER 3

METHODOLOGY

DESCRIPTION OF METHODOLOGY

3.1 Sample Collection

E. longifolia root was obtained from reliable 'orang asli' in the state of Pahang. The dried raw plant material was chopped into chip form or were ground into fine powder using a waring blender. Twenty three marketed *E. longifolia* products were selected and purchased from various drugstores, pharmacies, herbal stores in Pahang, Malaysia as well as online shopping i.e Amazon.com.

3.2 Chemicals Preparation

Eurycomanone standard reference was purchased from ChromaDex, USA. Solvent of HPLC grade were purchased from Sigma-Aldrich. Deionized water for HPLC analysis was prepared using PURELAB Flex Ultra-Pure Water System.

3.3 Extraction Procedure

The water extract was prepared by mixing 50 grams of *E. longifolia* powder or chips formed with 500mL deionized water under reflux for five hours, followed by filtration with Whatman No 1 filter paper and freeze dried.

3.4 Instrumentation of HPLC Analysis

Reverse phase HPLC equipped with the basic set-up, HPLC Agilent 1260 Infinity with photodiode array fitted with a Zorbex SB-C18 column (5 µm, 250 mm X 4.6 mm). The mobile phase consist of isocratic mixture of water and acetonitrile (86:14) with a flow rate is 0.8ml/min for 10 minutes. Injection volume is 10µl of each sample. The detection wavelength is 245nm using UV-Vis wavelength. Data acquisition was performed by ChemStation Data Software. The analysis was performed in HPLC instrument room at FIST laboratory.

3.5 Eurycomanone Standard

Five milligram of eurycomanone standard were dissolved in 1 mL deionized water, filtered through the 0.45 µL syringe filter and diluted with the same solvent serially to produced 0.5, 0.25, 0.125, 0.0625, 0.01325 mg/ml of solution respectively. Eurycomanone content were determined from standard calibration curve that generated from eurycomanone standard compounds.

3.6 Sample Preparation and Analysis

5 mg of each sample were dissolved in 1 mL deionized water and filtered through a 0.45µm nylon membrane syringe filter. 10µl of each sample was injected into HPLC system and the analysis of each sample were done in triplicate.

3.7 Method validation

The method was validated according to the ICH Guidance for Industry (Q2B Validation of Analytical Procedures: Methodology). The validation characteristics such as linearity, selectivity, precision, accuracy and the limits of detection (LOD) and limits of quantification (LOQ) (Guideline, 2005).

3.7.1 Linearity

The linearity was determined as the regression coefficient (R^2) of the calibration curve plotting the peak area against concentration from eurycomanone standard.

3.7.2 Limits of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ for eurycomanone standard were calculated from eurycomanone standard calibration curve in Microsoft Excel sheet using the formula;

LOD = 3.3 X (σ/S)

LOQ = 10 X (σ/S)

Where σ = the standard deviation of the response

S = the slope of the calibration curve

3.7.3 Selectivity

Selectivity was determined by spiking the Tongkat Ali water extract with eurycomanone standard and comparing the retention time of eurycomanone standard with the time of eurycomanone obtained from the sample. The t-test statistical analysis was applied and carried out using Excel and the significant different were considered at $P < 0.05$ (α).

3.7.4 Precision

The precision of the retention time and peak area were determined as the relative standard deviation (%RSD). The eurycomanone standard was analyzed at 5 concentration which is 0.5, 0.25, 0.125, 0.0625, 0.01325 mg/ml. The repeatability (same operating condition over short period of time) and

intermediate precisions (variation within laboratories such as days) were calculated.

3.7.5 Accuracy

Accuracy was determined by the percentage recovery of eurycomanone standard in 5 concentration which is 0.5, 0.25, 0.125, 0.0625, 0.01325 mg/ml. Percent recovery was calculated by using this formula:

Recovery % = (found concentration/ known concentration)*100

3.8 Determination of eurycomanone Concentration in *E. longifolia* Extract and Their Herbal Product

Eurycomanone level in each product were calculated in w/w%. The peak area of each sample injected was recorded. The contents of eurycomanone were determine from the regression equation generated from the eurycomanone standard compound.

Concentration (w/w%) = (Cf/Cs)*100

Cf = found concentration (from eurycomanone standard calibration equation)

Cs = sample concentration.

According to the Malaysian Standard (MS 2409: 2011), the eurycomanone level in *E. longifolia* dried extract should be around 0.8-1.5% (Standard, 2011).

3.9 Isolation of Eurycomanone Compound from *E. longifolia* Extract

Eurycomanone compound was isolated from *E. longifolia* extract by using prep-HPLC. The known reference material which is eurycomanone standard will be added into *E. longifolia* extract. An increase in height or area signifies presence of interested peak. The eurycomanone was isolated by collecting the sample at this peak. The presence of eurycomanone compound in was confirmed

by injecting back the sample to analytical HPLC. The sample was boiled with rotary evaporator at 80^{O}C to remove the trace of acetonitrile and freeze dried.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 INTRODUCTION

Eurycomanone was selected as a standard marker because it has been reported as one of the major quassionoid compound in *E. longifolia* (Wong et al., 2012). The RP-HPLC is used because it is one of the most common approach for the quantification and qualification of compound and water can be used as a mobile phase. In addition, reversed phase column (RP) column may be the most popular column used in the analytical separation of herbal medicines which provide better separation for some extracts of herbal medicines and its versatility for the analysis of chemical compounds in herbal medicines (Liang et al., 2004).

The acetonitrile (14%) is used as mobile phase because has a strong elution strength, which resulted in short elution time that is less than 10 minutes and the retention time of eurycomanone is 7.99±0.00 min. Furthermore, acetonitrile has lowest absorbance especially for shorter wavelengths and it result in lower noise in UV detection since short wavelength (245nm) is used for this method for eurycomanone detection. Isocratic elution was used as a separation technique whereas the composition of mobile phase remains constant, isocratic method is the simplest separation technique and should be the first choice when developing a separation (Kupiec, 2004). Isocratic has an edge over gradient as the HPLC equipment will be cheaper due to use of single pump rather than a single pump with a gradient mixer or dual pumps. This facilitates cheap solution for small industry usages.

The process of proving whether the method is acceptable and reproducible for its purpose for pharmaceutical intentions is called validation. Method

validation is one of the critical factors affecting the quality control of herbal products which is a major public concern in both resources-poor and developing countries where certain unscrupulous manufacturers selling adulterated herbal medicine (Kunle et al, 2012). In this regard, despite of the existence of certain guidelines in some individual countries and those outlined by WHO, there is still no adequate control by the government agencies (Kunle et al, 2012). Linearity, accuracy, specificity, precision, range, LOD and LOQ must be included in validation investigation depending whether the analytical method used is qualitative or quantitative (De Smet et al, 1997).

The appropriate quantitative analytical method with following chromatograms is desirable, the main objective of the methods is to provide a validated method which can be used to quantify the compounds most correlated with quantitative marker or pharmacological markers (Wani, 2007).

4.2 METHOD VALIDATION

4.2.1 Linearity

Linearity of the eurycomanone standard curve is 0.9997 and was presented in regression coefficient (R^2). The value of $R^2 > 0.999$ indicates a strong positive correlation coefficient of the procedure. The linearity of an analytical procedure is its ability (within the range) to obtain the results which are directly proportional to the concentration of analyte in the sample (Guideline, 2005). The increase in peak areas of eurycomanone detected ideally should be accurate and in proportion to concentration if R^2 close to the value of one.

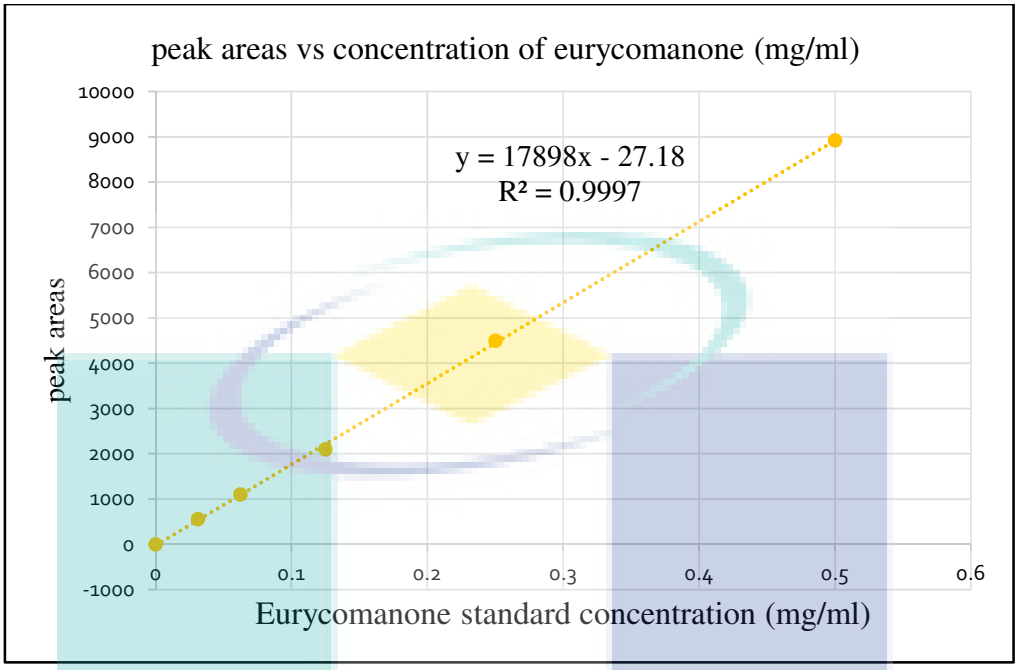


Figure 4.1: Calibration curve of eurycomanone standard.

4.2.2 Limits of Detection and Limits of Quantification

According to Guideline (2005), LOD is the lowest amount of analyte in a sample which can be detected but not quantitated as an exact value, meanwhile LOQ is the lowest amount of analyte in the sample can be quantitated. The LOD and LOQ were calculated from the standard deviation of y-intercepts of regression line and the slope of calibration curve. The LOD is 0.0196 and the LOQ is 0.0593. The amount of analyte in *E. longifolia* crude water extract and products contain the detectable amount since the concentration of crude water extract and products higher that LOQ.

4.2.3 Selectivity

The selectivity was determined by spiking the *E. longifolia* extract with eurycomanone standard and comparing the retention time of eurycomanone

standard with eurycomanone obtained in *E. longifolia* extract. The spiking method show there is no new addition of peaks or any shift of retention time. The peak which is identified as eurycomanone compound in the *E. longifolia* extract are increase in peak area and peak height. The retention time of eurycomanone standard compound was 7.99±0.00 min, and the retention time of *E. longifolia* extract is 8.00±0.01 min. The statistical analysis by t-test was calculated using Excel and indicated that there is no significant difference in retention time between eurycomanone standard compound and eurycomanone compound from *E. longifolia* extract (P = 0.3986). Altogether, both results confirm the eurycomanone identity and prove the selectivity of the proposed method towards eurycomanone in *E. longifolia* extract.

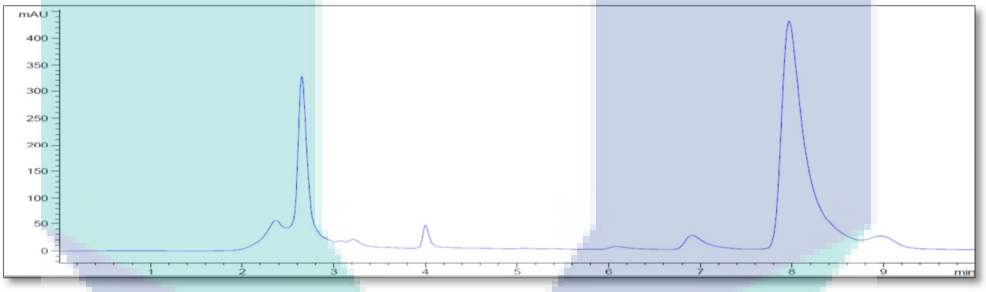


Figure 4.2: Chromatograms of *E. longifolia* crude spike with eurycomanone standard

4.2.4 Precision

The precision of an analytical procedure is the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed condition (Guideline, 2005). There are two levels of precision were conducted in this method which is repeatability and intermediate precision. Repeatability (intraday) is the precision under the same operating procedure over short period of time (every 15 minutes) and the intermediate precision (interday) is the variations within laboratories which is different days (in 3 days). The precision is presented in the percentage relative

standard deviation (coefficient of variation) and according to the Guideline (2005), confidence interval should be reported for the each type of precision investigated. Confidence interval is formed from the sample which depicted the value of a certain population parameter with a specified probability within the interval.

Confidence level is the end points of the interval are the confidence limits and the specified probability. Confidence limits represent the lower and upper boundaries which define the range of a confidence interval. The precision of the retention time and peak area (n=5) of eurycomanone standard was calculated. The %RSD of retention time was less than 0.30%, which indicated the good reproducibility. The %RSD of retention time for intermediate precision is 0.43%, which is under acceptance criteria RSD≤ 2%. For the peak area, %RSD of the peak area was calculated in the range of 0.03125-0.5mg/ml and the average was less than 0.25% which also shows a good reproducibility and for the intermediate precision of the peak area, the average of %RSD was 5.48%. At 95% confidence level, the mean confidence interval of retention time for repeatability and intermediate precision of the eurycomanone compound is 7.99- 8.02 min and 7.88-8.07 min, respectively.

Precision	Concentration (mg/ml)	RT (min)		Peak area (mAU)	
		Mean±SD	%RSD	Mean±SD	%RSD
Repeatability (Intraday)	0.03125	7.99±0.00	0.06	558.23±0.56	0.10
	0.0625	8.00±0.01	0.11	1098.94±0.13	0.01
	0.125	8.01±0.00	0.11	2089.29±10.16	0.49
	0.25	8.00±0.00	0.01	4486.70±9.15	0.20
	0.5	8.01±0.01	0.13	8898.30±23.06	0.26
Intermediate precision (interday)	0.03125	7.98±0.023	0.30	589.59±28.22	4.79
	0.0625	7.99±0.035	0.43	1163.05±57.46	4.94
	0.125	7.98±0.04	0.53	2189.32±107.50	4.91
	0.25	7.98±0.04	0.47	4862.16±318.13	6.54
	0.5	7.97±0.03	0.43	9605.89±597.05	6.21

Table 4.1: Precision analysis of retention time and peak area of eurycomanone standard.

4.2.5 Accuracy

Accuracy can be assessed by analyzing a sample of known concentration which is the standard compound (Shabir, 2004 and Guideline, 2005). Accuracy was determined as percent recovery of the eurycomanone standard compound at 5 concentrations. The found concentrations of eurycomanone standard were obtained from the regression equation of eurycomanone standard curve. The mean percentage recovery at 0.03125, 0.0625, 0.125, 0.25 and 0.5 mg/ml was 104.67±0.10, 100.67±0.01, 94.60±0.45, 100.88 ±0.20 and 99.74±0.26 %, respectively. This approach shows a high accuracy for the eurycomanone standard compound.

Concentration (mg/ml)	Peak areas	Found conc.	Recovery (%)	Mean % recovery	Standard deviation (%recovery)	%RSD (recovery)
0.03125	557.71	0.03	104.57	104.67	0.10	0.10
	558.15	0.03	104.65			
	558.83	0.03	104.77			
0.0625	1099.08	0.06	100.68	100.67	0.01	0.01
	1098.84	0.06	100.66			
	1098.88	0.06	100.67			
0.125	2100.47	0.12	95.10	94.60	0.45	0.48
	2086.80	0.12	94.49			
	2080.60	0.12	94.21			
0.25	4495.77	0.25	101.08	100.88	0.20	0.20
	4486.86	0.25	100.88			
	4477.48	0.25	100.67			
0.5	8922.15	0.50	100.00	99.74	0.26	0.26
	8896.63	0.49	99.72			
	8876.12	0.49	99.49			

Table 4.2: Recovery analysis of eurycomanone standard.

4.3 Determination of Eurycomanone Concentration in *E. longifolia* Extract and Their Herbal Products.

E. longifolia water extract and 23 of *E. longifolia* commercial products were analyzed by using the RP-HPLC with the proposed method. The chromatograms of the *E. longifolia* water extract shows the presence of the eurycomanone at the retention of 8.00±0.01 min and the retention time of eurycomanone standard is 7.99±0.00 min. The t-test statistical analysis using Excel indicated that there is no significant difference of retention time of eurycomanone standard and eurycomanone compound in *E. longifolia* products

($p = 0.8632$) in three days of injection duration of *E. longifolia* product. The peak area of eurycomanone was recorded by integration using the ChemStation software offline. The content of eurycomanone in *E. longifolia* water extract and its commercial products were calculated by applying the linear regression equation of the eurycomane standard compound, the results of eurycomanone concentration in *E. longifolia* water extract and its herbal products were presented as mean w/w% ±SD.

The figure below shows the chromatograms of eurycomanone standard, *E. longifolia* water extract, *E. longifolia* product with eurycomanone compound, *E. longifolia* product without eurycomanone compound and negative control. The negative control in this studies is herbal product without *E. longifolia* extract which possessed the aphrodisiac properties. HPLC chromatograms of eurycomanone standard, *E. longifolia* water extract, *E. longifolia* product with eurycomanone compound shows there was a peak at the retention time 7.88-8.07 min which indicates the presence of eurycomanone compound. In addition, HPLC chromatograms of *E. longifolia* product without eurycomanone compound and negative control do not shows the presence of the peak at retention time between 7.88-8.07 min.

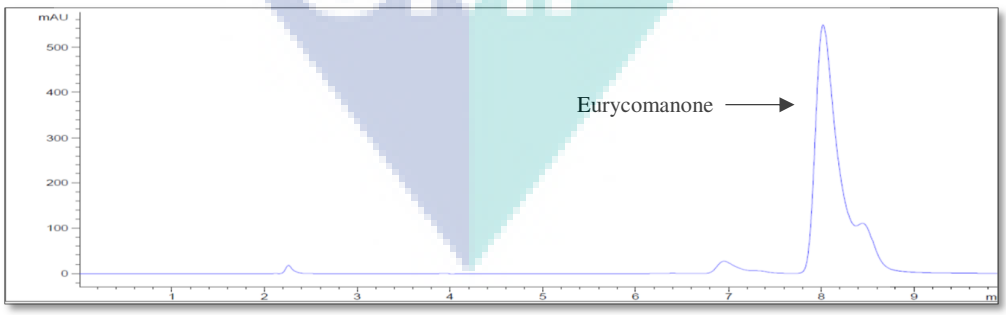


Figure 4.3: Chromatograms of eurycomanone standard.

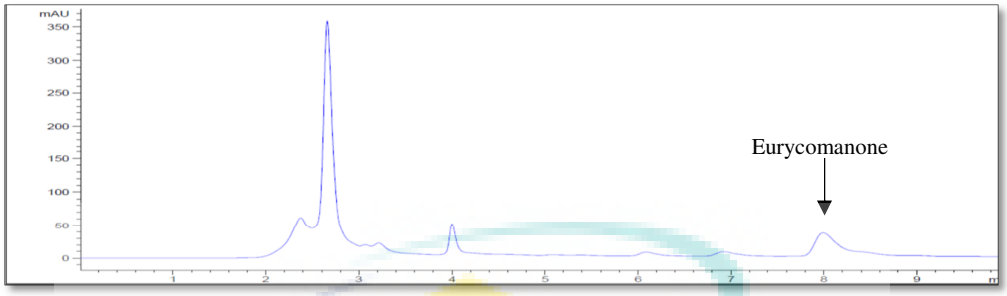


Figure 4.4: Chromatograms of *E.longifolia* crude water extract

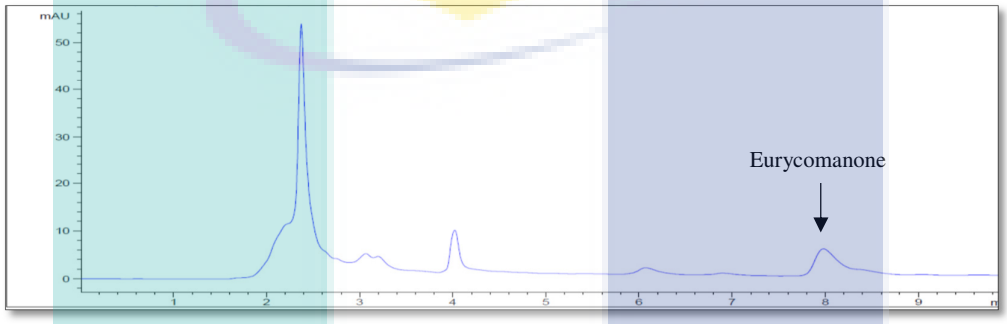


Figure 4.5: Chromatograms of *E. longifolia* product with eurycomanone (C1)

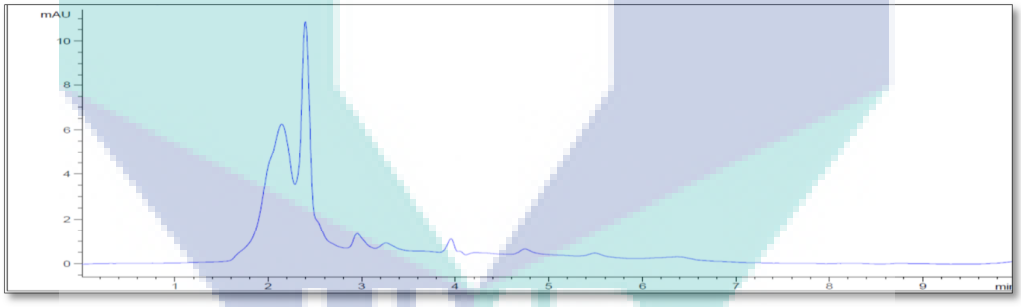


Figure 4.6: Chromatograms of negative control (Gali-Gali- Herbal product without *E. longifolia*)

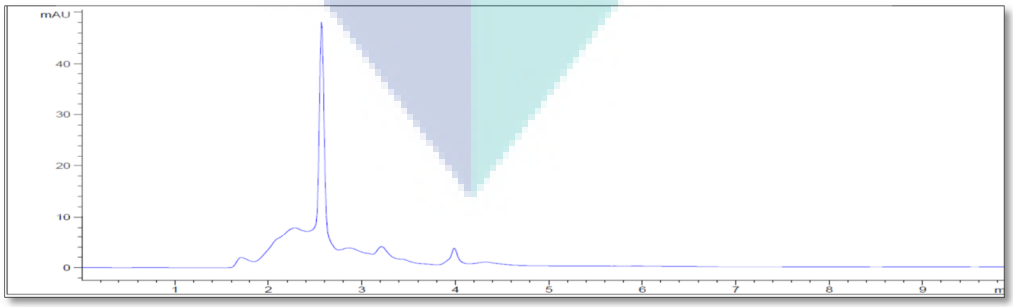


Figure 4.7: Chromatograms of *E. longifolia* product without eurycomanone (C33).

Table 4.4 below shows the content of eurycomanone in *E. longifolia* water extract and its commercial products. From the Table 4.4, the *E. longifolia* crude water extract contain the highest concentration of eurycomanone from all of the samples tested. The concentration of eurycomanone in *E. longifolia* water extract is 0.82±0.05 (w/w%), the percentage of eurycomanone in *E. longifolia* water extract falls within the allowed content of eurycomanone compound that was set by Malaysian Standard (MS 2409:2011). According to MS 2409:2011 Phytopharmaceutical Aspect of Freeze Dried Water Extract from *E. longifolia,* the standardization of freeze dried water extract in *E. longifolia* for four marker compounds is 0.80-1.50% for eurycomanone, total polysaccharides >30%, total protein >20 % and total glycosaponin >40% (Standard, 2011). There are several reasons that affects the concentration of eurycomanone or the bioactive compounds in the *E. longifolia* root water extract which is the extraction parameter such as temperature, extraction time and particle size (powder or chips form) (Mohamad et al., 2013). The location, the soil composition and weather (rainforest herb 2015) also can have the minor effects in plant chemistry (Vlahakis, C. & Hazebroek, J., 2000) when tested with HPLC. Mohamad et al. (2013) investigated the effects of extraction parameter of *E. longifolia*, among these parameters, the temperature was the most influential parameter compared to the other parameters. Temperature has a strong influence to the mass transfer and decomposition of the bioactive compounds. 100^{O}C was found to be the optimum boiling point for eurycomanone. In addition, the powder was preferred and the optimum extraction of the *E. longifolia* to be carried out is 45 minutes since most of the bioactive compound are sensitive to elevated temperature, by keeping them for a longer period of time would lead to the thermal decomposition of biological compound (Ma et al., 2009). Since the temperature is the most influential parameter, the deionized water was used as a solvent under reflux to obtain the good extraction of eurycomanone.

The location, weather and soil composition also affected the composition of eurycomanone. Khari et al. (2014) investigated the concentration of eurycomanone from the root of *E. longifolia* crude water extract obtained from five different locations (Pahang, Perak, Selangor, Kedah and Kelantan) in Malaysia, the results show the different of the eurycomanone content in *E. longifolia* root water extracts using RP-HPLC. The highest eurycomanone concentration was obtained from root from Selangor which is 3.28±0.01 w/w% and the lowest was obtained from root from Kelantan which is 0.89±0.01 w/w% which proved that the location and soil composition also affect the chemical compound of the plant.

23 commercialized *E. longifolia* products has been purchased and injected to RP-HPLC system to detect and quantified the level of eurycomanone content. From 23 products, 15 are herbal supplement capsules or tablet and the rest are premix coffees from various brands. The *E. longifolia* products were categorized into three categories which is a Malaysian Registered product (MRP), Malaysian Unregistered Product (MUP) and IP (International Product). The table 4 below shows the summary of *E. longifolia* tablet and capsule products with eurycomanone and without eurycomanone.

Product status	Eurycomanone (+)	Eurycomanone (-)
MRP[1]	2	4
MUP[2]	2	1
IP[3]	2	4

Table 4.3: Summary of the eurycomanone presence in *E. longifolia* based products (Capsule and tablet forms only).

[1] MRP (Malaysian Registered Product)
[2] MUP (Malaysian Unregistered Product)
[3] IP (International Product)

From 23 products only 6 contain the eurycomanone marker. On the other hand, 2 out of 6 Malaysian registered products contains eurycomanone which is C1 and C7, both have the highest content of eurycomanone among the other products which is 0.14±0.00 w/w% and 0.30±0.00 w/w%. C7 has the highest content of eurycomanone in *E. longifolia* products among all the products that has been analyzed for eurycomanone using HPLC. The eurycomanone has been detected from 2 out of 3 of Malaysian unregistered products which is C16 and C19. The eurycomanone content in both Malaysian unregistered products is 0.07±0.00 w/w% and 0.08±0.00 w/w%. Even these products were not registered with the Ministry of Health and Drug Control of Malaysia, because of the simple processing steps of the *E. longifolia* root, the cottage industry ("Perniagaan Kampung" in Malay) can even get involved easily in the manufacturing of *E. longifolia* herbal product and they do not aware or not to be bothered about the requirement to register their products (Norhidayah et al, 2015). In addition, 2 out of 6 International products contain eurycomanone marker which is C14 and C40. Eurycomanone level in C14 is 0.06±0.00 w/w% and C40 is 0.08±0.00 w/w%. The percentage of eurycomanone concentration in 6 products was lower than the lower limit set by Malaysian Standards, which is 0.8-1.5 w/w%. In this studies, none of the premix coffee detected with the presence of eurycomanone.

There is abundance of *E. longifolia* products in the market and the number are increasing day by day due to the aphrodisiac properties in *E. longifolia*. In addition there is a scientific studies that reported on the therapeutics properties of *E. longifolia*. Since the current mandatory regulatory parameter for herbal product only tested for the heavy metal, pesticide, microbial contamination and adulterant only (Sahoo et al., 2010), there is regulatory requirements to confirm the presence of the bioactive component of the herbal product. At present, the commercial preparations of *E. longifolia* has not been standardized and some commercial products may not even contain the any bioactive component of *the E. longifolia* (Rahman et al., 2004). In addition, health claims, medical claims and

nutrient content claims are the most common types of claims with which the herbal medicine may legally be sold in the market (Sahoo et al., 2010). There is a need for the regulatory requirement of the detection and quantification of eurycomanone marker in *E. longifolia* products to verify the products contained bioactive component before selling it to the market even though the existing herbal products on the market will be affected by this parameter. To achieve the desired benefit from herbal product, a person must take the required dose over a certain period of time because some biologically active compounds from herbs can be toxic (Kunle et al., 2012). In addition, until now, no conclusion that eurycomanone has linked to aphrodisiac properties, therefore only leading the eurycomanone as a chemical standard to confirm the presence *E. longifolia*. Because of these reasons, the definitive qualitative and quantitative of eurycomanone level in *E. longifolia* products need to be added as a mandatory regulatory parameter since Malaysian Standard regulation has set the amount of eurycomanone level in the *E. longifolia* water extract to be 0.8-1.5%. If the concentration of eurycomanone or bioactive component is lower than the range that has been set, there is maybe no desired effect on health and if it is overdose, it may cause a negative side effect.

The case for the *E. longifolia* herbal product in the market now days because there is no parameter to prove that the *E. longifolia* products in the market are made from *E. longifolia* extract or just a potato starch with the food colouring. The result in this studies shows that eurycomanone content level in the *E. longifolia* products are far from the lower set regulations by Malaysian Standard which may concern whether the product supplement can give the desired effect to the body. In Malaysia, *E. longifolia* now is become scarce and has been declared as a protected plant in most of the cultivated area including Malaysia (Bhat and Karim, 2010), harvesting activity of this plant is highly restricted and was guarded by the forest rangers and there is restriction importing *E. longifolia* overseas. This leads to the question on how overseas companies

obtained these *E. longifolia* roots. Consequently, *E. longifolia* is already hard to find and very expensive, yet there is abundance of *E. longifolia* product are sell at the market, for example, premix coffee is sold at the low price and the customer can get it with only RM2 to RM5 per sachet. This makes us wonder whether this *E. longifolia* based product really contain *E. longifolia* extract or there is the addition of adulterant such as Generic Viagra (sildenafil) which is sold at a low price by manufacturers. For the herbal product, the availability and quality of the raw materials are frequently problematic, which the active principles are diverse and may be unknown, and the quality of different batches of preparation may be difficult to control. In addition, in most countries, without proper scientific evaluation, without mandatory safety and toxicological studies, the herbal products are launched into the market (Kunle et al., 2012).

The adequate analytical method has to be applied for example Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GS) to prove the constant composition of herbal preparations (Bauer, 1998).

Table 4.4: Concentration of eurycomanone in *E. longifolia* water extract and its commercialized product.

CODE [4]	SAMPLE	DOSAGE FORM	HERBS CONTENT (as stated at the packaging label)	STATUS	EURYCOMANONE LEVEL MEAN (W/W%)±SD
+VE CONTROL	*Eurycoma longifolia* water extract	Powder	*Eurycoma longifolia*		0.82±0.05
-VE CONTROL	Gali-Gali	Capsule	*Cornus officinalis, Turnera diffusa, Ptychopetalum olacoides* and *Piper retrofractie*		nd [5]
C1	Tongkat Ali Plus	Capsule	*Eurycoma longifolia* and *Epimedium Sagittatum*	MRP	0.14±0.00
C5	Pure Tongkat Ali 200:1 Extract	Capsule	*Eurycoma longifolia*	IP	nd
C7	Nu Prep Lelaki	Capsule	*Eurycoma longifolia*	MRP	0.30±0.00
C8	Tongkat Ali Maca Plus	Capsule	*Eurycoma longifolia*, Maca, Ginger, Rice Bran and Black seed	MRP	nd
C9	Hurix's Tongkat Ali Plus	Capsule	*Eurycoma longifolia, Cistanche deserticola, Actinolitum, Cynomorium songaricum*,	MRP	nd

[4] Code C: Capsule and tablets dosage form B: Premix coffee
[5] nd : not detected

			Cuscuta chinensis, Cistanche Deserticola and *Epimedium brevicornum.*		
C1	Unleash Your Beast	Tablet	Tongkat Ali, Ginseng (Siberian/Korean) and Maca	IP	nd
C1	Pure D *Eurycoma longifolia*	Capsule	*Eurycoma longifolia*	IP	0.06±0.00
C1	100% Tongkat Ali and Ubi Jaga	Capsule	Tongkat Ali and Ubi Jaga	MUP	0.07±0.00
C1	Ranjang Besi, Tongkat Ali	Capsule	Tongkat Ali, Ranjang besi, Ranjang tembaga, Ubi jaga and Gajah beranak	MUP	nd
C1	Tongkat Ali	Capsule	Tongkat Ali	MUP	0.08±0.00
C2	Tongkat Ali Hitam Plus	Capsule	*Eurycoma longifolia, Foeniculum vulgare, Nigella sativa, Globia pendula, Curcuma domestica, Languas galangal* and *Nigris fructus.*	MRP	nd
C2	Kapsul Tongkat Ali Hitam	Capsule	*Eurycoma longifolia, Zingiber officinale, Eugenia aromatica, Piper longum, Acorus calamus, Nigella sativa,*	MRP	nd

			Coriamdrum sativum, Trachyspermum ammi, Pimpinella anisum and *Crodon caudatum*		
C3	Longjack	Capsule	Tongkat Ali, sarsaparilla, pumpkin powder, Muira puama, oat straw, nettle, Catauba, licorice, Tribulus terrestris, orchic oyster, cayenne paper and astragalus	IP	nd
C3	Make My Pepper Big	Capsule	Tongkat Ali, Maca, Ginseng (Siberian/Korean)	IP	nd
C4	Herb Natural Tongkat Ali	Capsule	*Eurycoma longifolia*	IP	0.08±0.00
B1	Raja Herba Coffee	Premix coffee	Tongkat Ali, Misai Kucing, Mengkudu Liar, Raja Satong, Halia Padi, Ubi Jaga, rempah Gunung, Akar Seruntum, Rancang Tembaga, Hulubalang Jantan.	MRP	nd
B2	Natural Herbs Coffee Kopi Panggung Al-Ambiak	Premix Coffee	Tongkat Ali, Guarana, Maca	MUP	nd
B3	Kopi Jantan Tradisional	Premix Coffee	Tongkat Ali, Guarana, Maca		nd
B4	Power Root	Premix	Tongkat Ali extract	MRP	nd

	Alicafe	coffee	and instant Ginseng extract		
B5	Super Jantan	Premix Coffee	Tongkat Ali, Ganoderma, Ubi Jaga	MRP	nd
B6	A-Taqwa Skyline Natural Herbs Coffee.	Premix coffee	Tongkat Ali, Maca, Guarana	MUP	nd
B7	CNI Tongkat Ali with Ginseng Coffee.	Premix coffee	Tongkat Ali powder, Ginseng extract powder	MRP	nd
B8	G.L.E Tongkat Ali with Ginseng Coffee super.	Premix coffee	Tongkat Ali powder, Ginseng extract powder	MUP	nd

CHAPTER 5

CONCLUSION AND RECOMMENDATION

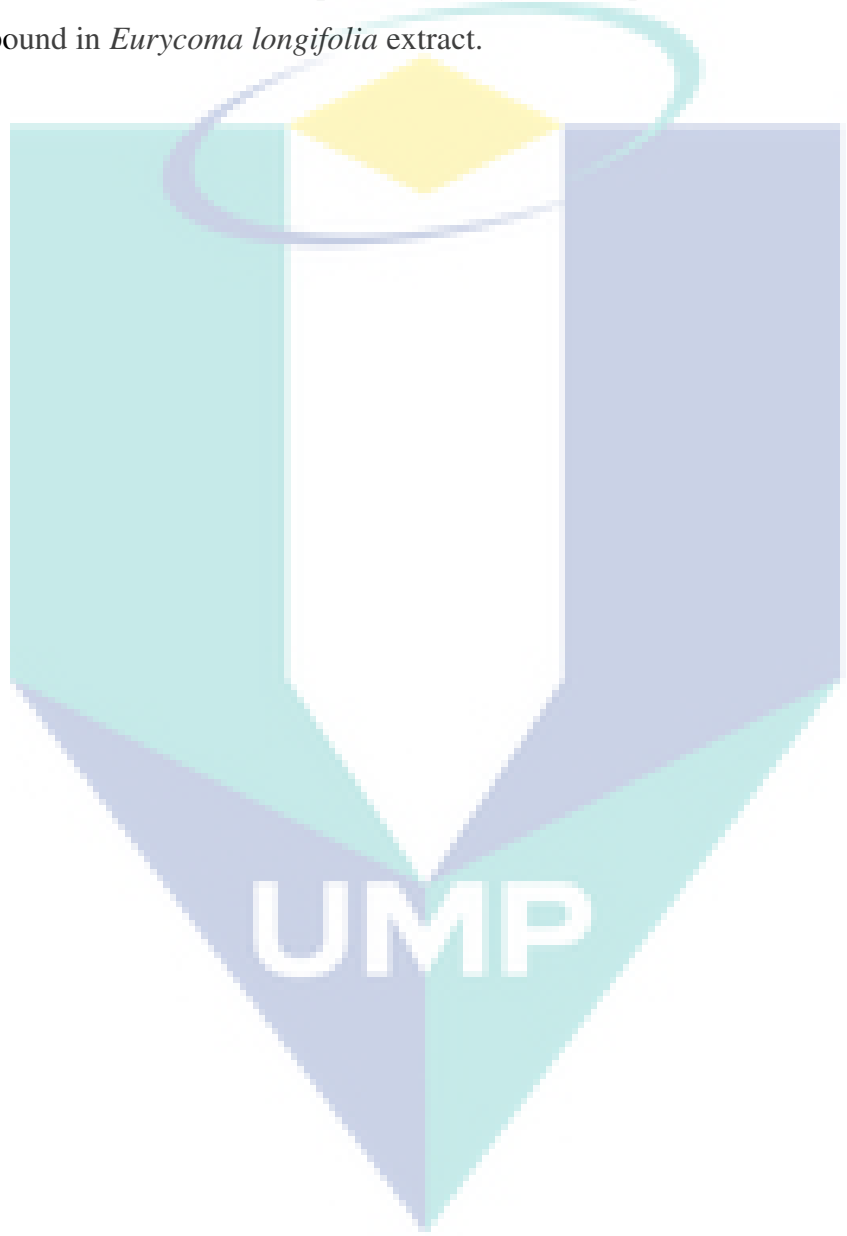
5.1 CONCLUSION

In conclusion, eurycomanone is a chemical marker that can be used to detect the presence of *E. longifolia* extract, it was selected as reference because it is one of the major quassinoid and concentration of this compound contributes to some biological activities of the plant, but as up to now no compound been conclusively linked to the aphrodisiac activity. This studies suggests that the eurycomanone level should be regulated as a mandatory regulatory parameter in *E. longifolia* herbal product and water extract since less than 30% of the randomly selected products shows the presence of eurycomanone compound and the eurycomanone content in the product is lower than regulation set by Malaysian standard which is 0.8-1.5%. This developed method is rapid and high in reproducibility, selectivity, precision and accuracy. The elution time for the marker compound is less than 10 minutes which leads to lower analysis cost for quality control of *E. longifolia* product in pharmaceutical industry or as an important contribution to the research in *E. longifolia*. The amount of eurycomanone in *E. longifolia* extract and in certain commercialized product need to be standardized and validated to the level that can be beneficial to health and to prevent unwanted health issues which is between 0.8-1.5%.

5.2 RECOMMENDATION

E. longifolia based product is a common Malaysian supplement which have many pharmaceutical properties that can give an advantages to human health. This research proved that there is a need in method validation to check the quality of *E. longifolia* based product. This method validation is suitable and can

be selected as one of the mandatory parameters in herbal drug production industry to promote the quality of the herbal drugs into a higher level. In addition, there is a need to studies the optimal parameter for eurycomanone such as extraction time and temperature, to get the optimum level of bioactive compound in *Eurycoma longifolia* extract.



REFERENCES

Ang, H. H. and Lee, K. L. 2002. Effect of Eurycoma longifolia Jack on orientation activities in middle-aged male rats. *Fundamental & clinical pharmacology*. **16**(6): 479-483.

Ang, H. H., and Lee, K. L. 2002. Effect of *Eurycoma longifolia* jack on orientation activities in middle-aged male rats. *Fundamental & clinical pharmacology*. **16**(6): 479-483.

Ang, H. H., Hitotsuyanagi, Y., Fukaya, H., and Takeya, K. 2002. Quassinoids from Eurycoma longifolia. *Phytochemistry*. **59**(8): 833-837.

Ang, H. H., Ngai, T. H., and Tan, T. H. 2003. Effects of Eurycoma longifolia jack on sexual qualities in middle aged male rats. *Phytomedicine*. **10**(6): 590-593.

Bauer, R. 1998. Quality criteria and standardization of phytopharmaceuticals: Can acceptable drug standards be achieved?. *Drug Information Journal*. **32**(1): 101-110.

Benslman, S. A. A. 2008. *A Pharmacokinetic Interaction between Propranlol and Eurycome Longifolia (Tongkat Ali).* Doctoral dissertation, Universiti Sains Malaysia, Malaysia.

Bhat, R., and Karim, A. A. 2010. Tongkat Ali (Eurycoma longifolia Jack): a review on its ethnobotany and pharmacological importance. *Fitoterapia*. **81**(7): 669-679.

Chan, K. L., Choo, C. Y., Abdullah, N. R. and Ismail, Z. 2004. Antiplasmodial studies of Eurycoma longifolia Jack using the lactate dehydrogenase assay of Plasmodium falciparum. *Journal of ethnopharmacology*. **92**(2): 223-227.

Choudhary, Y. K., Bommu, P. R. A. V. E. E. N., Ming, Y. K., and Zulkawi, N. B. 2012. Acute, sub-acute, and subchronic 90-days toxicity of *Eurycoma longifolia* aqueous extract (physta) in wistar rats. *International Journal of Pharmacy and Pharmaceutical Sciences*. **4**: 232-238.

De Smet, P. A. G. M., Keller, K., Hansel, R. and Chandler, R.F. 1997. *Adverse Effects of Herbal Drugs.* Springer-Verlag, Heidelberg. **129**(3): 137-145.

Farouk, A. E., and Benafri, A. 2007. Antibacterial activity of *Eurycoma longifolia* jack. A Malaysian medicinal plant. *Saudi medical journal*. **28**(9): 1422-1424.

Forest Research Institute Malaysia. 2014. *Technical Service.*

Guideline, I. H. T. 2005. Validation of analytical procedures: text and methodology. *Q2 (R1)*, *1*.

Guo, Z., Vangapandu, S., Sindelar, R. W., Walker, L. A., and Sindelar, R. D. 2005. Biologically active quassinoids and their chemistry: potential leads for drug design. *Current Medicinal Chemistry*, **12**(2), 173-190.

Hajjouli, S., Chateauvieux, S., Teiten, M. H., Orlikova, B., Schumacher, M., Dicato, M., Choo, C. H., and Diederich, M. 2014. Eurycomanone and eurycomanol from *Eurycoma longifolia* jack as regulators of signaling pathways involved in proliferation, cell death and inflammation. *Molecules*. **19**(9): 14649-14666.

Husen, R., Pihie, A. H. L., and Nallappan, M. 2004. Screening for Antihyperglycaemic Activity in Several Local Herbs of Malaysia. *Journal of Ethnopharmacology*. **95**(2): 205-208.

Jiwajinda, S., Santisopasri, V., Murakami, A., Hirai, N., and Ohigashi, H. 2001. Quassinoids from Eurycoma longifolia as plant growth inhibitors. *Phytochemistry*. **58**(6): 959-962.

Jiwajinda, S., Santisopasri, V., Murakami, A., Kawanaka, M., Kawanaka, H., Gasquet, M., Eilas, R., Balansard, G., and Ohigashi, H. 2002. In vitro anti-tumor promoting and anti-parasitic activities of the quassinoids from *Eurycoma longifolia*, a medicinal plant in Southeast Asia. *Journal of ethnopharmacology*. **82**(1): 55-58.

Kardono, L. B., Angerhofer, C. K., Tsauri, S., Padmawinata, K., Pezzuto, J. M. and Kinghorn, A. D. 1991. Cytotoxic and antimalarial constituents of the

roots of Eurycoma longifolia. *Journal of Natural Products*. **54**(5): 1360-1367.

Khanam, Z., Wen, C. S., and Bhat, I. U. H. 2015. Phytochemical screening and antimicrobial activity of root and stem extracts of wild Eurycoma longifolia Jack (Tongkat Ali). *Journal of King Saud University-Science*. **27**(1): 23-30.

Khari, N., Aisha, A. F., and Ismail, Z. 2014. Reverse phase high performance liquid chromatography for the quantification of eurycomanone in *Eurycoma longifolia* Jack (Simaroubaceae) extracts and their commercial products. *Tropical Journal of Pharmaceutical Research*. **13**(5): 801-807.

Kunle, O. F., Egharevba, H. O. and Ahmadu, P. O. 2012. Standardization of herbal medicines-A review. *International Journal of Biodiversity and Conservation*. **4**(3): 101-112.

Kunle, O. F., Egharevba, H. O. and Ahmadu, P. O. 2012. Standardization of herbal medicines-A review. *International Journal of Biodiversity and Conservation*. **4**(3): 101-112.

Kuo, P. C., Damu, A. G., Lee, K. H., and Wu, T. S. 2004. Cytotoxic and antimalarial constituents from the roots of *Eurycoma longifolia*. *Bioorganic & medicinal chemistry*. **12**(3): 537-544.

Kuo, P. C., Shi, L. S., Damu, A. G., Su, C. R., Huang, C. H., Ke, C. H., Wu, J. B., Lin, A. J., Bastow, K. F., Lee, K. S., and Wu, T. S. 2003. Cytotoxic and antimalarial β-carboline alkaloids from the roots of Eurycoma longifolia. *Journal of natural products*. **66**(10): 1324-1327.

Kupiec, T. 2004. Quality-control analytical methods: High-performance liquid chromatography. *International journal of pharmaceutical compounding*. **8**: 223-227.

Liang, Y. Z., Xie, P. and Chan, K. 2004. Quality control of herbal medicines. *Journal of chromatography B*. **812**(1): 53-70.

Ma, W., Lu, Y., Dai, X., Liu, R., Hu, R. and Pan, Y. 2009. Determination of anti-tumor constitute mollugin from traditional Chinese medicine Rubia

cordifolia: Comparative study of classical and microwave extraction techniques. Separation Science and Technology. **44**(4): 995-1006.

Mahfudh, N., & Pihie, A. H. L. 2008. Eurycomanone induces apoptosis through the up-regulation of p53 in human cervical carcinoma cells. *Journal of cancer molecules*. **4**(4): 109-115.

Meng, D., Li, X., Han, L., Zhang, L., An, W. and Li, X. (2014). Four new quassinoids from the roots of Eurycoma longifolia Jack. *Fitoterapia*. **92**:105-110.

Mitsunaga, K., Koike, K., Tanaka, T., Ohkawa, Y., Kobayashi, Y., Sawaguchi, T., and Ohmoto, T. 1994. Canthin-6-one alkaloids from *Eurycoma longifolia*. *Phytochemistry*. **35**(3): 799-802.

Mohamad, M., Ali, M. W., Ripin, A. and Ahmad, A. 2013. Effect of Extraction Process Parameters on the Yield of Bioactive Compounds from the roots of Eurycoma longifolia. *Jurnal Teknologi*. **60**(1): 51-57.

Mohamad, M., Ali, M. W., Ripin, A., and Ahmad, A. 2013. Effect of extraction process parameters on the yield of bioactive compounds from the roots of *Eurycoma longifolia*. *Jurnal Teknologi*. **60**(1): 51-57.

Morita, H., Kishi, E., Takeya, K. and Itokawa, H. 1992. Biphenylneolignans from wood of Eurycoma longifolia. *Phytochemistry*, **31**(11): 3993-3995.

Mustafa, K. 2011. Beware of wonder cures. *New Straits Times*. 13 September: 2.

Produk Tongkat Ali Di Pasaran, Tulen Atau Tiruan. 2013. Video. Malaysia: NTV7 Media Prima Berhad. Adapted from https://www.youtube.com/watch?v=i_m8a2rvwKY (watched on 19 March 2015)

Norhidayah, A., Vejayan, J., Yusoff, M. M. and Gambang, K. 2015. Detection and Quantification of Eurycomanone Levels in Tongkat Ali Herbal Products. *Journal of Applied Sciences*. **15**(7): 999-1005.

Ong, E. S. 2004. Extraction methods and chemical standardization of botanicals and herbal preparations. *Journal of Chromatography B*. **812**(1): 23-33.

Park, S., Nhiem, N. X., Van Kiem, P., Van Minh, C., Tai, B. H., Kim, N., Yoo, H.H., Song, J. H., Ko, H. J. and Kim, S. H. 2014. Five new quassinoids and cytotoxic constituents from the roots of Eurycoma longifolia. *Bioorganic & medicinal chemistry letters*. **24**(16):3835-3840.

Rahman, A. A., Yap, M. S., Shakaff, A. M., Ahmad, M. N., Dahari, Z., Ismail, Z. and Hitam, M. S. 2004. A microcontroller-based taste sensing system for the verification of *Eurycoma longifolia*. *Sensors and Actuators B: Chemical*. **101**(1): 191-198.

Razak, F. F. A., and Aidoo, K. E. 2011. Toxicity studies of *Eurycoma longifolia* (jack)-based remedial products. *Asian Journal of Pharmaceutical and Clinical Research*. **4**(3): 23-27.

Sahoo, N., Manchikanti, P. and Dey, S. 2010. Herbal drugs: standards and regulation. *Fitoterapia*. **81**(6): 462-471.

Shabir, G. A. 2004. A practical approach to validation of HPLC methods under current good manufacturing practices. *Journal of validation technology*. **10**: 210-218.

Shuid, A. N., Siang, L. K., Chin, T. G., Muhammad, N., Mohamed, N., and Soelaiman, I. N. 2011. Acute and subacute toxicity studies of Eurycoma longifolia in male rats. *International Journal of Pharmacology*. **7**(5): 611-646.

Standard, M. 2011. Phytopharmaceutical Aspects of Freeze Dried Water Extract from Tongkat Ali Roots-Specification. *Jabatan Standard Malaysia, USA., Pages*, *12*.

Tambi, M. I., and Imran, M. K. 2009. Efficacy of the 'Malaysian Ginseng', US patented, standardised water soluble extract of Eurycoma longifolia Jack in managing idiopathic male infertility. In *Papers contributed to the 9th International Congress of Andrology. Medimond International Proceedings, Bologna, Italy*: 165-168.

Thiengsusuk, A., Chaijaroenkul, W., and Na-Bangchang, K. 2013. Antimalarial activities of medicinal plants and herbal formulations used in thai traditional medicine. *Parasitology research*. **112**(4): 1475-1481.

Thoai, L. V., and Suong, N. N. 1970. Constituents of *Eurycoma longifoh* Jack. *The Journal* of *Organic Chemistry*. **35**:*1104-1109.*

Tzar, M., Hamidah, Y., Hartini, M., Marianayati, M., and Nazrun, A. 2011. The antibacterial or antifungal effects of Eurycoma longifolia root extract. *The Internet Journal of Herbal and Plant Medicine*. **1**(1).

Varghese, C. P., Ambrose, C., Jin, S. C., Lim, Y. J., and Keisaban, T. 2013. Antioxidant and anti-inflammatory activity of Eurycoma longifolia jack, a traditional medicinal plant in Malaysia. *Int J Pharm Sci Nanotechnol*. **5**: 1875-1878.

Vlahakis, C. and Hazebroek, J. 2000. Phytosterol accumulation in canola, sunflower, and soybean oils: effects of genetics, planting location, and temperature. *Journal of the American Oil Chemists' Society*. **77**(1): 49-53.

Vlahakis, C. and Hazebroek, J. 2000. Phytosterol accumulation in canola, sunflower, and soybean oils: effects of genetics, planting location, and temperature. *Journal of the American Oil Chemists' Society*. **77**(1): 49-53.

Wani, M. S. 2007. Herbal medicine and its standardization. *Pharmaceutical Reviews*. **5**(6).

Wong, P. F., Cheong, W. F., Shu, M. H., Teh, C. H., Chan, K. L. and Abu Bakar, S. 2012. Eurycomanone suppresses expression of lung cancer cell tumor markers, prohibitin, annexin 1 and endoplasmic reticulum protein 28. *Phytomedicine*. **19**(2): 138-144.

World Health Organization. 2014. World cancer report, 2014. *WHO Report. Geneva: WHO*.

Yadav, N. P. and Dixit, V. K. 2008. Recent approaches in herbal drug standardization. *Int J Integr Biol*. **2**(3): 195-203.

Yusuf, H., Mustofa, Wijayanti, M. A., and Susidarti, R. A. 2013. Semisynthesis and biological evaluation of eurycomanone derivatives as new antimalarial. *International Research Journal of Pharmaceutical and Applied Sciences*. **3**(6): 4-7.

Zakaria, Y. 2009. *Suppression Effect of Eurycomanone on the Growth of Human Hepatoma Cells (Hepg2) by Inducing P53-Mediated Apoptotic Pathway.* Doctoral dissertation. Universiti Putra Malaysia, Serdang, Selangor, Malaysia.

APPENDIX A

Table 4.5: List of selected *E.longifolia* products.

C1	C5	C7	C8
			
C9	C13	C14	C16
			
C17	C19	C20	C22
			
C33	C37	C40	B1
			
B2	B3	B4	B5
			
B6	B7	B8	
			

APPENDIX B

HPLC CHROMATOGRAMS OF *E. LONGIFOLIA* CRUDE WATER EXTRACT AND ITS HERBAL PRODUCTS

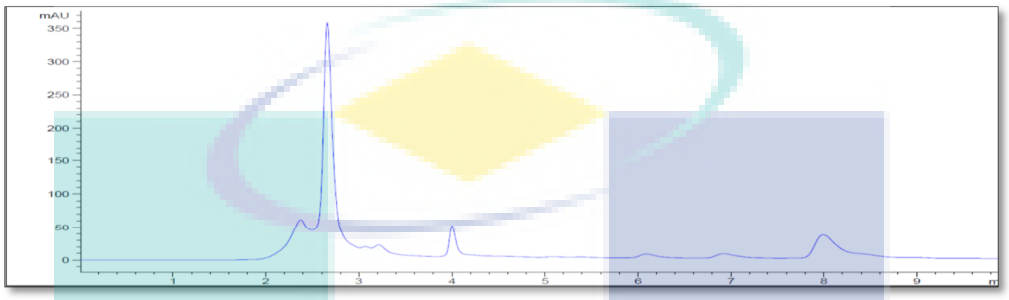


Figure 4.6.1: HPLC chromatograms of *E. longifolia* crude water extract

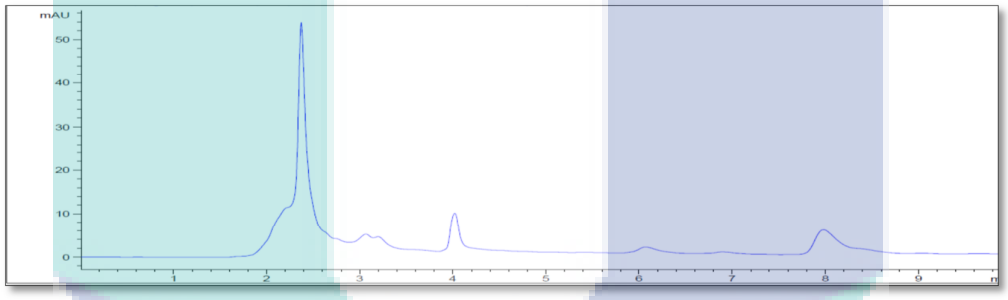


Figure 4.6.2: HPLC chromatograms of *E. longifolia* herbal product (**C1**)

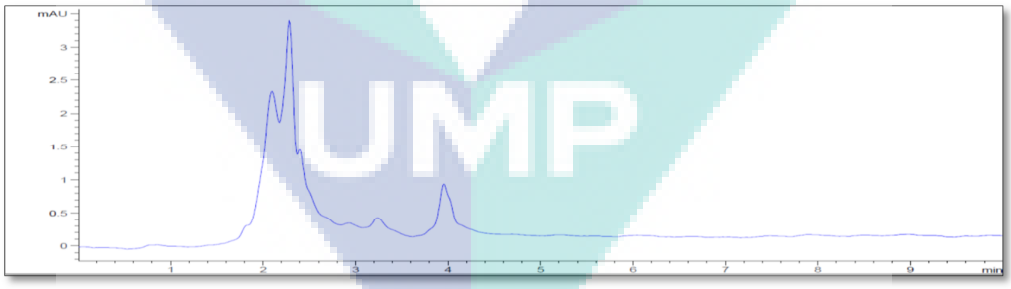


Figure 4.6.3: HPLC chromatograms of *E. longifolia* herbal product (**C5**)

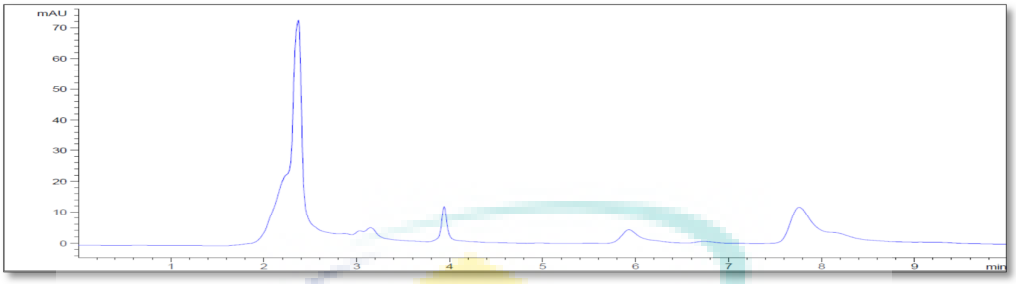


Figure 4.6.4: HPLC chromatograms of *E. longifolia* herbal product (**C7**)

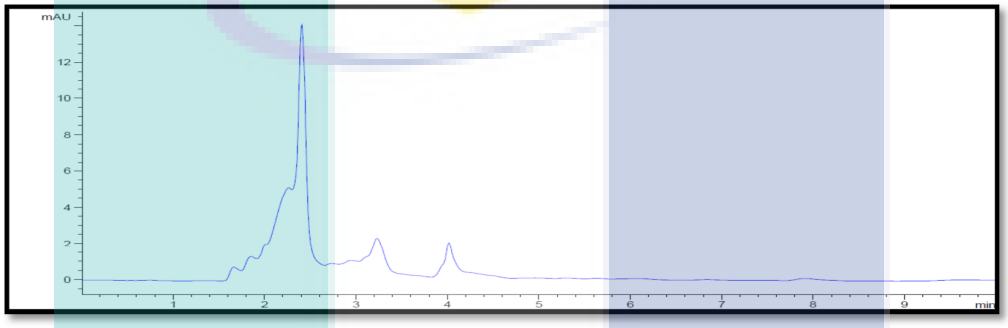


Figure 4.6.5: HPLC chromatograms of *E. longifolia* herbal product (**C8**)

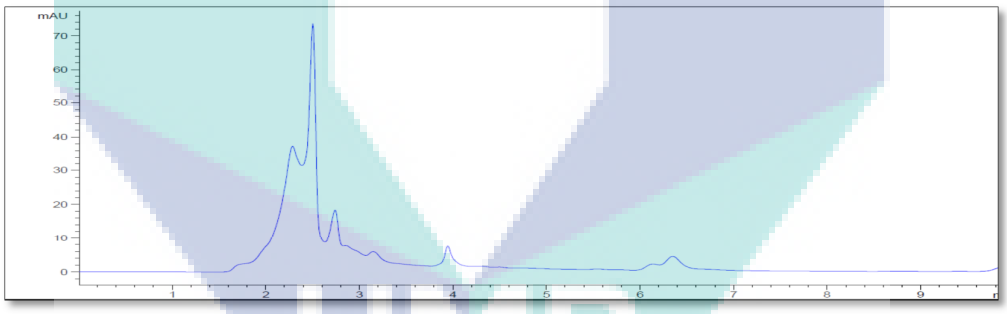


Figure 4.6.6: HPLC chromatograms of *E. longifolia* herbal product (**C9**)

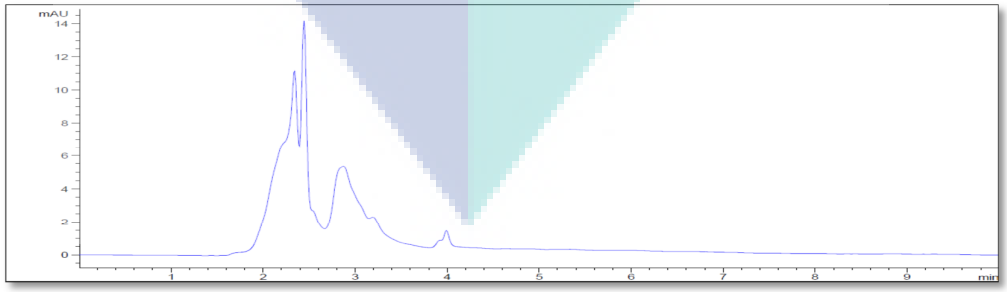


Figure 4.6.7: HPLC chromatograms of *E. longifolia* herbal product (**C13**)

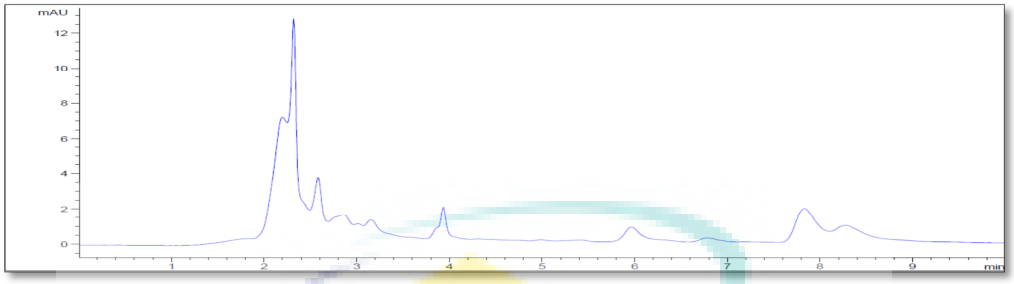


Figure 4.6.8: HPLC chromatograms of *E. longifolia* herbal product (**C14)**

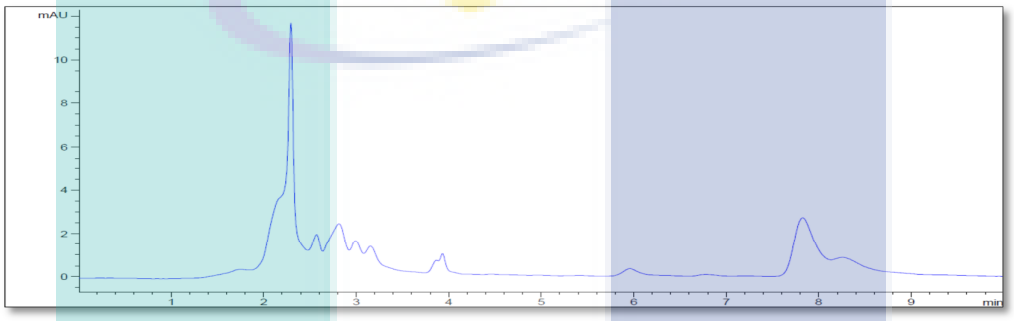


Figure 4.6.9: HPLC chromatograms of *E. longifolia* herbal product (**C16)**

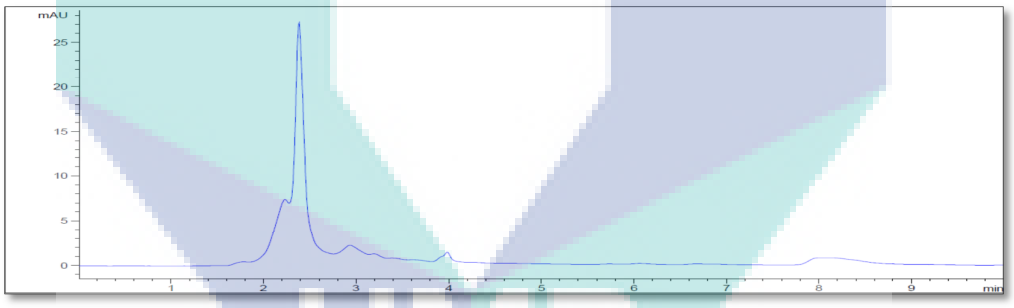


Figure 4.6.10: HPLC chromatograms of *E. longifolia* herbal product (**C17)**

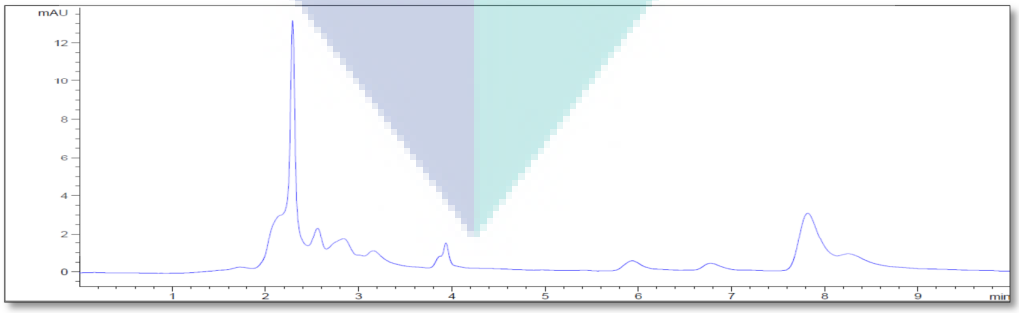


Figure 4.6.11: HPLC chromatograms of *E. longifolia* herbal product (**C19)**

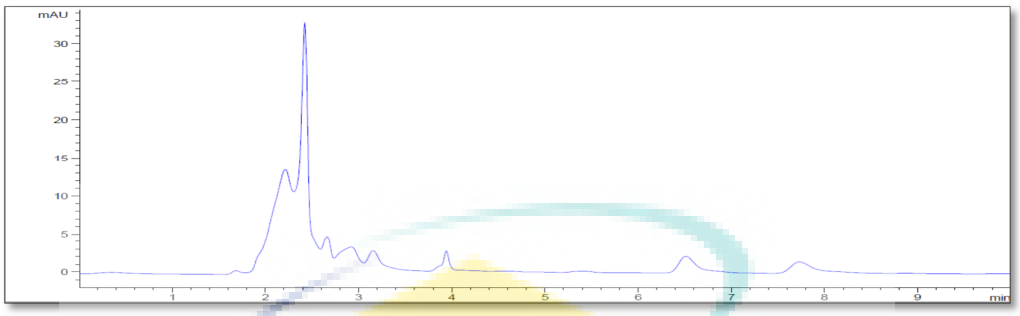


Figure 4.6.12: HPLC chromatograms of *E. longifolia* herbal product (**C20)**

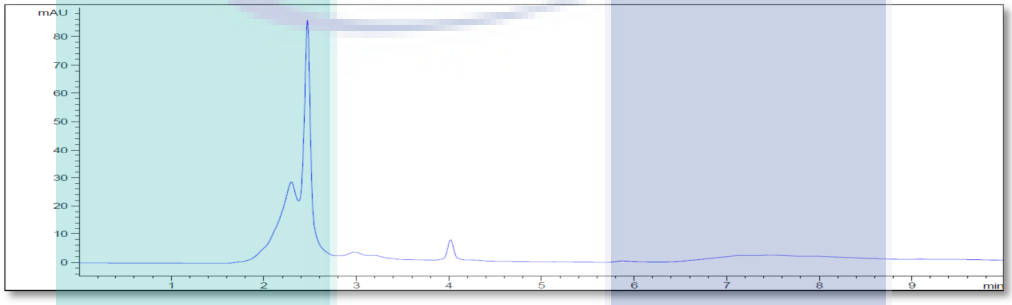


Figure 4.6.13: HPLC chromatograms of *E. longifolia* herbal product (**C22)**

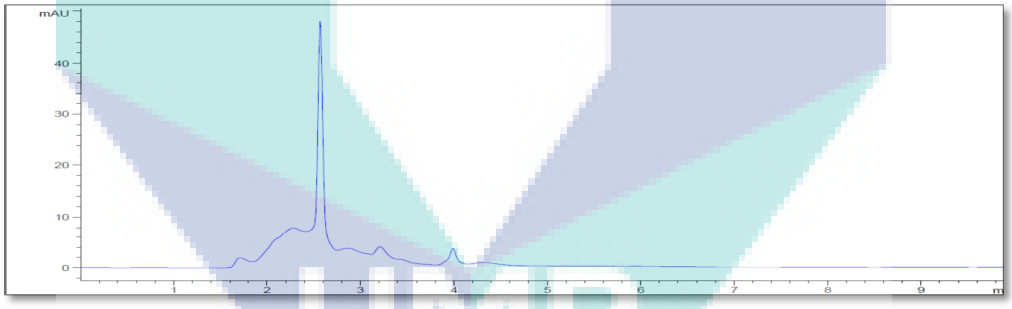


Figure 4.6.14: HPLC chromatograms of *E. longifolia* herbal product (**C33)**

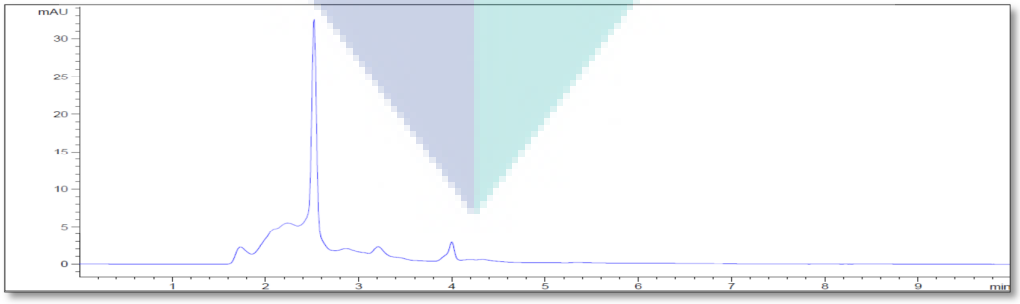


Figure 4.6.15: HPLC chromatograms of *E. longifolia* herbal product (**C37)**

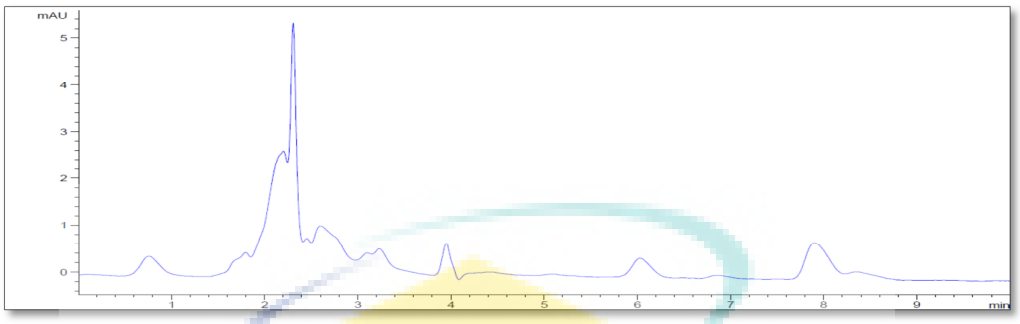


Figure 4.6.16: HPLC chromatograms of *E. longifolia* herbal product (**C40)**

HPLC Chromatograms of *E. longifolia* premix coffees

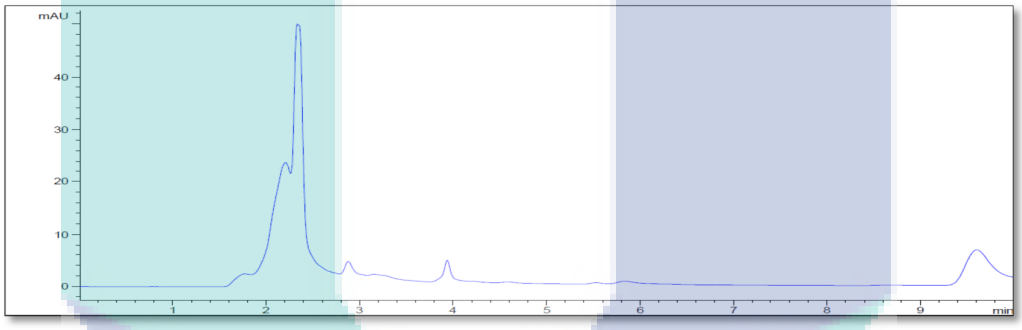


Figure 4.6.17: HPLC chromatograms of *E.longifolia* premix coffee (**B1**)

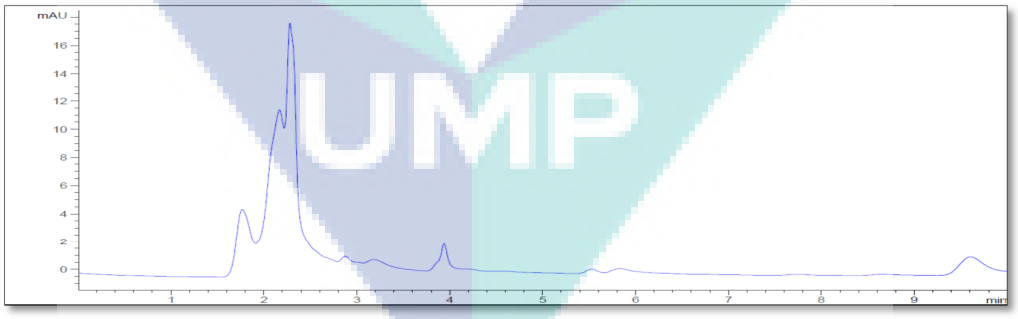


Figure 4.6.18: HPLC chromatograms of *E.longifolia* premix coffee (**B2**)

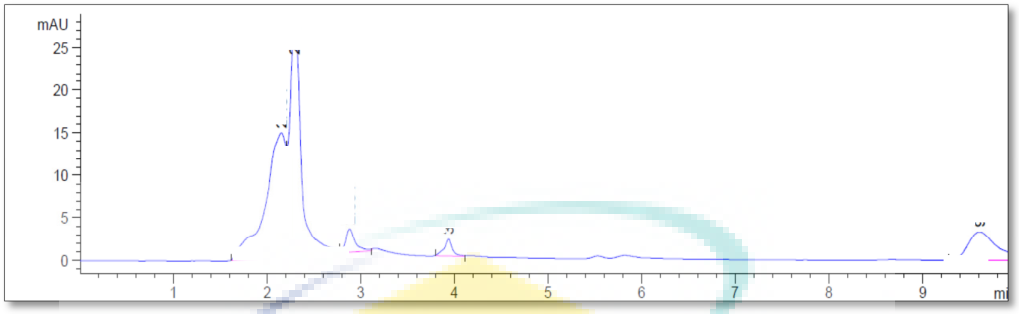


Figure 4.6.19: HPLC chromatograms of *E.longifolia* premix coffee (**B3**)

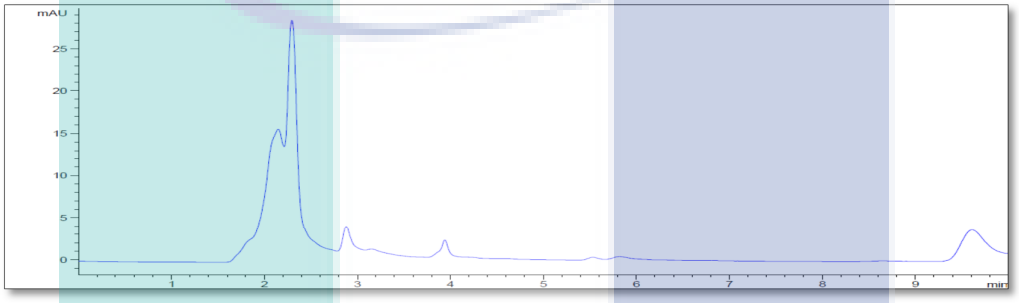


Figure 4.6.20: HPLC chromatograms of *E.longifolia* premix coffee (**B4**)

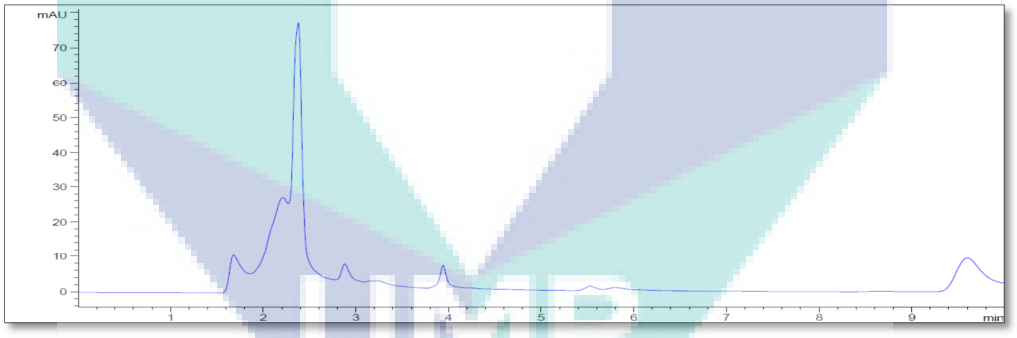


Figure 4.6.21: HPLC chromatograms of *E.longifolia* premix coffee (**B5**)

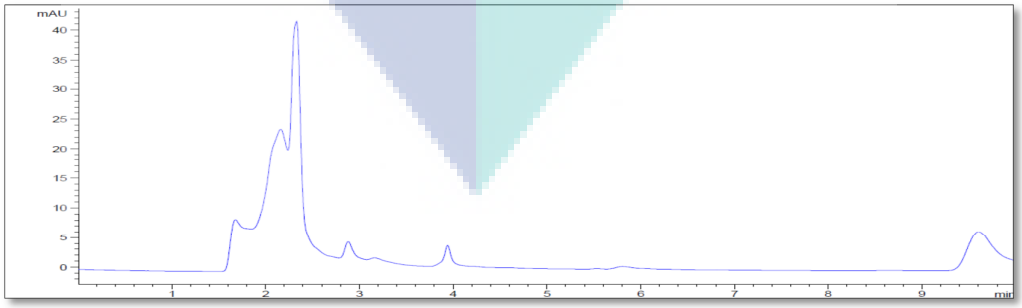


Figure 4.6.22: HPLC chromatograms of *E.longifolia* premix coffee (**B6**)

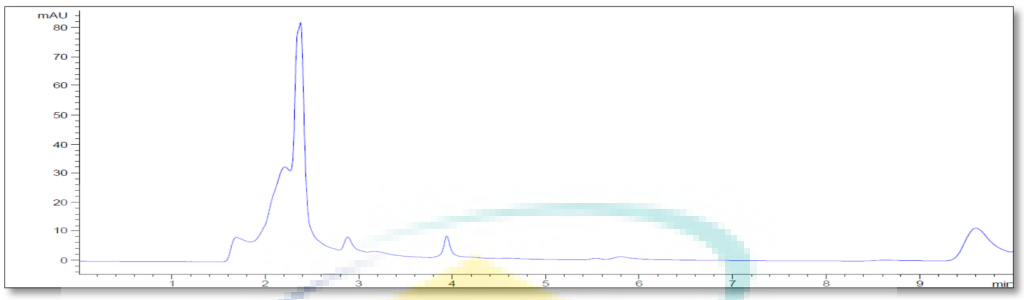


Figure 4.6.23: HPLC chromatograms of *E.longifolia* premix coffee (**B7)**

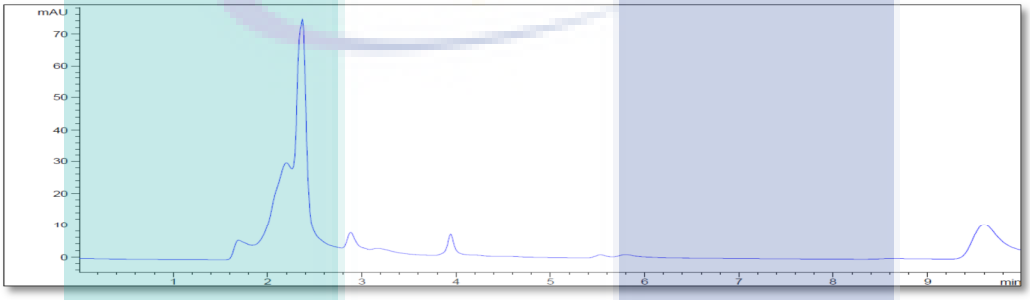


Figure 4.6.24: HPLC chromatograms of *E.longifolia* premix coffee (**B8)**

APPENDIX C

Concentration (mg/ml)	Retention time (min)	Mean	Standard deviation	%RSD	Confidence interval at 95%	
					Lower	Upper
0.03125	8.001 7.993 7.992	7.995	0.005	0.062	7.983	8.008
0.0625	8.012 8.002 7.994	8.003	0.009	0.113	7.980	8.025
0.125	8.012 8.011 8.01	8.011	0.001	0.113	8.009	8.013
0.25	8.009 8.007 8.009	8.008	0.001	0.014	8.005	8.011
0.5	8.002 8.022 8.005	8.010	0.011	0.135	7.983	8.036
				Mean	7.992	8.019

Table 4.1 (a): Precision (repeatability) analysis of retention time of eurycomanone standard

Conc. (mg/ml)	Peak areas (mAU)	Mean	Standard deviation	%RSD	Confidence interval at 95%	
					Lower	Upper
0.03125	557.710 558.151 558.830	558.230	0.564	0.101	556.829	559.631
0.0625	1099.083 1098.842 1098.882	1098.936	0.129	0.012	1098.616	1099.256
0.125	2100.467 2086.803 2080.604	2089.291	10.162	0.486	2064.046	2114.536
0.25	4495.771 4486.856 4477.484	4486.704	9.145	0.204	4463.988	4509.420
0.5	8922.146 8896.630 8876.118	8898.298	23.059	0.259	8841.015	8955.581
				Mean	3404.899	3447.685

Table 4.1 (b): Precision (repeatability) analysis of peak area (mAU) of eurycomanone standard.

Conc. (mg/ml)	Retention time (min)			Mean	Standard deviation	%RSD	Confident interval at 95%	
	day 1	day 2	day 3				Lower limit	Upper limit
0.03125	8.001	7.994	7.957	7.984	0.024	0.296	7.875	8.0836
0.0625	8.012	7.997	7.946	7.985	0.035	0.433	7.899	8.0709
0.125	8.012	7.994	7.932	7.979	0.045	0.526	7.875	8.0836
0.25	8.009	7.992	7.937	7.979	0.038	0.472	7.886	8.0728
0.5	8.002	7.962	7.933	7.966	0.035	0.435	7.880	8.0517
						mean	7.883	8.0725

Table 4.1 (c): Precision (intermediate precision) analysis of retention time of eurycomanone standard

Table 4.1 (d): Precision (intermediate precision) analysis of peak area (mAU) of eurycomanone standa

Conc. (mg/ml)	Peak areas			Mean	SD	%RSD	confident interval at 95%	
	day 1	day 2	day 3				Lower	Upper
0.03125	557.71	599.71	611.35	589.59	28.22	4.79	519.49	659.68
0.0625	1099.08	1179.80	1210.27	1163.05	57.46	4.94	1020.32	1305.78
0.125	2100.47	2308.81	2158.67	2189.32	107.50	4.91	1922.27	2456.36
0.25	4495.77	5022.46	5068.26	4862.16	318.13	6.54	4071.88	5652.44
0.5	8922.15	9871.33	10024.20	9605.89	597.05	6.22	8122.73	11089.06

UMP