QUALITY CONTROL ANALYSIS, STANDARDIZATION AND STABILITY STUDIES OF *EURYCOMA LONGIFOLIA* AND EFFECT OF ITS SAPONIN CONTENT ON SOLUBILITY AND TOXICITY OF EURYCOMANONE

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By

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LIST OF ABBREVIATION

µg	Microgram
µg/mL	Microgram per milliliter
µL	Microliter
µM	Micro molar
µm	Micrometer
AAS	Atomic Absorption Spectroscopy
As	Arsenic
ATR-FTIR	Attenuated Total Reflectance- Fourier Transform Infrared Spectroscopy
BSA	Bovine Serum Albumin
Cd	Cadmium
cm	Centimetre
CO_2	Carbon Dioxide
DMSO	Dimethyl sulfoxide
ED_{50}	Effective Dose for 50%
g	Gram
g/L	Gram per liter
GC	Gas chromatography
h	Hour
H2O	Water
HCl	Hydrochloric Acid
Hg	Mercury
HNO_3	Nitric Acid
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin-Layer Chromatography

IC_{50}	Inhibitory Concentration 50
ICH	International Conference on Harmonisation
kJ/mol	Kilo Joule per Mole
LC_{50}	Lethal Concentration
LD_{50}	Lethal Dose 50
LD_{95}	Lethal Dose 95
LOD	Limit of Detection
LOQ	Limit of Quantification
mg	Milligram
mg/kg	Milligram per Kilogram
MIC	Minimum Inhibitory Concentration
min	Minutes
mL	Milliliter
mL/min	Milliliter per Minutes
mm	Millimeter
mM	Millimolar
N	Normal
nm	Nanometer
ºC	Degree Celsius
Pb	Lead
PCA	Principal Component Analysis
PCS	Photon Correlation Spectroscopy
ppm	Part per Million
PSI	Pounds per Square Inch
RH	Relative Humidity
rpm	Round per Minutes
RSD	Relative Standard Deviation

RT	Room Temperature
SD	Standard Deviation
$t_{1/2}$	Half Life
TEM	Transmission Electron Microscopy
TGA	Thermo gravimetric Analysers
UV-Vis	Ultra Violet and Visible
v/v	Volume per Volume
W	Watt
w/v	Weight per Volume
wt/wt	Weight per Weight

ANALISIS KAWALAN KUALITI, PEMIAWAIAN DAN KAJIAN KESTABILAN *EURYCOMA LONGIFOLIA* DAN KESAN KANDUNGAN SAPONINNYA TERHADAP KELARUTAN DAN KETOKSIKAN EURIKOMANON

ABSTRAK

Ujian kawalan kualiti dilakukan pada bahan mentah akar, batang dan daun *E. longifolia* daripada lima lokasi yang berbeza untuk menentukan julat nilai kawalan kualiti. Kandungan kelembapan, abu dan abu tidak larut asid bagi akar berada di dalam julat yang ditetapkan oleh Standard Perubatan Herba Asean dan Monograf Herba Malaysia Jilid 1. Julat nilai bagi kawalan kualiti bahan mentah batang dan daun telah ditetapkan. Peratusan ekstrak larut air adalah lebih tinggi berbanding ekstrak larut alkohol untuk semua bahagian tumbuhan. Peratusan ekstrak yang diperolehi dengan kaedah panas adalah lebih tinggi berbanding dengan kaedah sejuk untuk kedua-dua ekstrak larut air dan alkohol. Kandungan Pb, Cd, As dan Hg dalam bahan mentah *E. longifolia* didapati berada dalam julat yang diterima Pertubuhan Kesihatan Dunia. Kaedah pengekstrakan telah dioptimumkan dengan menggunakan nisbah serbuk akar: pelarut dan masa pengekstrakan yang berbeza. Keputusan menunjukkan pengeluaran ekstrak yang terbaik ialah pada nisbah serbuk akar: air 1:15 selama 4 jam setiap kitaran dan diulang sehingga 3 kitaran refluk. Peratusan hasil dan profil kimia yang melibatkan analisis kualitatif (HPTLC, UV dan HPLC) dan analisis kuantitatif (HPLC) telah dijalankan untuk semua ekstrak.

Kaedah analisis spektroskopi dan kromatografi digunakan untuk pemprofilan kimia dan pemiawaian ektrak air bagi akar, batang, daun termasuk enam sampel

komersial *E. Longifolia* berdaftar. Kaedah HPLC memberi peratusan eurycomanone bagi akar, batang, daun dan sampel komersial di dalam julat 0.89 ± 0.01% sehingga 3.28 ± 0.01% , 0.23 ± 0.00% sehingga 1.10 ± 0.00%, 0.10 ± 0.01% sehingga 0.34 ± 0.02% dan 0.07 ± 0.00% sehingga 0.16 ± 0.00%. Peratusan jumlah fenol, glycosaponins, polisakarida dan protein bagi akar adalah masing-masing dari julat 5.94 ± 0.23% sehingga 10.10 ± 0.08%, 40.12 ± 1.05% sehingga 44.45 ± 0.54%, 25.30 ± 0.15% sehingga 51.30 ± 2.56% dan 21.42 ± 0.6% sehingga 44.92 ± 2.5%. Peratusan jumlah fenol, glycosaponins, polisakarida dan protein bagi batang adalah masing-masing dari julat 4.2 ± 0.13% sehingga 8.76 ± 0.3%, 24.09 ± 1.06% sehingga 47.24 ± 1.07%, 20.53 ± 0.48% sehingga 68.18 ± 0.22% dan 12.17 ± 0.6% sehingga 48.20 ± 2.0%. Peratusan jumlah fenol, glycosaponins, polisakarida dan protein bagi daun adalah masing-masing dari julat 15.88 ± 0.1% sehingga 22.33 ± 0.28%, 52.26 ± 0.31% sehingga 61.58 ± 0.44%, 7.59 ± 0.04% sehingga 12.59 ± 0.11% dan 58.90 ± 1.6% sehingga 81.58 ± 2.3%. Peratusan jumlah fenol, glycosaponins, polisakarida dan protein bagi sampel komersial adalah masing-masing dari julat 2.53 ± 0.00% sehingga 3.34 ± 0.01%, 3.31 ± 0.31% sehingga 16.44 ± 0.21%, 5.98 ± 0.54% sehingga 66.17 ± 3.96% dan 6.4 ± 0.2% sehingga 7.8 ± 0.3 %.

Dalam kajian kestabilan dipercepatkan, ekstrak (BR, BS dan BL) adalah lebih stabil pada 30ºC berbanding suhu lain yang dikaji dan anggaran hayat (t_{90}) BR, BS dan BL adalah 0.31, 0.23 dan 0.36 bulan, masing-masing, pada 30ºC. Eurycomanone mempunyai jangka hayat yang lebih panjang dalam ektrak daun (BL) berbanding ekstrak akar (BR) dan batang (BS). Penanda sebatian kimia mengikuti degradasi tertib pertama dan degradasinya menjadi lebih cepat pada suhu dan kelembapan yang lebih tinggi. Oleh itu, untuk jangka hayat yang lebih panjang, adalah disyorkan untuk

menyimpan *E. longifolia* ekstrak pada suhu lebih rendah dari 30ºC di dalam bekas yang tertutup rapat.

Saponins meningkatkan kebolehlarutan eurycomanone melalui pembentukan miseles seperti yang ditunjukkan oleh kehadiran zarah sub-mikron. Kepekatan Misel Kritikal (CMC) dianggarkan bernilai 267 μg/mL untuk ekstrak air akar mentah (TA), 57 μg/mL untuk ekstrak kaya saponin dan 847 μg/mL untuk ekstrak kaya eurycomanone. Keputusan ini menunjukkan kehadiran miseles dan menunjukkan peranan saponin dalam pembentukan miseles, seperti yang ditunjukkan oleh nilai CMC rendah dalam ekstrak kaya saponin berbanding dengan nilai CMC ekstrak kaya eurycomanone. Kesan sitotoksik ekstrak kaya eurycomanone terhadap HCT 116 sel karsinoma kolorektal menunjukkan kepekatan perencatan median (IC_{50}) 22.14 ± 1.0 μg/mL, manakala ekstrak kaya saponins pada julat kepekatan yang sama tidak menunjukkan kesan sitotoksik terhadap sel HCT 116. Kesan sitotoksik gabungan ekstrak kaya saponin dan ekstrak kaya eurycomanone terhadap sel HCT 116 telah meningkatkan peratusan perencatan, bergantung kepada dos. Didapati bahawa dengan penambahan ekstrak kaya saponin dengan ekstrak kaya eurycomanone, kesan sitotoksik dapat dikurangkan terutamanya pada ekstrak kaya saponins yang berkepekatan rendah (25, 50 dan 75 μg/mL), dan tidak mempunyai kesan pada kepekatan tinggi (100 μg / mL). Ini menunjukkan kehadiran saponins di dalam ekstrak *E. longifolia* dapat melindungi kesan sitotoksik eurycomanone. Tambahan pula, penemuan yang diperolehi menunjukkan bahawa penggunaan ekstrak mentah *E. longifolia* lebih selamat berbanding penggunaan sebatian tulen atau ekstrak eurycomanone pekat.

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ABSTRACT

Quality control test was done on *E. longifolia* root, stem and leaf from five different localities in order to determine the range of quality control values. Moisture, ash and acid insoluble ash content of all the root raw material were found to be within acceptable limit set by Standard of Asean Herbal Medicine and Malaysian Herbal Monograph Volume 1. The range of quality control values of the stem and leaf raw material have been set. The percentages of water soluble extracts were higher as compared to alcohol soluble extracts in all parts of the plant. Percentage extractive values by the hot method were higher as compared to the cold method for both water and alcohol soluble extracts. The amounts of Pb, Cd, As and Hg in *E. longifolia* raw material were found within acceptable limit according to World Health Organization. Extraction method was optimized by different root powder: solvent ratio and extraction time. Results suggest the best extraction conditions at the root powder: water ratio of 1:15 and at 3 reflux cycles each for 4 h. The percentage yield and chemical profile involving qualitative analysis (HPTLC, UV and HPLC) and quantitative analysis (HPLC) were carried out for all the extracts.

Analytical spectroscopy and chromatographic methods were used for chemical profiling and standardization of *E. longifolia* root, stem, leaf water extracts

and six registered commercial products. HPLC validated methods give the percentage of eurycomanone for root, stem, leaf and commercial products in a ranges 0.89 ± 0.01% to 3.28 ± 0.01%, 0.23 ± 0.10% to 1.10 ± 0.00%, 0.10 ± 0.00% to 0.34 ± 0.01% and 0.07 ± 0.00% to 0.16 ± 0.00%, respectively. The percentage of total phenolics, glycosaponins, polysaccharides and protein for root ranging from 5.94 ± 0.23% to 10.10 ± 0.08%, 40.12 ± 1.05% to 44.45 ± 0.54%, 25.30 ± 0.15% to 51.30 ± 2.56% and 21.42 ± 0.6% to 44.92 ± 2.5%, respectively. The percentage of total phenolics, glycosaponins, polysaccharides and protein for stem ranging from 4.20 ± 0.13% to 8.76 ± 0.30%, 24.09 ± 1.06% to 47.24 ± 1.07%, 20.53 ± 0.48% to 68.18 ± 0.22% and 12.17 ± 0.6% to 48.20 ± 2.0%, respectively. The percentage of total phenolics, glycosaponins, polysaccharides and protein for leaf ranging from 15.88 ± 0.10% to 22.33 ± 0.28%, 52.26 ± 0.31% to 61.58 ± 0.44%, 7.59 ± 0.04% to 12.59 ± 0.11% and 58.90 ± 1.6% to 81.58 ± 2.3%, respectively. The percentage of total phenolics, glycosaponins, polysaccharides and protein for commercial products ranging from 2.53 ± 0.00% to 3.34 ± 0.01%, 3.31 ± 0.31% to 16.44 ± 0.21%, 5.98 ± 0.54% to 66.17 ± 3.96% and 6.4 ± 0.2% to 7.8 ± 0.3 %, respectively.

In accelerated stability study, extracts (BR, BS and BL) were more stable at 30ºC compared to other temperatures studied, and the estimated shelf life (t_{90}) of BR, BS and BL were 0.31, 0.23 and 0.36 months, respectively, at 30ºC. Eurycomanone has higher shelf life in the leaf (BL) as compared to root (BR) and stem (BS). The marker compound followed the first order degradation and its degradation rate was increased by increasing storage temperature and humidity. Therefore, in order to guarantee a longer shelf life of the products, it is recommended to store the products containing *E. Longifolia* extract at below 30ºC in a tightly closed container.

Saponins increase the solubility of eurycomanone through the formation of micelles as indicated by the presence of sub-micron particles. The Critical Micellar Concentration (CMC), was estimated to be 267 μg/mL for the crude root water extract (TA), 57 μg/mL for saponins-rich extract and 847 μg/mL for eurycomanone-rich extract. These results indicate the presence of micelles and show the role of saponins in the formation of these micelles as indicated by the lower CMC value in saponins-rich extract compared to the CMC value of eurycomanone-rich extract. Cytotoxicity effect of eurycomanone-rich extract on HCT 116 colorectal carcinoma cells showed dose dependent growth inhibitory effect with median inhibitory concentration (IC_{50}) of 22.14 ± 1.0 μg/mL, whereas the saponins-rich extract at the same concentration range did not show any cytotoxic effect on the same cells. Cytotoxicity effect of combination saponins and eurycomanone-rich extract on HCT 116 cells was increased in a dose dependent manner. It was found that adding the saponins-rich extract to the eurycomanone-rich extract reduced the cytotoxic effect of the later particularly at low concentration of the saponins-rich extract (25, 50 and 75 μg/mL), and has no effect at higher concentration (100 μg/mL). These findings indicate that the presence of saponins in the *E. longifolia* extracts protect against the cytotoxic effect of the eurycomanone. Furthermore, these findings indicate that using the crude extracts of *E. longifolia* could be safer than using pure compounds or concentrated fractions of eurycomanone.

CHAPTER 1
INTRODUCTION

1.1 Significance of Medicinal Plants

Since historically, medicinal plants have played an important role in human life. Plants have been used extensively as food and medicine for the treatment of a range of diseases. The sale and use of herbal medicines have increased significantly over the past 20 years. The use of herbal medicines in the US increased from 2.5% in 1990 to 12.1% in 1997, with a total of $5 billion being spent (Eisenberg *et al*., 1998). Within the UK, over 30% of a random sample of 5010 adults were taking, or had been taken, herbal remedies (Thomas *et al.*, 2001). Furthermore, 80% of the world's population in developing countries depend on the use of herbal and other traditional medicines for their primary health care (Khalid *et al.*, 2009).

Medicinal plants are available from a variety of plant material which is from leaf, stem, root and bark. They contain many biologically active compounds and can be used to treat mild or chronic diseases. Herbs are easy to be obtained, prepared and consumed in many ways, using either fresh raw materials or dried ingredients. Herbal remedies are also available in the form of capsules, tablets or powder.

1.2 Efficacy, Safety and Quality Control in the Standardization of Herbal Medicine

Determination of quality aspects of herbal products can be a problem because of the variation in the content and concentrations of constituents of plant material, different range of extraction techniques and processing steps used by different manufacturers. Many of the herbal products are prepared from a single plant or a combination of two or more different types of medicinal plants. It is unusual to have different herbal ingredients in one product but the problem is to determine which components contribute to biological activities. Pharmacological activities and consistency in herbal products are based on chemical constituent's changes which possess the activity of the herbs. To overcome this problem, consistent products can be produced through standardization.

Standardization of herbal materials is carried out to ensure the consistency and repeatability of a particular extract in ensuring guaranteed potency through acceptable levels of active compounds. This process can be performed by chemical (HPTLC, HPLC/GC and FTIR) or biological (antioxidant) analysis. Standardization is an important step where the active constituents are known. However, for many herbs the active constituents are not known. In these cases, products may be standardized on the content of certain 'marker' compound (chemical characteristic of the herb or present in large quantities). Although there are many benefits proven, natural products are not getting wider acceptance in the mainstream of pharmaceuticals due to lack of standardization. In order to bring these remedies into the mainstream pharmaceutical market, strong scientific evidence is required to support the efficacy claims of these products (Barnes, 2003).

Statement that herbal drugs are very safe and free from side effects is not absolutely true. The plants contain hundreds of constituents and some are highly toxic such as the most cytotoxic anti-cancer plant-derived drugs, digitalis, the pyrrolizidine alkaloids, ephedrine, phorbol esters, etc. There are two kinds of side effects reported. The first, considered to be intrinsic to herbal drugs themselves, for example related to predictable toxicity, over-dosage and interaction with conventional drugs, as reported for modern medicines. The most known side effects reported for herbal drugs are extrinsic to the preparation for example because of several manufacturing problems; misidentification of plants, lack of standardization, failure of good manufacturing practice, contamination, substitution and adulteration of plants and incorrect preparation or dosage (Calixto, 2000).

Due to increasingly widespread use of herbs, it is important to ensure the safety of the herbal products. Therefore, the quality control and standardization of raw material and the herbal preparation should be permanently conducted. Standardization and quality control of herbals is the process involved in the evaluation of crude drugs physicochemical aspects such as selection and handling of crude material, safety, efficacy and stability assessment of finished products, documentation, safety and risk based on experience, provision of product information to consumer and product promotion. However, more attention is given to the following quality index such as macro and microscopic examination, foreign organic matter, ash values, moisture content, extractive values, crude fibre, qualitative chemical evaluation, chromatography's examination, quantitative chemical evaluation and toxicological studies (WHO, 1996a; WHO, 1996b; WHO, 1992).

Several factors can also affect the quality control and consequently the therapeutic value of herbal medicines such as the use of fresh plants, light, temperature, nutrients, water availability, duration and time collection, methods of collecting, drying, packaging, storage, transportation of raw material, age, part of the collected plant and other factors. Method of extraction, contamination with microorganism, heavy metals, pesticides and etc, can also affect the quality, safety and efficacy of herbal drugs (Calixto, 2000).

1.3 Problem Statements and Justification of the Research

In Malaysia, dietary supplements are widely consumed for general health and well-being and it is easily available in community pharmacies, health food stores, night markets, grocery stores and also obtained via the internet. Currently, 183 products manufactured from *E. longifolia* are available and registered with the National Pharmaceutical Control Bureau (Appendix A). However, there is still a lack of information on its chemical profile in the commercial products. All parts (root, stem and leaf) of E. longifolia are useful and contributed to different biological activities, thus indicating the need to perform quality control tests. The toxic potential of eurycomanone as a major marker compound makes it necessary to standardize the plant extracts in commercial products to comply with the set standards in order to protect consumers from possible toxic effects. Stability studies must be performed in order to estimate the shelf life, suitable temperature storage and stability of eurycomanone compound in all parts of the plants. The stability of *E. longifolia* extracts need to comply to ICH standard guidelines. The solubility and toxicity of eurycomanone needs improvements. Since saponins are the major primary metabolites in this plant, their effect on the solubility and toxicity of eurycomanone was investigated.

1.4 Objectives of the Study

The objectives of this study are as the following:

1. To perform quality control tests on *E. longifolia* root, stem and leaf raw material from five different locations and to optimize the extraction method for the root.
2. To develop and validate reliable analytical methods for chemical profiling and standardization of *E. longifolia* root, stem and leaf water extracts and several selected commercial products.
3. To perform and study the accelerated stability profile of *E. longifolia* water extract of the root, stem and leaf using eurycomanone as a marker.
4. To study the effect of saponins on the solubility and cytotoxicity of eurycomanone-rich extract.

CHAPTER 2
LITERATURE REVIEW

2.1 *Eurycoma longifolia* Jack

2.1.1 Taxonomy

Taxonomically, *E. longifolia* Jack is classified as the following scheme:

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Sapindales

Family: Simaroubaceae

Genus: Eurycoma

(Comprehensive information can be found at http://en.wikipedia.org/wiki/Eurycoma_longifolia)

2.1.2 Vernacular Names

Some of the common and popular names of this plant includes Long Jack, Malaysian Ginseng, Local Ginseng, Natural Viagra, Pasak Bumi, Payung Ali, Penawar Pahit, Setunjang Bumi, Bedara Pahit, Tongkat Baginda, Pokok Syurga, Tongkat Ali Hitam, Pokok Jelas, Cay ba binh, Ian- don, and Jelaih (Bhat and Karim, 2010).

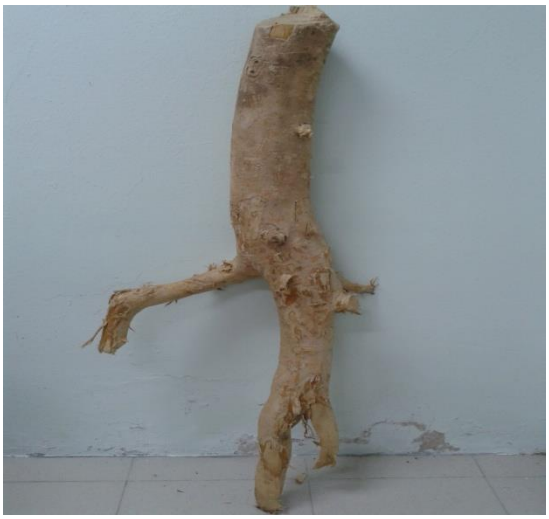
2.1.3 Plant Description

E. longifolia Jack is a slender, evergreen flowering tree belongs to the family Simaroubaceae, is occurring naturally in jungles of Malaysia and Indonesia. The female and male trees reach a height of up to 15 meters (Wernsdorfer *et al.*, 2009) and bearing fruits after nearly 2–3 years of cultivation. However, it is generally believed that for complete maturation of the plant, it might take up to 25 years. But, for commercial uses, most of the times, the roots are harvested after 4 years of cultivation. The fruits are green in color, 2–3 cm long and turn to dark red after ripening. The leaves are pinnate, spirally arranged, long (10–15 inches) with 10–30 leaflets. The flowers are produced in large panicles and the plant is dioecious with male and female flowers borne on different trees. The plant grows profusely in sandy, well-drained soil in the presence of partial shade and with adequate quantity of water. This plant is widely sought 'singly' or as an 'essential component' in herbal remedies for a variety of illnesses and as health supplements. Almost all the parts of Tongkat Ali have been used traditionally for therapeutic purposes (Bhat and Karim, 2010). There are four different species of Tongkat Ali, namely *Eurycoma longifolia, Eurycoma apiculata, Polyathia bullata* and *Goniothalamus sp.* (Aziz *et al.*, 2003). Among the four, *Eurycoma longifolia* is the most commonly used species for the extract production (Athimulam *et al*., 2006).

(a)

(b)

(c)



(d)

Figure 2.1: Pictures of *Eurycoma longifolia* (a) - (b) whole plant, (c) leaves and (d) root

2.1.4 Review of Chemical Constituents of *Eurycoma longifolia*

Various chemical constituents have been identified from the different parts of *E. longifolia*. Reviews on the identified chemicals of *E. longifolia* are summarized in Table 2.1.

Table 2.1: Chemical constituents of *Eurycoma longifolia*

Plant part	Extract	Chemical constituents	References
Root	Aqueous	quassinoids (e.g. eurycolactones A-E, eurycomalides A-B, eurycomalactone, 6α-hydroxyeurycomalactone, 7α-hydroxyeurycomalactone, eurycomanone, 13α(21)-epoxyeurycomanone, 12,15-diacetyl-13α(21)-epoxy-eurycomanone,12-acetyl-13,21-dihydroeurycomanone, 15-acetyl-13α(21)-epoxyeurycomanone, 3,4ε-dihydroeurycomanone, 13,21-dihydroeurycomanone, eurycomanol, 13β,18-dihydroeurycomanol, 13β, 21-dihydroxyeurycomanol, eurycomanol-2-O-β-D-glycopyranoside, 11-dehydroklaineanone, 15β-hydroxyklaineanone, 14,15β-dihydroxyklaineanone, 5α,14β,15β-trihydroxyklaineanone,15β-O-acetyl-14-hydroxyklaineanone, 6α-acetoxy-14,15β-dihydroxyklaineanone, 6α-acetoxy-15β-hydroxyklaineanone, laurycolactones A-B, longilactone, dehydroxylongilactone, 2,3-dehydro-4α-hydroxylongilactone, ailanthone, (α/β-epoxide) ailanthone, chaparrinone (α-methyl), 3,4ε-dihydrochaparrinone, picrasinoside B, klaineanolide B, iandonoside B, eurycomaoside, 16-α-O-methylneoquassin, samaderin B and glaucarubolone), canthin-6-one alkaloids (e.g. canthin-6-one, 9-methoxycanthin-6-one, 5,9-dimethoxycanthin-6-one, 9,10-dimethoxycanthin-6-one, 11-	(Chua *et al.*, 2011; Chua *et al.*, 2012; Yusmazura *et al.*, 2009)

	hydroxycanthin-6-one, 1-hydroxy-11-methoxycanthin-6-one, 10-hydroxy-9-methoxycanthin-6-one, 11-hydroxy-10-methoxycanthin-6-one, 11-O-β-D-glucopyranosylcanthin-6-one, canthin-6-one-3*N*-oxide, 9-methoxycanthin-6-one-3*N*-oxide and 9-methoxy-3-methylcanthin-5,6-dione), β-carboline alkaloids (e.g. β-carboline-1-propionic acid, 7-hydroxy-β-carboline-1-propionic acid, 7-methoxy-β-carboline-1-propionic acid, and 1-methoxymethyl-β-carboline), squalene-type triterpene (e.g. eurylene and 11/14-deacetyl eurylene), biphenylneolignans (e.g. 2,2'-dimethoxy-4-(3-hydroxy-1-propenyl)-4'-(1,2,3-trihydroxypropyl) diphenyl ethers (isomer), 2-hydroxy-3,2',6'-trimethoxy-4'-(2,3-epoxy-1-hydroxypropyl)-5-(3-hydroxy-1-propenyl)-biphenyl and 2-hydroxy-3,2'-dimethoxy-4'-(2,3-epoxy-1-hydroxypropyl)-5-(3-hydroxy-1-propenyl)-biphenyl) and others (e.g. isoleucine, calcium, magnesium and potassium)	
Methanol	quassinoids (e.g. eurycolactones A-F, eurycomalides A-B, eurycomalactone, 6α-hydroxyeurycomalactone, 6-hydroxy-5,6-dehydroeurycomalactone, 5,6-dehydroeurycomalactone, eurycomanone, 13β,21-dihydroxyeurycomanol, 14,15β-dihydroxyklaineanone, 5α,14β,15β-trihydroxyklaineanone, laurycolactones A-B, longilactone, 6-dehydroxylongilactone, 2,3-dehydro-4α-hydroxylongilactone and pasakbumin B-C), canthin-6-one alkaloids (e.g. canthin-6-one, 1-hydroxycanthin-6-one, 9-hydroxycanthin-6-one, 5-hydroxymethylcanthin-6-one, 5-methoxycanthin-6-one, 9-methoxycanthin-6-one, 10-methoxycanthin-6-one, 1-hydroxy-9-	(Kardono *et al.,* 1991; Chan *et al.*, 1991; Chan *et al.*, 1992; Ang *et al*., 2000; Ang *et al.,* 2002; Kuo *et al.,* 2003b; Teh *et al*., 2010; Kuo *et al.,* 2003a)

	methoxycanthin-6-one, 4-hydroxy-5-methoxycanthin-6-one, 8-hydroxy-9-methoxycanthin-6-one, 5-hydroxymethyl-9-methoxycanthin-6-one, 4,5-dimethoxycanthin-6-one, 9,10-dimethoxycanthin-6-one, canthin-6-one 9-*O*-β-glucopyranoside, canthin-6-one-3*N*-oxide, 9-hydroxycanthin-6-one-3*N*-oxide and 9-methoxycanthin-6-one-3*N*-oxide), β-carboline alkaloids (e.g. β-carboline-1-propionic acid, 7-methoxy-β-carboline-1-propionic acid, methyl β-carboline-1-carboxylate, *n*-pentyl β-carboline-1-propionate and picrasidine L), triterpenes (e.g. eurylene, mixture of β-sitosterol and stigmasterol, and β-sitosteryl glucoside) and others (e.g. scopoletin, fraxidin, scopolin, ρ-hydroxybenzaldehyde, syringic aldehyde, 2,4'-dihydroxy-3'-methoxyacetophenone, 2,3-dihydroxy-1-(4'-hydroxy-3'-methoxyphenyl)-propan-1-one, 3-hydroxy-1-(4'-hydroxy-3'-methoxyphenyl)propan-1-one, *threo*-1,2-bis-(4-hydroxy-3-methoxyphenyl)propane-1,3-diol, vanillic acid, protocatechuic acid, nicotinic acid, syringic acid, sodium syringate, sodium ρ-hydroxybenzoate, lariciresinol, *erythro*-1-*C*-syringylglycerol, *threo*-1-*C*-syringylglycerol, *erythro*-guaiacylglycerol, *threo*-guaiacylglycerol, iandonone, adenosine, guanosine, thymidine, alanine, proline, arginine, serine, glucose and fructose).	
Ethanol (50%)	quassinoids (e.g. eurycomanone, 13,21-dihydroeurycomanone, 13α(21)-epoxyeurycomanone, longilactone, eurycomalactone, 14,15β-dihydroxyklaineanone, eurycomanol, eurycomanol-2-*O*-β-glucopyranoside) and a canthin-6-one alkaloid (e.g. 9-methoxycanthin-6-one).]	(Chan *et al.*, 1989; Chan and Choo, 2002; Chan *et al*., 2004)

	Aqueous and Methanol	Volatile components were also detected in the aqueous and methanol root extracts, e.g. 3-methylbutanal, 1-butanol, 1-pentanol, 2-hexadecanol, acetol, nonanal, acetic acid, 2-methylhexanol, benzaldehyde, -2,3-butanediol, butyrolactone, 2-furanmethanol, 3-methylbutanoic acid, 2(5H)-furanone, curcumene, hexanoic acid, butylated hydroxytoluene, 1-(1H-pyrrol-2-yl)-ethanone, (*R*)-(−)-massoilactone, 1H-pyrrole-2-carboxaldehyde, 3-phenoxy-1-propanol, octanoic acid, [1*R*,2*S*,5*R*]-1'-[butyn-3-one-1-yl]-menthol, 2-phenoxyethanol, ethyl *p*-ethoxybenzoate, nonanoic acid, 4-ethynyl-4-hydroxy-3,5,5-trimethyl-2-cyclohex-1-enone, 2,4-bis(1,1-dimethylehtyl)phenol, diethyl phthalate, benzoic acid, 2,3,6,7-tetrahydro 4a,8a-butano-[1,4]dioxino[2,3-*b*]-1,4dioxin.	(Shafiqul *et al*., 2006)
Leaves	Ethanol	Quassinoids (lonilactone, 6-dehydro lonilactone,11-dehydroklaineanone, 12-epi dehydroklaineanone, 15β-hydroxyklaineanone, 14,15 β-dihydroxyklaineanone, and 15-β-O-acetyl-14-hydroxyklaineanone)	(Jiwajinda *et al.*, 2001)

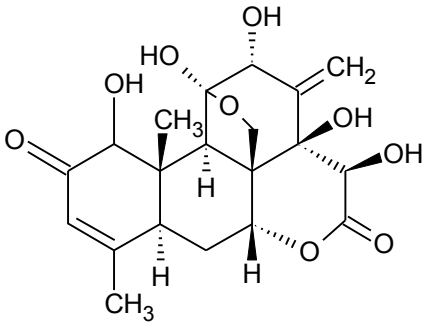


Figure 2.2: Chemical structure of Eurycomanone

2.1.5 **Review of Pharmacological Activities of *Eurycoma longifolia***

E. longifolia Jack (Tongkat Ali) is one of the most popular herbs in Malaysia. This plant is a symbol of man's ego and strength because it has been claimed by Malaysians to improve strength and power during sexual activities; it increases male virility and sexual prowess and is usually taken as a decoction of the roots in water (Gimlette and Thomson, 1977). The root has also been traditionally used for fever, afterbirth medication, boils, wounds, ulcer, syphilis, bleeding gums, ache, dysentery, glandular swelling, oedema and tonic (Gimlette and Burkill, 1930; Burkill and Haniff, 1930). The leaves of the plant have been used by traditional healers to cure malaria, ulcers, prevent gum diseases and for the treatment of sexually transmitted diseases such as syphilis and gonorrhoea (Bhat and Karim, 2010).

E. longifolia is reported to have many pharmacological activities. Antimalarial activity of *E. longifolia* has been investigated by several studies. Eurycomanone, pasakbumi B and 7-methoxy-ρ-carboline-1-propionic acid isolated from *E. longifolia* root exhibited potent anti-plasmodial activity against *P. falciparum* (Chan *et al.*, 1986). In another study, it was shown that eurycomanone, 13,21 dihydroeurycomanone, 13**α**(21)-epoxyeurycomanone, eurycomalactone, and 9-methoxycanthin-6-one isolated from 50% aqueous ethanol root extracts displayed

anti-plasmodial activity against chloroquine-resistant Gombak A isolate of *P. falciparum* with IC_{50} in the range of 0.23-1.56 µg/mL (Chan *et al*., 2004). A mixture of semi-purified root extract (13β,18-dihydroeurycomanol, eurycomanol-2-O-β-D-glucopyranoside, eurycomanol and eurycomanone) of 0.07-5.00 µg/mL demonstrated more than 50% inhibition of *P. falciparum* strain (Kuo *et al.,* 2004; Ang *et al.*, 1995). The standardized methanol extract of *E. longifolia* root at the concentration of 10, 30 and 60 mg/kg BW in combination with artemisinin given orally to mice for 3 days showed suppression of 63, 67 and 80% of *Plasmodium yoelii* infection in mice, respectively, whilst 80% suppression of *P. yoelii* infection was observed with subcutaneous treatment of *E. longifolia* (10 mg/kg BW) combined with artemisinin (Mohd *et al.,* 2007).

Cytotoxicity of *E. longifolia* has been reported in many studies. Eurycomanone isolated from *E. longifolia* roots exhibited potent cytotoxicity with IC_{50} values of 45 ± 0.15 µg/mL of hepatoma (HepG2) cells through apoptosis mechanism (Yusmazura *et al.*, 2009). In another study, Eurycomalactone, 6-dehydroxylongilactone, 9-methoxycanthin-6-one, canthin-6-one, longilactone, 14,15β-dihydroxyklaineanone, pasakbumin C and canthin-6-one 9-O-β-glucopyranoside isolated from *E. longifolia* roots exhibited potent cytotoxicity towards human lung cancer (A549) cell lines with ED_{50} in the range of <2.5 to 4.6 µg/mL, while eurycomalactone, 6-dehydroxylongilactone, 9-methoxycanthin-6-one**,** 14,15β-dihydroxyklaineanone**,** eurycomanone**,** pasakbumin B and pasakbumin C exhibited potent cytotoxicity towards human breast cancer MCF-7 cells (ED_{50} <2.5-4.5 µg/mL) (Kuo *et al.*, 2004). 9-methoxycanthin-6-one, 9-methoxycanthin-6-one-N-oxide, 9-hydroxycanthin-6-one and 9-hydroxycanthin-6-one-N-oxide isolated from

E. longifolia roots exhibited cytotoxic effects against human cancer cell types including breast, colon, fibrosarcoma, lung, melanoma, KB and murine lymphocytic leukemia (P-388) with ED_{50} in the range of 1.4 to 11.7 µg/mL. Eurycomanone was active against all cells above including vincristine-resistant KB cells (ED_{50} 0.2-11.3 µg/mL), except P-388 cells (Kardono *et al.*, 1991). A different study reported that eurycomanone, 21-dihydroeurycomanone and 14,15β-dihydroxyklaineanone from *E. longifolia* roots showed a potent cytotoxic effect against KB cells with IC_{50} values of 0.98, 0.81 and 0.96 µM, respectively (Chan *et al.*, 2004).

Many studies reported the effect of aphrodisiac activity in *E. longifolia*. Aqueous extract of *E. longifolia* root (30, 60, 90, 150 mg/kg) given via oral gavage to Sprague Dawley male rats for 28 days enhanced sexual activities and sperm quality (sperm count, motility, morphology and viability) of the treated rats. In another study, chloroform, methanol, butanol and water fractions of *E. longifolia* root given orally to 3-4 month old male Sprague Dawley albino rats for 10 days showed a dose-dependent increase in mounting frequency from 5.3 (400 mg/kg) to 5.4 (800 mg/kg), 4.9 to 5.4, 4.8 to 5.2 and 5.2 to 5.3, respectively (Ang and Sim, 1997). These fractions (500 mg/kg) contributed towards sexual motivation activity in adult, middle-aged male albino mice and in retired breeders (Ang *et al*., 2003a), as well as enhanced sexual qualities of middle-aged male rats (Ang *et al*., 2003b). Different studies reported that methanol, chloroform, water and butanol fractions of *E. longifolia* root (800 mg/kg) given via oral gavage to 9-month old Sprague Dawley rats for 10 days showed changes in sexual behaviour such as increased orientation activities towards the receptive female rats (anogenital sniffing, licking and mounting), increased genital grooming towards themselves and restricted movements

to a particular area of the cage (Ang and Lee, 2002). The fractions (800 mg/kg) were also found to promote sexual arousal in sexually sluggish old male rats (Ang *et al.*, 2004). Additionally, these fractions (800 mg/kg) given orally for 12 weeks to testosterone-stimulated castrated intact male rats revealed pro-androgenic property as evidenced by the enhanced growth of the laevator ani muscle (Ang and Cheang, 2001).

Anti-diabetic property of *E.longifolia* was reported. In this study, antihyperglycaemic activity of aqueous extract of *E. longifolia* root (150 mg/kg body weight) was observed in streptozotocin-induced hyperglycaemic Sprague Dawley rats after 10 days of treatment (Husen *et al*., 2004).

Anxiolytic activity effect of *E. longifolia* was investigated. The result showed that chloroform, methanol, butanol and water fractions of *E. longifolia* root (0.3 g/kg) was administered by oral gavage to inbred adult albino mice (35-40 g) exerted anxiolytic effect (Ang and Cheang, 1999).

The effect of *E.longifolia* on anti-osteoporosis was investigated. In this study, supplementation of *E. longifolia* root aqueous extract given to 12-month old orchidectomised Sprague Dawley rats for 6 weeks showed maintenance of bone calcium level (Shuid *et al*., 2011).

2.2 Stability Studies

Stability is defined as the time during which a drug to maintains its chemical integrity and labelled potency within the certain limits. The stability of a pharmaceutical preparation is its degree of resistance to chemical and physical changes. Products should be consistent in its effectiveness and claimed potency or may change only within the limits set by the legal provisions until the expiration date (Racz, 1989). Stability is one of the most important factors that determine whether a compound or mixture of compounds can be developed into a pharmaceutical product. Stability studies provide evidences on how the quality of a drug substance varies with the passage of time under the influence of environmental factors (WHO, 1996). Stability studies before developing a dosage form are the first quantitative assessment of chemical stability of a new product and its possible formulation. Stability studies are used to recommend storage conditions and predict shelf life of medicinal products.

The stability studies are important for three main reasons. Firstly, it is for the patient safety by making sure that the patient receives a uniform dose of drug throughout the whole shelf life of the product. Even though a drug may have been shown to be safe for use, this is not necessarily true for the decomposition products. Secondly, consideration must be given to the relevant legal requirements concerned on the identity, strength, purity and quality of the drug. Finally, stability study is important to prevent the economic impact of marketing an unstable product. The stability of a product is influenced by a number of factors, which may be classified into two main categories, physical and chemical factors.

2.2.1 Physical Factors Effecting Stability

2.2.1.1 Temperature

Temperature increases the rate of degradation of active ingredients due to the increase in kinetic energy, which increases the number of colliding molecules. The decomposition normally increases by two to three times for every 10°C temperature rises. Therefore, it is important to be aware of it while keeping raw material and in production of a product especially when heating is required. It is important especially in the case of thermo labile and volatile constituents.

2.2.1.2 Moisture

Moisture content increases the rate of decomposition and makes the product susceptible to hydrolysis. For raw herbal powders or extract, it allows the growth of microbes that not only deteriorate the constituents but can produce toxic substances.

2.2.1.3 Light

Sunlight as an energy form can initiate and accelerates decomposition. Photo labile constituents of extracts deteriorate on expose to sunlight (Rawlins, 1977; Pugh, 2002).

2.2.2 Chemicals Factors Effecting Stability

The constituents of extracts also undergo chemical degradation over the time. The chemical degradation is further classified as follows:

2.2.2.1 Hydrolysis

Hydrolysis, reaction of the substance with water is the most common cause of drug degradation (Waterman *et al*., 2002). It is considered as the main cause of deterioration of drugs, especially for aqueous-based preparations.

2.2.2.2 Oxidation

Oxidation is defined as the removal of an electropositive atom, radical or electron, or addition of an electronegative atom, radical or electron. Decomposition of pharmaceutical preparation due to oxidation is nearly as probable as that of hydrolysis. The rate of oxidation is depends on the temperature. For example, peroxidation of fatty acids, break down of fatty acids into aldehydes and ketones, accelerates as the temperatures exceeds 50°C (Rawlins, 1977).

2.2.2.3 Isomerisation and Polymerization

Polymerization is defined as the addition of similar molecules whereas isomerisation is the change in isomeric forms. These phenomena change the biological activity of compounds.

2.2.3 Accelerated Stability

The stability study of pharmaceuticals performed at exaggerated conditions is called accelerated stability. The accelerated stability study is performed for fast prediction of stability. Degradation is usually slow at room temperature and shelf life may go up to several years. Stability testing for this type will be time consuming and expensive since the period can be as long as two years. Therefore, accelerated stability testing is devised for rapid prediction of long term stability of a product. The stability of drugs at room temperature is the main purpose of this study but it would take a long time to conduct. Therefore, stability studies are conducted at elevated temperatures and the data is evaluated by applying Arrhenius equation (Sungthongjeen, 2004).

An estimation of shelf life (t_{90}), a time in which a product retains 90% of its original potency, may be made by accelerating the decomposition process and extrapolating the results to normal storage conditions. Therefore, stability tests are often conducted under elevated temperatures to accelerate the degradation process in order to get a fast stability prediction. Application of the principles of chemical kinetics on the results of accelerated stability tests enables us to make prediction and estimate the shelf life of a product at normal temperature.

2.3 Saponins

2.3.1 Definition and Chemical Compositions

'Saponin' name is derived from the Latin word sapo, which means 'soap', because saponin molecules form a soap- like foams when shaken with water (Vincken *et al*., 2007). Saponins are found in great number of plant species and usually found in roots, tubers, leaves, blooms or seeds. Saponins can be classified into two groups based on the nature of their aglycone skeleton. The first group consists of the steroidal saponins and the second group consists of the triterpenoid saponins. Steroidal saponins consist of a steroidal aglycone, a C27 spirostane skeleton, generally comprising of a six-ring structure (Figure 1.1 (a)). Triterpenoid saponins consist of a triterpenoid aglycone, which consists of a C30 skeleton, comprising of a pentacyclic structure (Figure 1.1 (b)) (Sparg *et al*., 2004).

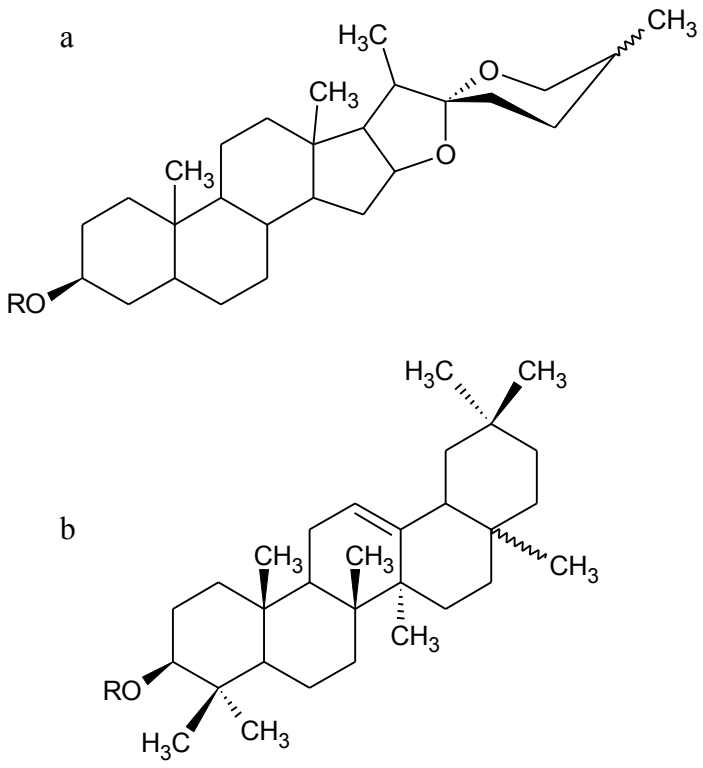


Figure 2.3: Aglycone skeletons of (a) steroidal spirostane and (b) triterpenoid saponins. R = sugar moiety

2.3.2 A Review of the Pharmacological Activities of Saponins

Pharmacological activities of saponins have been reported in several studies. Haemolytic activity in a mixture of saponins isolated from *Maesa lanceolata* Forssk. (Myrsinaceae) was reported. In this study, the maesasaponin mixture showed very high haemolytic activity, haemolysing 50% of the human erythrocytes (1% suspension in phosphate buffer saline) at a concentration of 1.6 µg/mL (Sindambiwe *et al*., 1998).

The Molluscicidal activity of saponins has been reported in many studies. A six-oleanane-type triterpenoid saponin mixture (maesasaponin mixture, isolated from *Maesa lanceolata*) was tested for molluscicidal activity against *Biomphalaria glabrata* snails. The saponin mixture showed high toxicity, with LD95 and LD50 values of 4.1 and 2.3 µg/mL, respectively (Sindambiwe *et al.*, 1998). In another

study, the biodegradability of molluscicidal water-extracted saponins from the berries of *Phytolacca dodecandra* was reported. From the study, the saponins in an aqueous extract of *Phytolacca dodecandra* readily biodegraded ($t1/2$ = 15.8 h) when consumed within 10 days in aquatic environments under aerobic conditions. As a result, the use of *Phytolacca dodecandra* berries for snail control in schistosomiasis infested water bodies are environmentally acceptable (Mølgaard *et al*., 2000). One of the ten isolated saponins from the leaves of *Maesa lanceolata* showed molluscicidal activity against *Biomphalaria glabrata* snails. Maesasaponin VI2 is responsible for a large part of the activity of the mixture. This saponin had a LC50 value of 0.5 mg/mL in its isolated form (Apers *et al.*, 2001).

The effect of anti-inflammatory activity of saponins has been reported by several studies. Fruticesaponin B, a bidesmosidic saponin with an unbranched saccharide moiety isolated from *Bupleurum fruticescens* L. (Apiaceae), was shown to have the highest anti inflammatory activity of the all the saponins tested in the mouse oedema assays (Just *et al*., 1998). Saponins isolated from *Bupleurum rotundifolium* L. (Apiaceae) were reported to have anti-inflammatory activity against both 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced ear oedema and chronic skin inflammation (Navarro *et al.*, 2001) Kalopanaxsaponin A and pictoside A, both two isolated triterpenoid saponins from the stem bark of *Kalopanax pictus* Nakai (Araliaceae) showed significant anti-inflammatory activity at the oral dose of 50 mg/mL (Li *et al*., 2002).