THE EFFECTS OF *EURYCOMA LONGIFOLIA* EXTRACT AND EURYCOMANONE ON THE STEROIDOGENESIS IN MOUSE LEYDIG CELLS

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by

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DEDICATIONS

This thesis is dedicated to my beloved parents, Khurshid Ahmed B. Ayoob and Noridah Bt Pakwanchee who have always been a great source of inspiration and motivation.

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LIST OF ABBREVIATIONS

17β-HSD	17β-hydroxysteroid dehydrogenase
1-D	One-dimensional
2-D NMR	Two-dimensional nuclear magnetic resonance spectroscopy
7-MCPA	7-methoxy-(9H-β-carbolin-1-yl)-(E)-1-propenoic acid
A549	Lung cancer cells
ABP	Androgen-binding protein
ABTS	2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
ALC	Adult Leydig cells
ANOVA	One-way analysis of variance
ANX1	Annexin 1
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CYP11A	Cholesterol side-chain cleavage enzyme
CYP17	17α-hydroxylase
ddH2O	Double distilled water
DHEA	Dehydroepiandrosterone
DHY	13, 21-dihydroeurycomanone
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E. longifolia	*Eurycoma longifolia*
EL	Eurycomanol
ELISA	Enzyme-linked immunosorbent assay

EN	Eurycomanone
EP	13α (21)-epoxyeurycomanone
ERp28	Endoplasmic reticulum protein 28
F2	Standardised E. longifolia extract
FBS	Fetal bovine serum
FM	Formestane
FSH	Follicle stimulating hormones
GnRH	Gonadotropin-releasing hormone
hCG	Human chorionic gonadotropin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hnRNP	Heterogeneous nuclear ribonucleoprotein
HRP	Horseradish peroxidase
HS	Horse serum
IgG	Immunoglobulin G
ILC	Immature Leydig cells
KCl	Potassium chloride
LH	Luteinizing hormone
mAb	Monoclonal antibody
MCF-7	Breast cancer cells
MDBK	Normal kidney cell line
mRNA	Messenger Ribonucleic Acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na2CO3	Sodium carbonate
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide

NADPH	Nicotinamide adenine dinucleotide phosphate
NaHCO3	Sodium bicarbonate
NMR	Nuclear magnetic resonance
OD	Optical density
P. falciparum	*Plasmodium falciparum*
PBS	Phosphate buffer saline
Pen/Strep	Penicillin/Streptomycin
pH	Potential of hydrogen
PHB	Prohibitin
PI	Propidium iodide
PLC	Progenitor Leydig cells
p-value	Probability value
RIPA	Radioimmunoprecipitation assay
SD	Standard deviation
SER	Smooth endoplasmic reticulum
SHBG	Sex hormone binding globulin
SQ40	Standardised E. Longifolia extract
StAR	Steroidogenic acute regulatory protein
TA164	*E. longifolia* root standardized extract
TAF273	*E. longifolia* root methanolic extract
TM3	Leydig cell

LIST OF SYMBOLS

°C	Degree Celsius
g	Gram
kg	Kilogram
L	Litre
M	Molar
mg	Miligram
min	Minutes
mL	Millilitre
mM	MilliMolar
mm2	Square millimetre
ng	Nanogram
nm	Nanometre
pg	Picogram
rpm	Revolutions per minute
α	Alpha
β	Beta

KESAN EKSTRAK *EURYCOMA LONGIFOLIA* DAN EURIKOMANON PADA STEROIDOGENESIS DALAM SEL LEYDIG MENCIT

ABSTRAK

Eurycoma longifolia Jack (Tongkat Ali) adalah salah satu tumbuhan tradisional yang paling popular di hutan hujan tropika Asia Tenggara, terutamanya di Malaysia dan Indonesia. Walaupun *E. longifolia* telah digunakan secara meluas sebagai perubatan tradisional, hanya sedikit pengetahuan yang difahami tentang kesannya terhadap sel-sel Leydig iaitu sel-sel yang menghasilkan testosteron. Oleh itu, kesan ekstrak *E. longifolia* (F2) terpiawai dan sebatian tulennya (eurikomanon, EN) terhadap penghasilan testosteron, estrogen dan aromatase dalam sel-sel Leydig tikus telah dikaji di mana Formestan (FM) digunakan sebagai kawalan positif dalam kajian ini. Kesan F2, EN dan FM terhadap kemandirian sel-sel telah ditentukan terlebih dahulu dengan menggunakan ujian MTT. Kitaran sel-sel Leydig yang dirawat kemudiannya menjalani pewarnaan dengan Propidium iodide (PI) sebelum dianalisis secara Sitometri-Aliran. Tahap testosteron, estrogen dan aromatase dalam sel-sel Leydig yang dirawat kemudiannya ditentukan secara ELISA. Akhirnya, aktiviti enzim aromatase disaring dengan menggunakan kit saringan rencatan daya pemprosesan tinggi. Kajian ini mendapati bahawa kesan rencatan pertumbuhan meningkat secara dos-berkeperluan dalam sel Leydig yang dirawat dengan F2. Walau bagaimanapun, fenomena ini tidak dilihat dalam sel-sel yang dirawat dengan EN dan FM melainkan kepekatan yang tinggi digunakan. Analisis kitaran sel menunjukkan bahawa kemandirian sel yang dirawat dengan F2 berkurangan kerana kitaran sel-sel terbantut/terhenti pada fasa S pada 96 jam rawatan. Penghentian kitaran sel-sel yang jelas pada fasa S juga dapat diperhatikan pada sel-sel yang dirawat dengan EN pada

rawatan 24 jam dan 48 jam . Walau bagaimanapun, sel-sel yang terencat telah berkurang pada 72 jam dan 96 jam rawatan. Sementara itu, sel-sel yang dirawat FM tidak menunjukkan perencatan kitaran sel yang signifikan. ELISA membuktikan bahawa sel-sel Leydig yang dirawat dengan F2, EN dan FM meningkatkan paras testosteron, mungkin disebabkan oleh pengurangan penukaran testosteron kepada estrogen. Penambahan hCG di dalam medium pertumbuhan telah merangsang proses ini di mana peningkatan pengumpulan testosteron di dalam medium dapat diperhatikan seawal 48 jam rawatan. Hasil lanjut menunjukkan bahawa ekspresi gen aromatase telah direncat dalam sel-sel yang dirawat dengan F2 dan FM. Hal ini mungkin menyebabkan pengurangan kemandirian sel-sel memandangkan ekspresi gen aromatase tidak dipengaruhi oleh sel yang dirawat dengan EN. Hasil kajian menunjukkan terdapat perencatan yang jelas apabila EN dan FM digunakan sebagai sebatian ujian. Bagaimanapun, F2 tidak menunjukkan sebarang kesan rencatan terhadap akitiviti enzim aromatase, oleh itu dapat disimpulkan bahawa ekspresi enzim aromatase yang lebih rendah berkemungkinan disebabkan oleh jalur penghasilan steroid yang lain seperti enzim 5α-reduktase. Hasil kajian ini telah meningkatkan pemahaman bahawa ekstrak piawai *E. longifolia*, F2 dan sebatian tulen, EN mungkin berguna untuk kajian masa hadapan dalam merawat masalah kesihatan manusia, seperti ketidaksuburan.

THE EFFECTS OF *EURYCOMA LONGIFOLIA* EXTRACT AND EURYCOMANONE ON THE STEROIDOGENESIS IN MOUSE LEYDIG CELLS

ABSTRACT

Eurycoma longifolia Jack (Tongkat Ali) is one of the most popular traditional plants found in the tropical rainforest of Southeast Asia, especially in Malaysia and Indonesia. Although *E. longifolia* is widely used as traditional medicine, little is understood of the *in vitro* effect on Leydig cells; the testosterone-producing cells. Therefore, the effect of a standardised *E. longifolia* extract (F2) and pure compound, eurycomanone (EN) on the productions of testosterone, estrogen and aromatase in mouse Leydig cells were determined in this study, whereby the Formestane (FM) was used as a positive control. The effect of F2, EN and FM on the cell viability was first determined using MTT assay. The cell cycle of the treated Leydig cells was then subjected to Propidium iodide (PI) staining prior analysed by Flow Cytometry. The levels of testosterone, estrogen and aromatase in treated Leydig cells were then determined by ELISA. Finally, the aromatase enzyme activity was screened using a high throughput inhibitor screening kit. The study found that the growth inhibitory effect increased in a dose-dependent manner in the Leydig cells treated with F2. However, this phenomenon was not observed in EN- and FM-treated cells despite when higher concentrations were used. Cell cycle analysis revealed that F2-treated cells reduced viability due to the cell cycle arrest at S phase within 96 hours treatment. A significant S phase cell cycle arrest at 24 hours and 48 hours treatments could also be observed in EN-treated cells. However, the arrested cells were reduced at 72 hours and 96 hours treatments. Meanwhile, FM-treated cells did not show any significant

cell cycle arrest. The ELISA revealed that F2-, EN- and FM-treated Leydig cells increased the level of testosterone may be due to the reduction of the conversion of testosterone to estrogen. The hCG supplemented growth medium enhanced this process, whereby the increased testosterone accumulation in conditioned medium was observed as early as at 48 hours treatment. Further results showed the aromatase gene expression was inhibited in the F2- and FM-treated cells. This may be attributed to the cell viability reduction as the aromatase gene expression was not affected by the EN treatment. The results showed a clear inhibition when EN and FM were used as the test compounds. However, F2 did not show any promising inhibitory effect on the aromatase enzyme activity, which concludes that the lower aromatase enzyme expression might be due to other steroidogenesis pathways, such as by 5α-reductase enzyme. The results have contributed to the understanding that the standardized *E. longifolia* extract, F2 and pure compound, EN might be useful for future study in treating human health problems, such as infertility.

CHAPTER ONE

INTRODUCTION

1.0 General introduction

Malaysia is categorized as a mega biodiversity-rich country (Ang, 2004) that is blessed with the abundance of valuable herbal plants. Herbal medicine can be defined as an alternative approach to anyone who uses these herbaceous remedies to treat the sick (Kamboj, 2000). The herbal medicine from local plants has been traditionally employed decades ago by our ancestors. This medicine is getting worldwide attention due to the neutraceutical values and persistent beneficial role in human health (Graham et al., 2000). Apart from that, the medicinal plants are believed as safe to use and are not prone to cause dependency or addiction as compared to the synthetic drugs (Olaku & White, 2011).

Eurycoma longifolia Jack or locally known as Tongkat Ali is one of the most popular herbal plants that have been used extensively in Southeast Asia (Jaganath & Ng, 2000). Besides, the plants have also contributed as a complementary and alternative herbal therapy medicine in the Western countries. *E. longifolia* is the most well-known herbs for their aphrodisiacs property and the ability to reduce infertility or erectile dysfunction (Bhat & Karim, 2010). The root of the plant is very valuable, where it is widely used for treating fever, aches and glandular swelling (Darise et al., 1982). Normally, the old folks boil the chipped roots and take it as a decoction or sometimes mix it with honey or dates to reduce the taste of bitterness. Currently, *E. longifolia* are supplied in the form of crude powder, in a capsule mixed with other herbs or even as an additive in coffee or tea.

Infertility is generally defined as incompetency of a couple to reproduce after unprotected sexual intercourse for a year (Prakash, 2007). Infertility is a global issue affecting one in ten couples that usually due to the problem of the male partner (Burns & Covington, 2006). The main factor leading to male infertility is the lifestyle, which includes heavy electronic devices usage, alcohol consumption, horse riding and wearing close-fitting attire. Improper diet and a stressful working environment could also lead to infertility (Sharpe & Franks, 2002). Currently, many advanced therapeutic approaches and technologies are used for treating infertility, such as *in vitro* fertilization (IVF), intra-cytoplasmic sperm injection (ICSI) and hormonal therapy. However, these methods are very costly and time-consuming, which eventually leads to socio-economic problems because not everyone can afford it (Katz et al., 2002). Hence, the studies on herbal medicines, such as *E. longifolia* that is able to increase the testosterone level, and hence improve male sexual libido have been done extensively. This is to increase the consumption of the herbs through scientific proofs and to overcome the current problem, as well as to reduce male infertility.

In this study, a standardised *E. longifolia* extract (F2) was used in order to investigate the effects of the plant as a testosterone booster, and hence combating male infertility problem. The effects of the extract were then compared with the pure *E. longifolia* compound; eurycomanone (EN) and a known aromatase inhibitor; formestane (FM). The standardised extract F2 consists of four major compounds, which includes eurycomanone (EN), 13α (21)-epoxyeurycomanone (EP) and 13, 21-dihydroeurycomanone (DHY) and eurycomanol (EL). These methanolic root extracts of *E. longifolia*, F2 and EN were provided by Prof. Dr. Chan Kit Lam from the School of Pharmaceutical Sciences, USM. The investigation of the effects of F2 and EN was

carried out as below. Firstly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done to identify the cytotoxic effect of F2 and EN on the primary testosterone producing cell, the Leydig cell (TM3). Next, the cell cycle analysis was done using Flow Cytometry to detect the phases of arrested Leydig cells in the cell cycle post-treatment with F2 and EN.

Moving to the levels of testosterone and estrogen released from the F2 and EN-treated Leydig cell into the conditioned media, the investigation was checked using enzyme-linked immunosorbent assay (ELISA). Human chorionic gonadotropin (hCG) was incorporated into the treatment media during treatments as it mimics the role of Luteinizing hormone (LH), which triggers the Leydig cells to secretes testosterone (Chen et al., 1994). The expression level of aromatase that is responsible for converting testosterone to estrogen in the F2 and EN-treated Leydig cells was also observed using ELISA. Finally, the aromatase inhibition activity was detected by screening the effect of F2, in comparison to EN and FM using CYP19/MFC high throughput inhibitor screening kit.

1.1 Problem Statement

Enhanced testosterone level and spermatogenetic effect by *E. longifolia* are mainly due to the presence of quassinoids in the plant extracts (Talbott et al., 2013). Quassinoid is a biological compound that contributes to *E. longifolia* as the testosterone production booster, whereby the extracts enriched by quassinoid in *E. longifolia* is more effective as the booster. The effects of *E. longifolia* extracts have been determined in the animal model previously. However, despite the preliminary effect of quassinoid-rich *E. longifolia* was determined in the rat interstitial cells (Low et al., 2013a), the *in vitro* effects of *E. longifolia* extracts have not been investigated at the molecular level in depth previously especially using Leydig cells. Therefore, it is important to determine the effects of the extract isolated from *E. longifolia* root, F2, in comparison of the effects with the pure *E. longifolia* compound (EN) and the aromatase inhibitor (FM) using mouse Leydig cells. It is crucial to investigate the mechanisms and actions of the extract to further identify the potency of *E. longifolia* for human use.

1.2 Objectives

This study aimed to determine the *in vitro* effects of extracts from the root of *E. longifolia*, F2 and EN using mouse Leydig cells. The specific objectives of this study are:

1. To determine the inhibitory effects of F2 and EN on the viability of Leydig cell using MTT assay.
2. To detect the phases of the cell cycle that were arrested in the Leydig cells post F2 and EN treatment using flow cytometry.
3. To investigate the effects of F2 and EN on the production of testosterone and estrogen, as well as the expression of aromatase in Leydig cells using ELISA.
4. To study the potential of F2 and EN on the aromatase activity inhibition using an inhibitor screening kit.

The aromatase inhibitor (FM) were used as the controls for the investigation. The effects of F2 and EN were then compared with FM in Leydig cells in this study.

CHAPTER TWO

LITERATURE REVIEW

2.1 The *Eurycoma longifolia* Jack

2.1.1 The characteristics of *E. longifolia*

Eurycoma longifolia Jack is a plant belongs to the family Simaroubaceae. The plant is widely known as 'Tongkat Ali' in Malaysia, 'Pasakbumi' in Indonesia, 'Cay ba binh' in Vietnam and 'Ian-don' in Thailand (Meng et al., 2014). According to Puri (2011), the Genus *Eurycoma* consists of almost 200 species as a whole. The plants are found in tropical rainforests throughout Southeast Asia especially in Malaysia and Indonesia. The plants can also be found in Thailand, Vietnam, Laos and Philippines to a lesser extent. *E. longifolia* is well known as Malaysian ginseng (Zanoli et al., 2009) or national treasure (Mohamed et al., 2015) and hence it is the focus of our present study.

E. longifolia is a slow growing, an evergreen herbal tree that is able to grow in poor nutrients soil, but better in proper shades and sunlight. According to Puri (2011), the plant grows slender up until 12-15 m high (Figure 2.1). *E. longifolia* bears green coloured ovoid fruits, which will gradually turn to dark red upon ripening after 2-3 years of cultivation. The complete maturation of this plant may take up to 25 years, however, the roots are mostly harvested after 4 years of cultivation for commercial purposes. The leaves are roughly 5-20 cm long, pinnated and spirally arranged. Each leaves consist of numerous obovate-lanceolate leaflets. This dioecious plant born male and female flowers on different trees (Bhat & Karim, 2010). The reddish flowers are tiny and densely arranged. Harvesting of Tongkat Ali is highly restricted since they

have been declared as a protected plant in Malaysia. Currently, *E. longifolia* root extracts are commercialized by mixing them with tea, coffee and carbonated beverages as an alternative to improve general health.

Figure 2.1: The *E. longifolia* tree is growing rapidly in the natural habitat at the main campus of Universiti Sains Malaysia (Bhat & Karim, 2010).

2.1.2 The compounds isolated from *E. longifolia* extract

The effectiveness of *E. longifolia* is mainly due to the presence of various bioactive constituents in the roots, stems, leaves and even bark of the plant. The plant mainly contains quassinoids, quassinoid diterpenoids, tirucallene-type triterpenes, squalene derivatives, biphenylneolignans, anthraquinones, canthinone alkaloids and dimeric dihydrobenzofuran (Teh et al., 2010). Quassinoids, the most common bioactive compounds found indigenously in the plant are naturally occurring degraded triterpenes.

Four quassinoids; pasakbumin-A, -B, -C, and -D from *E. longifolia* were separated and identified by Tada et al. (1991). The authors eventually found that pasakbumin-A (eurycomanone) and pasakbumin-B are actually responsible for exhibiting potent anti-ulcer activity. A few quassinoids were isolated from the leaves of *E. longifolia*, which includes lonilactone, 6-dehydrolonilactone, 11-dehydroklaineanone, 12-epi-dehydroklaineanone, 15β-hydroxyklaineanone, 14,15β-dihydroxyklaineanone, and 15-β-O-acetyl-14-hydroxyklaineanone (Jiwajinda et al., 2001). These compounds were reported to possess anti-tumor effects and anti-parasitic activities.

Kuo et al. (2004) reported the isolation of nearly 65 compounds from the roots of *E. longifolia*. The structures of the compounds have also been determined by extensive spectroscopic methods, including one-dimensional (1-D) and two-dimensional nuclear magnetic resonance spectroscopy (2-D NMR), apart from mass spectral data. For the first time, the authors identified 4 quassinoids diterpenoids

obtained from natural sources, which includes eurycomalideA, eurycomalide B, 5α-14β, 15β-trihydroxyklaineanone and 13β, 21-dihydroxyeurycomanol.

Park et al. (2014) identified 5 new quassinoids; eurylactone E, eurylactone F, eurylactone G, eurycomalide D and eurycomalide E together with 10 known quassinoids, which were isolated from the roots of *E. longifolia* and characterized the compounds as above approaches.

Rehman et al. (2016) classified the different type of quassinoids based on their number of carbon atoms forming their basic skeleton ($C_{18,}$ C_{19} and C_{20}). Laurycolactone A, laurycolactone B, eurycolactone B and eurycolactone D are categorized as $C_{18.}$ On the other hand, 6α-hydroxyeurycomalactone, 7α-hydroxyeurycomalactone, eurycomalide A and eurycolactone E are a few examples of quassinoids under the C_{19} category. These C-19 quassinoids portrays a wide range of biological activities, such as anti-plasmodial, anti-malarial, anti-leukemic, anti-tubercular and anti-viral. Finally, C_{20} quassinoids are the largest extent and gaining serious attention. They include eurycomanone, eurycomanol and 13α (21)-dihydroeurycomanone.

Using the wood of *E. longifolia*, Morita et al. (1992) isolated different biphenyl neolignans. This includes the novel isomeric 2,2'-dimethoxy-4'-(-3-hydroxy-1-propenyl)-4-(1,2,3-trihydroxypropyl) diphenyl ethers and another 2 novel biphenyls, 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.

Meanwhile, an isolation of 5 new canthin-6-one alkaloids were done by Mitsunaga et al. (1994) from the bark and wood of *E. longifolia*. The isolated compounds includes, 10-hydroxy-9-methoxycanthin-6-one, 11-hydroxy-10-methoxycanthin-6-one, 5,9-dimethoxycanthin-6-one and 9-methoxy-3-methylcanthin-5,6-dione, 9,10-dimethoxycanthin-6-one.

2.1.3 The aphrodisiacs and anti-infertility activities of *E. longifolia*

The plant is widely used as traditional medicine to treat various diseases in South-East Asia, and has been scientifically proven to possess medicinal and healing properties especially in enhancing sexual prowess. A research was conducted by Ang & Sim (1998) for evaluating the effects of *E. longifolia* root extracts on sexually naive male rats. In the process, different fractions of extracts (chloroform, water, butanol) were orally administered at different doses (200 mg/kg, 400 mg/kg and 800 mg/kg body weight). The authors revealed that the sexual performance of the treated animals increased in a dose-dependent manner. Besides, an improvement in mounting, intromission and ejaculation were also reported evidencing *E. longifolia* as a potential sexual stimulator.

The administration of different *E. longifolia* fractions on middle-aged male rats changes their sexual behaviour (orientation activities) towards receptive females. The changes includes anogenital sniffing, licking and mounting apart from an increment in genital grooming towards themselves. However, a reduced attraction towards the external environment, inclusive of climbing and raring was also observed (Ang & Lee, 2002). By using sexually stagnant old male rats, the effects of *E. longifolia* various fractions of extracts (methanol, butanol l, chloroform and water) were tested by Ang

et al. (2004). The tests were done on the rat's sexual arousal by observing the act of yawning and stretching. The result revealed an increase in yawning and stretching especially when the highest dose of extract (800 mg/kg) was used.

An *in vivo* study was conducted by Chan et al. (2009), to investigate the efficacy of *E. longifolia* standardized methanol extract with major quassinoid contents, 13α, 21-epoxyeurycomanone, 13α, 21-dihydroeurycomanone, eurycomanone and eurycomanol on infertile rats. The authors concluded that there was a significant increase in sperm count, morphology and plasma testosterone level in a dose dependent-manner. The results suggest that *E. longifolia* is crucial to enhance male fertility.

Since most of the study conducted on *E. longifolia* focused on rodents, Tambi & Imran (2009) used the standardized, water-soluble *E. longifolia* root extract on humans. The study investigated the semen volumes, sperm concentration, normal sperm morphology percentage and sperm motility of 75 sub-fertile male patients after given 200 mg of *E. longifolia* water extract daily for 3 months. An improvement in all of the semen parameters was observed, including the sperm quality, which allows 14.7% of spontaneous pregnancy. A total of 76 patients with late-onset hypogonadism were administered with 200 mg of a standardised water-soluble *E. longifolia* extract for 1 month. After the test period, the patients showed improved sign of ageing male symptoms and apparently, even the serum testosterone level increased (Tambi et al., 2012).

Low et al. (2013a) carried out an investigation on the effects of standardized *E. longifolia* bioactive fraction and its chemical contents on male fertility and the involved mechanism of action. The finding revealed that the sperm concentration of the male rats administered with *E. longifolia* extract was increased. Apart from that, the authors observed an elevation of the spermatocytes number, round spermatids and Leydig cells as compared to the control. When treated with 25 mg/kg of the extract, the rat's testicular testosterone hiked up while the estrogen level was significantly lower. It was also concluded that quassinoids (eurycomanone and 13α, 21-dihydroeurycomanone) increased the testosterone level of Leydig cells.

A recent study by Ebrahimi et al. (2016) observed the effectiveness of different quassinoid levels in *E. longifolia* extract on rats sperm count. The urinary metabolic changes post-treatment was evaluated as well. In the process, 24 male Sprague-Dawley rats were orally administered with different concentrations of extracts at the duration of 48 days. Post-treatment, NMR analysis of the urine samples was done and the sperm count was analysed. The result showed that rats given quassinoid-rich extract have higher sperm concentration compared to rats given the quassinoid-poor aqueous extract.

Apart from treating male infertility, Abdul-Ghani et al. (2012) proved that *E. longifolia* extract can also be used to treat female sexual disorders. The effect of *E. longifolia* extract on two reproductive disorders in female rats, which were the ovarian cystic follicles and irregular estrous cycle, were examined by first treating the rats with a hormone that is responsible in inducing reproductive disorder. Post-treatment, the

result clearly showed a reversal effect where the number of rats showed the disorder was reduced.

2.1.4 Anti-malarial and anti-cancer properties of *E. longifolia*

Malaria is well-known as one of the most deadly diseases in the world with over 2 million death per year. Moreover, the infesting *Plasmodium falciparum* has developed a resistance against chloroquine treatment, which has been extensively used previously (Miller et al., 1986). Therefore, an alternative research was taken using *E. longifolia* extracts considering the problem. Using *E. longifolia* that is traditionally used as medicine in Vietnam, Nguyen-Pouplin et al. (2007) and Le et al. (2003), the studies discovered that the extract portrayed a high anti-malarial activity and the potential to inhibit the growth of chloroquine-resistant *P. falciparum*.

Besides, a study was conducted by Chan et al. (1986) to test the efficacy of *E. longifolia* extract on anti-plasmodial activity against K-1, a Thailand strain multi-drug resistant to *P. falciparum*. The study was carried out under *in vitro* condition and the isolation of 10-hydroxycanthin-6-one, eurycomalactone, eurycomanone and eurycomanol from the plant showed anti-malarial activity. Replacing the culture medium containing quassinoids-rich *E. longifolia* extract daily against 6 isolates of *P. falciparum* revealed a complete inhibition as early as 3 days post-treatment (Ang et al., 1995). By using *in vivo* method, Mohd et al. (2007) evaluated the anti-malarial property of *E. longifolia* root standardized extract (TA164), and revealed that the TA164 combined with artemisinin repressed the *Plasmodium yoelii* infection in the experimental mice.

On the other hand, cancer is also another leading disease that causes death day by day; more cases are being reported every day. Plant-derived cancer medicine should be cytotoxic towards cancer cells, which will eventually cause cancer cell death (Rehman et al., 2016) and mild adverse effect on healthy cells. Eurylene, a new squalene-type triterpene that was isolated from *E. longifolia* by Itokawa et al. (1991), was found toxic towards cancer cells. In another study, Okano et al. (1995) investigated the anti-cancer promoting activity of quassinoids and revealed more than 50% inhibitory effect through a methylenedioxy bridge with a side chain enhancing the activity. On the other hand, Chuen & Azimahtol (2004) identified that semi-purified eurycomanone expressed cytotoxic activity towards MCF-7 breast cancer cells. Another study by Nurhanan et al. (2005) used the different type of extracts (methanol, n-butanol, chloroform and water) to identify their effects against various cancer cells. Eventually, the extracts were cytotoxic towards all of the cell lines, excluding a normal MDBK kidney cell line.

2.1.5 The common benefits of *E. longifolia*

The root, bark and stem of *E. longifolia* are used as traditional medicine locally (Meng et al., 2014). All parts of this plant particularly the roots have been extensively used since a long time to treat different illness, such as fever, intestinal worms, mouth ulcers and headache (Perry & Metzger, 1980). The roots of the plant are also used by older individuals as traditional 'anti-aging' remedy to improve the energy, mood and libido reduction problems that often comes with age (Zhari et al., 1999; Hassan et al., 2012). The roots of *E. longifolia* also benefits as the herbal ingredient for women after childbirth in a way to enhance blood flow and functions (Ismail et al., 1999). Besides, the leaves of this plant is used traditionally in curing ulcers, preventing gum diseases

and to treat sexually transmitted infections, such as syphilis and gonorrhoea (Bhat & Karim, 2010). *E. longifolia* also served as a health tonic and anti-stress medication (Zanoli et al., 2009). Additionally, the plant extracts are widely used as local traditional medicines for antipyretic and anti-ulcer (Park et al., 2014). Some of the constituents of *E. longifolia* have also been proven to possess anti-amoebic property (Le & Nguyen, 1970). On the other hand, the anti-diabetic property of *E. longifolia* aqueous extract has been proven by Husen et al. (2004). In the treatment of osteoporosis, *E. longifolia* enhanced the testosterone level apart from stimulating the proliferation of osteoblast and apoptosis of osteoclast. This phenomenon eventually maintained the bone remodelling activity and reduce the risk of bone loss (Mohd et al., 2012).

2.1.6 Mechanism and action of *E. longifolia*

Numerous studies have been undertaken in order to identify the potential valuable effects and biological activities of *E. longifolia*. However, only a few works of literatures recorded the mechanisms and actions of this plant. Table 2.1 summarizes some of the identified mechanisms behind the positive effects of *E. longifolia*.

Table 2.1: The mechanism and action of *E. longifolia* on human health problems and diseases. However, no information on the anti-malarial, anti-microbial and anti-diabetic activities was reported for this extract.

Effects	References	Mechanisms
Anti-tumor	Tong et al., 2015	Standardised *E. Longifolia* extract, SQ40, inhibited LNCaP cell development by suppressing the growth *via* G0/G1 phase arrest, and accompanied by the down-regulation of CDK2, CDK4, Cyclin D1 and Cyclin D3, but up-regulation of the protein level of p21Waf1/Cip1.
	Al-Salahi et al., 2014	The TAF273 from *E. longifolia* root methanolic extract showed a potent cytotoxic effect on K-562 cells *via* G_1 and S phases of cell cycle arrest.
	Hajjouli et al., 2014	Eurycomanone and eurycomanol of *E. longifolia* inhibited the viability of Jurkat and K-562 cells with no effects to the healthy cells. Eurycomanone inhibited NF-κB signalling through inhibition of IκBα phosphorylation and mitogen-activated protein kinase (MAPK) activation.
	Wong et al., 2012	Eurycomanone treatment on lung A549 cancer cells decreased the expression of lung cancer markers: heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1, p53 tumor suppressor protein and other cancer-associated genes, e.g. annexin 1 (ANX1), prohibitin (PHB) and endoplasmic reticulum protein 28 (ERp28). The mRNA expression of the studied genes was significantly down-regulated except PHB.
	Zakaria et al., 2009	Eurycomanone possessed anti-cancer effect against HepG2 cells by apoptosis induction *via* the up-regulation of p53 and Bax, and down-regulation of Bcl-2, which increased the levels of cytochrome C.

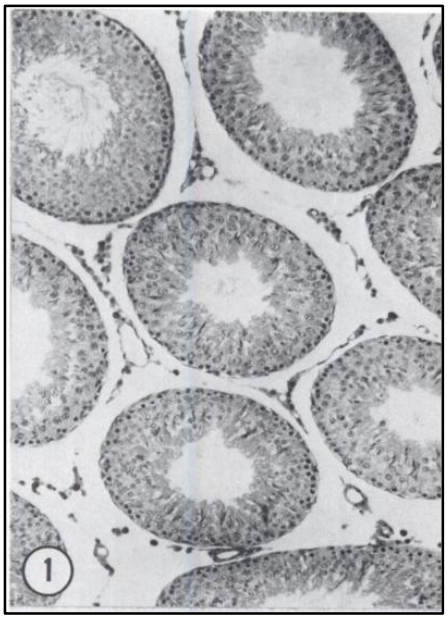
	Tee & Azimahtol, 2005	Purified extract of *E. Longifolia*, F16 induced apoptosis of MCF-7 *via* the modulation of Bcl-2 protein level.
Anti-infertility	Chen et al., 2015	*E. Longifolia* extract improved infertility *via* suppression of α-2-HS glycoprotein expression that enhanced the testosterone level and insulin sensitivity.
Anti-proliferative	Ohishi et al., 2015	9-hydroxycanthin-6-one, a compound extracted from *E. Longifolia* root reduced the expression of Wnt signal targeted genes; mitf and zic2a, in zebrafish embryos *via* the activation of GSK3β independent CK1α expression.
Anti-inflammatory	Nguyen et al., 2015	7-MCPA (7-methoxy-(9H-β-carbolin-1-yl)-(E)-1-propenoic acid) isolated from *E. longifolia* hairy-root cultures activated Nrf2 through ROS-dependent p38 MAPK pathway and enhanced the activation of the Nrf2/HO-1 pathway which showed anti-inflammatory effect.
Anti-osteoporosis	Shuid et al., 2012	*E. Longifolia* extract supplementation on male Sprague-Dawley rats elevated the testosterone level. The supplement also reduced the bone resorption marker and up-regulated OPG gene expression of the orchidectomised rats, which plays important role in the the protective effects of *E. Longifolia* extract against bone resorption due to androgen deficiency.

2.2 The Leydig cells

Leydig cells were first discovered by a German scientist named Franz Leydig in 1850, and hence the cells are named after him (Mendis-Handagama & Ariyaratne, 2005). Leydig cells are the cells that are located in the interstitial compartment of the testis, adjacent to the seminiferous tubules (Figure 2.2) and they are the sole source of androgens from the testis (Dong & Hardy, 2004). Generally, Leydig cells are morphologically epitheloid and ovoid with the presence of different organelles, such as cytoplasm, euchromatic round eccentric nuclei, nucleolus and mitochondria. There are two major types of Leydig cells; fetal Leydig cells and adult Leydig cells (Svechnikov et al., 2010).

During embryogenesis, fetal Leydig cells emerge and develop in the uterus. (Benton, 1995). Upon developing, the cell becomes terminally differentiated and competently produce testosterone before birth occurs (Shima et al., 2013). The testosterone released by fetal Leydig cells is very crucial for male reproductive system differentiation, especially for testis in gestation (Huhtaniemi & Pelliniemi, 1992). The development process of the cells is independent of Luteinizing hormone (LH) even though the cells express LH receptor and respond to LH stimulation (Zhang et al., 2001).

(a)



(b)

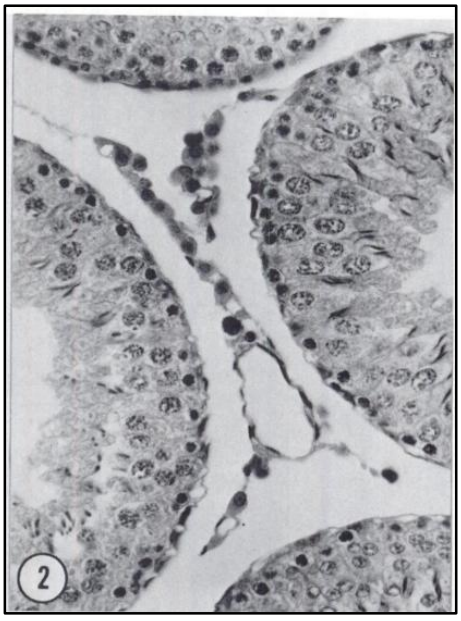


Figure 2.2: The Leydig cells. **(a)** Cross section of a rat testis showing interstitial tissue with Leydig cells. **(b)** The area of interstitial tissue shows a moderate size of Leydig cells between the seminiferous tubules (Christensen & Peacock, 1980).

On the other hand, adult Leydig cells developed upon puberty from the testicular mesenchymal-like stem cells (Ye et al., 2017). The adult Leydig cells constantly supply testosterone, which is important for the spermatogenesis process, sex gland accessories development and also the emergence of secondary sexual characteristics (Svechnikov et al., 2010). The adult Leydig cells continuously form with 4 distinct types of identified cells; stem, progenitor, immature and adult (Chen et al., 2009). The development of Leydig cells is shown in Figure 2.3.

After 7 days of birth, a spindle-shaped cell, known as stem Leydig cells (SLC) could be seen in the testis interstitial space (Ge et al., 2006). However, these cells are not under the lineage of Leydig cell since they do not express any of the Leydig-cell certain markers, such as LH receptor. A spindle-shaped population of progenitor Leydig cells (PLC) developed by 14 days of postnatal. PLC is recognized as Leydig cell lineage by expressing LH receptor and also by producing the low level of androgen (Shan et al., 1993). PLC has the low abundance of smooth endoplasmic reticulum (SER), where steroidogenic enzymes are located. At 28 days of birth, the PLC gradually converts from spindle-shaped to round-shaped immature Leydig cells (ILC) forming approximately 13-14 million cells (Hardy et al., 1989). The SER of ILC expands greatly and they have the intermediate capacity of steroidogenesis (Zirkin & Ewing, 1987). From postnatal day 28 to day 56, the adult Leydig cells (ALC), which are also round with small lipid droplets, undergo one time doubling process forming approximately 25 million cells per testis. The SER is the most abundant at this stage and the cells secrete the highest level of testosterone. Normally, ALC does not undergo proliferation however, the cells are regenerated once their population is diminished (Keeney et al., 1988).

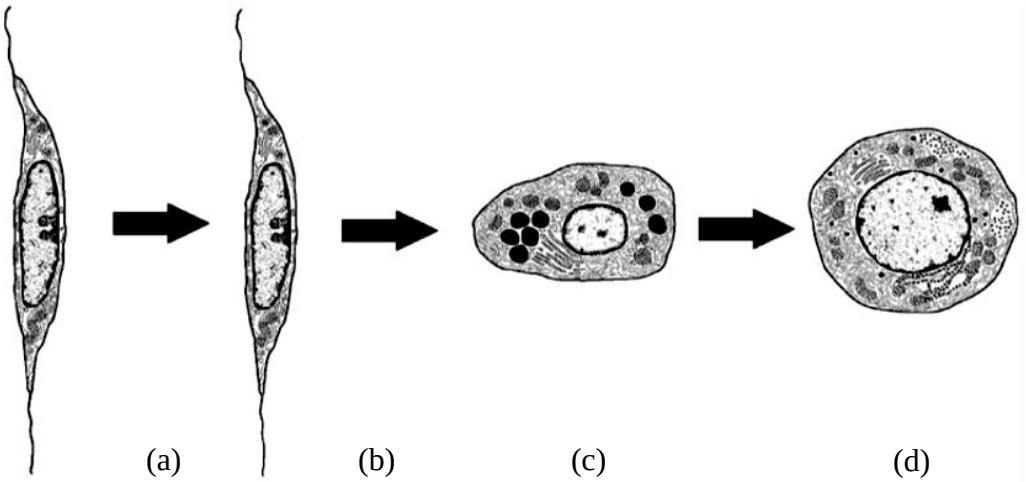


Figure 2.3: The development of Leydig cells; **(a)** stem Leydig cells, **(b)** immature Leydig cells, **(c)** progenitor Leydig cells, and **(d)** adult Leydig cells (Chen et al., 2009).

2.3 The hypothalamic-pituitary-gonadal axis

Leydig cells are able to carry out their primary function, which is testosterone secretion through hypothalamic-pituitary-gonadal axis (Erasmus et al., 2012). There are three major organs comprising the axis; the hypothalamus, the anterior pituitary gland and the testes (Figure 2.4).

The hypothalamus located in the brain first produces and secretes Gonadotropin-releasing hormone (GnRH) in a pulsatile manner. GnRH then triggers the release of Luteinizing hormones (LH) and Follicle stimulating hormones (FSH) in the anterior pituitary gland. LH and FSH are the two gonadotropic hormones released by both males and females. They share a common α-peptide chain with human chorionic gonadotrophin (hCG), but differs from each other by a specific β-peptide chain, which specifies their biological action (Gingrich, 2010).

The FSH functions on Sertoli cells, where the spermatogenesis process occurs, and is stimulated by the release of androgen-binding protein (ABP). This ABP bind with testosterone produced outside of the seminiferous tubules in order to maintain the growth of germ cells (Prante et al., 2008). Meanwhile, in the testis, LH stimulates the Leydig cells to secrete testosterone. When the testosterone level is more than needed, the Sertoli cells secrete inhibin that terminates the hypothalamus in releasing GnRH in a negative feedback. Therefore, the system is kept in equilibrium (Erasmus et al., 2012).

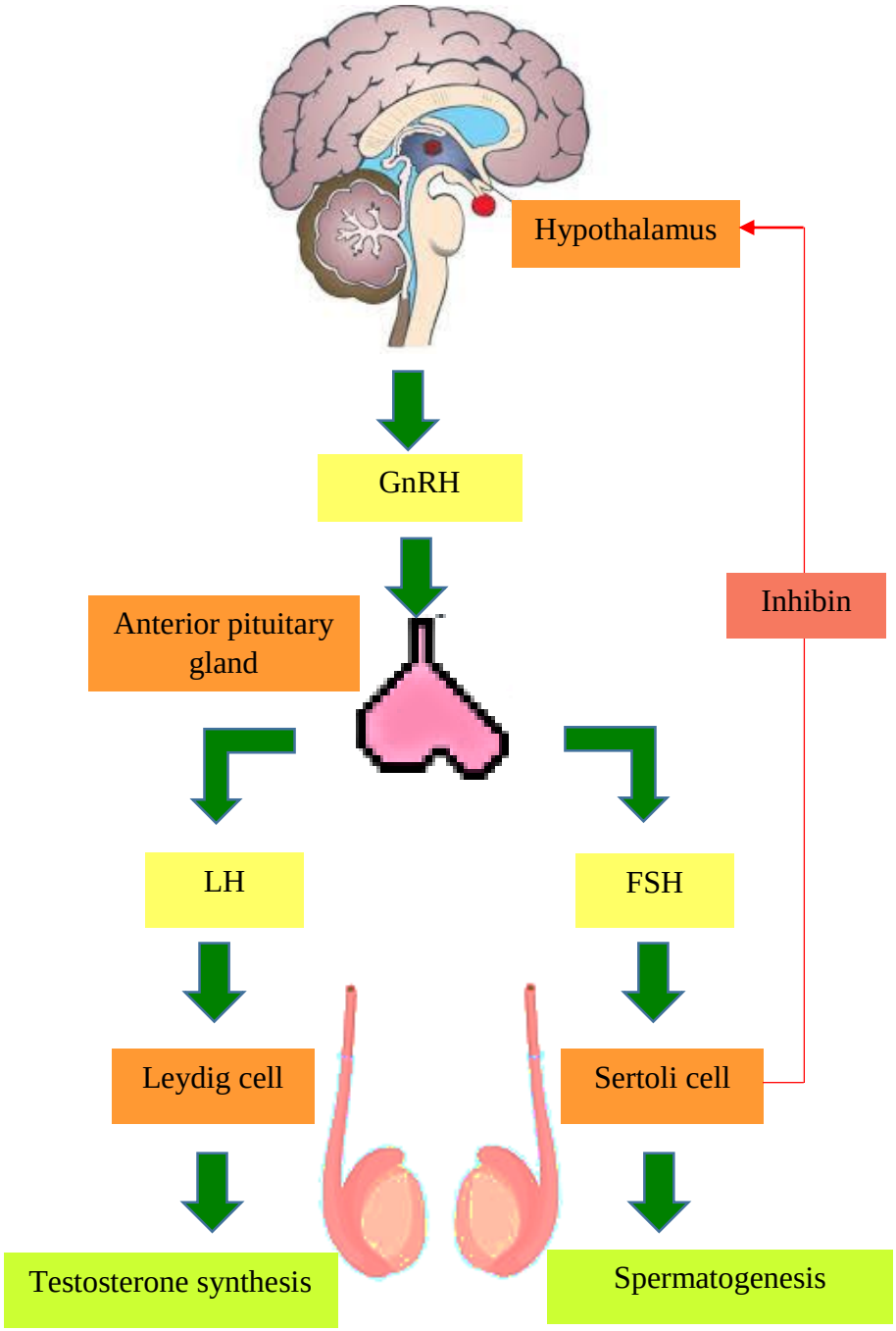


Figure 2.4: Diagrammatic illustration of the hypothalamic-pituitary-gonadal axis. Modified from (Gingrich, 2010).

2.4 Steroidogenesis

Steroidogenesis is the process of steroid hormones synthesis from the different part of human bodies, such as adrenal cortex, testis, ovaries and adipose tissue. Example of steroid hormones includes glucocorticoids, mineralocorticoids, androgens and estrogens. The biosynthesis of steroid is a series of the complex process with consecutive steps. Basically, all steroid hormones are derived from cholesterol synthesized by various tissues especially adrenal gland and gonads as their parent compound. Steroidogenic acute regulatory protein (StAR) is responsible for transporting cholesterol into the mitochondria (Hauet et al., 2006). Several steroidogenic enzymes are responsible for the steroid biosynthesis as shown in Table 2.2. These enzymes are mostly found in the inner mitochondria and smooth endoplasmic reticulum.

The steroidogenesis pathway of rodents differ slightly from humans in the production of androstenedione (Engeli et al., 2018). In humans, the cholesterol is first converted to pregnenolone by a cholesterol side-chain cleavage enzyme, CYP11A (Parker & Schimmer, 1995). The pregnenolone is then converted to 17-hydroxy pregnenolone and further down to dehydroepiandrosterone (DHEA) by the CYP17 enzyme. An enzyme that is known as 3β-hydroxysteroid dehydrogenase then converts DHEA to an androgen, androstenedione. In contrast, rodents prefer intermediate progesterone and 17-hydroxy progesterone enzyme for androstenedione conversion. This androstenedione is a weak androgen, which is converted to testosterone by 17β-hydroxysteroid dehydrogenase (17β-HSD) in both humans and rodents. The 17β-HSD types 3 and 5 are responsible to catalyse the conversion of androstenedione to testosterone, which is expressed in testis Leydig cells (Mindnich et al., 2004). Finally,