THE PHYTOPHARMACEUTICAL DEVELOPMENT OF *EURYCOMA LONGIFOLIA* JACK AND ITS MECHANISTIC STUDIES ON RAT SPERMATOGENESIS

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by

LOW BIN SENG

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To my dearest parents Mr. and Mrs. Low,
parents-in-law Mr. and Mrs. Lim,
sister Bin Kee, brother-in-law Khian Chuan, nephew Hong Ming,
my loving wife Lee Meng, sons Linjia and Linjian.

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LIST OF ABBREVIATIONS

1	Eurycomanone
2	Eurycomanol
3	13,21-Dihydroeurycomanone
4	13α(21)-Epoxyeurycomanone
α	Alpha
β	Beta
µg	Microgram
µM	Micromolar
%	Percentage
Å	Angstrom (10^{-10} m)
AGTM	Aminoglutethimide
Ala	Alanine
ANOVA	Analysis of variance
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
$AUC_{0\rightarrow\infty}$	Area under curve
°C	Degree of Celsius
cAMP	Cyclic adenosine monophosphate
CL	Clearance
C_{max}	Maximum concentration of certain chemical in the plasma
CYP11a	Cytochrome P450 side chain cleavage enzyme ($P450_{SCC}$)
CYP17a	17β-hydroxysteroid dehydrogenase
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
DPX	Mounting agent for histology containing distyrene and xylene
ED_{50}	Median effective dose
EC_{50}	50 % pharmacological effective concentration
EIA	Enzyme immunoassay
F1	Fraction 1 from *Eurycoma longifolia*
F2	Standardized eurycomanone-rich extract or Fraction 2 from *E. longifolia*

F3	Fraction 3 from *Eurycoma longifolia*
F4	Fraction 4 from *Eurycoma longifolia*
FEB	Free energy binding
FMS	Formestane
FSH	Follicle stimulating hormone
Glu	Glutamic acid
Gly	Glycine
GnRH	Gonadotropin releasing hormone
GnIH	Gonadotrpoin inhibiting hormone
GPCR	G protein-coupled receptor
h	Hour
H&E	Hematoxylin and eosin stain
hCG	Human chorionic gonadotropin
HO-TMX	Hydroxytamoxifen
HPLC	High-performance liquid chromatography
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	Median inhibition concentration
ID	Identity
Ile	Isoleucine
IV	Intravenous
k_e	Rate of elimination
kg	Kilogram
KTZ	Ketoconazole
LD_{50}	Median lethal dose
Leu	Leucine
LH	Luteinizing hormone
LOD	Limit of detection
LOQ	Limit of quantification
MCF-7	Human estrogen sensitive breast cancer cell line
Met	Methionine
mg	Milligram
mL	Milliliter
n	Animal sample size

nm	Nanometer
nM	Nanomolar
NFD	Nifedipine
OECD	Organization for Economic Cooperation and Development
$P450_{SCC}$	Cytochrome P450 side chain cleavage enzyme
PBSG	Phosphate buffer saline with gelatin
PDB	Protein Data Bank
PDE	Phosphodiesterase
pg	Picogram
Phe	Phenylalanine
RSD	Relative standard deviation
RT	Retention time
SD	Standard deviation
S.E.M.	Standard error mean
StAR	Steroidogenic acute regulatory protein
$t_{½}$	Half-life
T_{max}	Time to reach C_{max}
TAM	Methanol extract of *E. longifolia*
Thr	Threonine
TMX	Tamoxifen
USA	United State of America
USM	Universiti Sains Malaysia
V_d	Volume if distribution
W	Aqueous extract of *E. longifolia*
WHO	World Health Organization
w/v	Weight over volume
w/w	Weight over weight

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PEMBANGUNAN FITOFARMASEUTIKAL *EURYCOMA LONGIFOLIA* JACK DAN PENGAJIAN MEKANISTIK ATAS SPERMATOGENESIS TIKUS

ABSTRAK

Eurycoma longifolia Jack yang mempunyai kebolehan dalam peningkatan seksual and kesuburan lelaki merupakan suatu perubatan tradisional yang terkenal. Berdasarkan kepada kajian sebelumnya, ekstrak metanol *E. longifolia* yang bioaktif difraksinasikan melalui kromatografi turus pengisian resin menghasilkani empat fraksi. Di antara keempat-empat fraksi ini, didapati bahawa fraksi 2 (F2) meningkatkan spermatogenesis tikus dengan ketara. Analisis dengan kromatografi cecair prestasi tinggi (HPLC) pada F2 menunjukkan bahawa F2 mengandungi sejumlah 32.1 % (w/w) kuassinoid termasuk 14.5 % (w/w) eurikomanon. Pemberian F2 secara oral bagi tikus selama 52 hari adalah berkesan untuk meningkatkan spermatogenesis. Selain itu, peningkatan kepekatan sperma berikutan dengan pemberian F2 terus dibuktikan oleh kajian mikroskopik morfometri di mana populasi sel-sel germa, kadar penghasilan spermatozoa, ketinggian epithelium germina dan bilangan sel-sel Leydig telah ditingkatkan secara ketara. Kesan *E. longifolia* ke atas paksi hipotalamus-pituitari-gonadal juga ditunjuk apabila F2 meningkatkan paras hormon testosteron, hormon peluteinan (LH), hormon peransang folikal (FSH) tetapi sebaliknya menurunkan paras estrogen dalam plasma. Kajian *in vitro* membuktikan kesan eurikomanon ke atas sel-sel perantaraan testis tikus dalam meningkatkan penghasilan testosteron. Peningkatan paras testosteron dan pengurangan estrogen yang diakibatkan oleh eurikomanon secara *in vitro* adalah konsisten dengan kajian *in vivo*. Eurikomanon menambahkan bilangan cyclic AMP dengan merencatkan fosfodiesterase yang kemudiannya meningkatkan steroidogenesis testosteron. Eurikomanon juga mengurangkan penghasilan estrogen dengan merencatkan enzim aromatase selepas rawatan F2. Tiada apa-apa kesan ketoksikan ditunjukkan daripada system pembiakan betina dan perkembangan fetus-fetus. Paras ketidakjumpaan kesan buruk

(NOAEL) untuk toksikologi pembiakan dan teratologi tikus adalah 100 mg/kg. Di samping itu, kesan pengumpulan bahan kimia oleh fetus mungkin tidak menjadi disebabkan bioperolehan kuassinoid yang agak rendah disebabkan polariti yang tinggi, keterlarutan lipid yang lemah, separuh hayat yang pendek dan kadar pelupusan yang tinggi. Di samping peningkatan androgen selepas pengambilan F2 melalui mulut, tiada kesan toksik pada gonad jantan dijumpai. Lebih-lebih lagi, F2 tidak menunjukkan kesan apoptosis pada sel-sel germa berbanding dengan kawalan. Oleh itu, F2 atau fraksi *E. longifolia* yang kaya dengan kuasinoid mungkin bernilai untuk dibangunkan sebagai suatu fitofarmaseutikal bagi menangani ketidaksuburan lelaki.

THE PHYTOPHARMACEUTICAL DEVELOPMENT OF *EURYCOMA LONGIFOLIA* JACK AND ITS MECHANISTIC STUDIES ON RAT SPERMATOGENESIS

ABSTRACT

Eurycoma longifolia Jack is popularly used as a traditional medicine for various pharmacological properties including the improvement of sexual and male fertility. From a previous study, the bioactive methanol extract of *E. longifolia* was fractionated through resin-packed column chromatography and yielded four fractions. The fraction 2 (F2) significantly increased the spermatogenesis of rats. High performance liquid chromatography (HPLC) analysis revealed that the bioactive fraction F2 contained 32.1 % (w/w) of total major quassinoids comprising 14.5 % (w/w) of eurycomanone. The male rats orally administered with 25 mg/kg of F2 and 250 mg/kg of *E. longifolia* aqueous extract that contained almost similar in concentration of eurycomanone, significantly increased the sperm concentration when compared with that of the control animals. Upon oral administration of F2 for 52 days, male rats showed an improvement of the spermatogenesis. The increase of the sperm concentration upon treatment with F2 was further confirmed by the microscopic morphometrical studies showing that the population of the germ cells, spermatozoa production rate, the height of germinal epithelium and Leydig cells count was significantly elevated. The administration of F2 increased the levels of testosterone, luteinizing hormone, follicle stimulating hormone but reduced the estrogen level in plasma, further indicates the effect of *E. longifolia* on the hypothalamic-pituitary-gonadal axis. *In vitro* study showed that eurycomanone, the major quassinoid, significantly increased the testosterone production in the isolated rat testis interstitial cells. The increase in testosterone and decrease in estrogen induced by eurycomanone *in vitro* were consistent with the *in vivo* study. Eurycomanone inhibited phosphodiesterase resulting in the accumulation of cyclic AMP that subsequently modulated the steroidogenesis of testosterone, and also reduced

estrogen production by inhibiting the aromatase enzyme. Upon oral treatment of female rats, F2 was not toxic to the female reproductive system and the development of foetuses. The no-observed adverse effect level (NOAEL) for reproductive toxicology and teratology was 100 mg/kg in rats. The possible cumulative chemicals exposure of foetus probably may not arise as the bioavailability of the bioactive quassinoids was relatively low due primarily to the high polarity, poor lipid solubility, short half life and high elimination rate. Male rats orally administered with F2 did not show toxic effects on the gonads despite the androgen elevation property of the fraction. Furthermore, F2 showed no apoptotic effect on the germ cells when compared to control. Hence, *E. longifolia* quassinoid-rich fraction F2 may be worthy for the development as a phytopharmaceutical for alleviating the infertile male.

CHAPTER ONE

INTRODUCTION

1.1 Infertility: An Overview

Infertility is generally defined as the inability of a couple to conceive after trying through unprotected intercourse for a year (Prakash, 2007). In all cultures worldwide, infertility is recognized as a crisis that has a potential to agitate the stability of individuals, relationships among the family members as well as the community welfare (Burns and Covington, 2006). Globally, one in ten couple experienced infertility (Burns and Covington, 2006). According to World Health Organization (WHO), over 186 million couples in developing countries (except China) were affected by infertility (WHO, 2003). Among the infertile couples, about 20 % of the infertility problem originated from the male partner and 30-40 % was contributed by both male and female (Raman et al., 2005). Among infertile males, over 90 % were diagnosed as oligozoospermia, a clinical condition known as low or poor in sperm quality (Winston, 1986) with a population of $< 15 \times 10^6$ spermatozoa/mL compared to the normal of $> 15 \times 10^6$ spermatozoa/mL (WHO, 2010). Moreover, former meta-analyses data of the sperm count showed a global downward trend (Merzenich et al., 2010).

1.1.1 Oligozoospermic male infertility

Hitherto, the origin of the oligozoospermic male infertility is unclear and yet it was believed that the defect may be caused primarily by one or a combination of several factors including personal lifestyle (frequent uses of electronic devices, wearing tight attire, horse riding, etc.), diet, working habits (long hours of driving, stress, strenuous work), etc. (Sharpe and Franks, 2002). Besides, physiological changes such as immunological abnormalities, erectile dysfunction, anatomical abnormality and genetic disorders may also contribute to the male infertility (Winston, 1986).

1.1.2 Challenges of the treatment approach on male infertility

For decades, the investigation of female reproductive functions has stood in the foreground however the studies on male fertility have only been given an equal attention in the recent years (Nieschlag, 2010). Earlier study reported that glutathione, an antioxidant, protected the epididymis, spermatogenic epithelium and ejaculated spermatozoa from excessive generation of reactive oxygen species (Irvine, 1996). However, fertility studies on glutathione for male infertility treatment still remain obscured. Acupuncture improved the ultrastructure of spermatozoa for idiopathic male infertility but not to the number of spermatozoa (Pei et al., 2005). Medical treatments including human chorionic gonadotropin (hCG), antiestrogen, recombinant follicle stimulating hormone (rFSH), surgery and microsurgery involving the sperm intra-fallopian transfer and intra-cytoplasmic sperm injection (ICSI) have been developed to overcome the male infertility but not to enhance the sperm quality (Jungwirth et al., 2012). In fact, these treatments are not only costly but have also led to socio-economic problems (Katz et al., 2002). Besides, a potent aromatase inhibitor was developed to improve the reproductive activity and spermatogenic function of animals however, the route of administration that used surgically implanted osmotic pump was not appropriate as a treatment method (Kawakami et al., 2004). In addition, direct exogenous oral administration of testosterone did not improve the spermatogenesis on the idiopathic male infertility (Sheckter et al., 1989) but may in turn cause liver toxicity (Ishak and Zimmerman, 1987). Therefore, alternatives to overcome infertility that are more affordable and easily available such as traditional herbal treatment, are gaining in popularity today for the treatment of oligozoospermia.

1.1.3 Introduction to natural products

Natural products from herbs are recognized as one of the fastest growing industry all over the world. In fact, this industry has long been in practice as from pre-historic epic along with the growth of human civilization. In the ancient time, medicinal treatment was based on herbal as a practice and most of these practices in the herbal treatments indeed are being

vertically and verbally passed down from one generation to the next. As the ingredient of the herb was not very well studied, the practice was eventually challenged by synthetic drugs, produced following the development of the chemistry during 14 and 15 century in the West. To date, thousands of the herbal natural products that are manufactured in the form of capsule, soft-gel, powder in sachet and even in the canned beverages are available as over-the-counter supplements. Herbal natural products may possess various biological properties such as antineoplastic, antibacterial, antiviral, antipyretic, antidiabetic, antioxidant, antifertility, etc., but the safety and efficacy of the herbal natural products are still not fully studied.

1.2 Traditional herbal medicine on male infertility

Plant-based chemical ingredients have been resourceful as new therapeutic agents following the continuously broad research and development during the last century (Mérillon and Ramawat, 1999). Protodioscin, a phytochemical agent derived from *Tribulus terrestris* L. plant has been clinically proven to improve sexual desire and erectile dysfunction (Gauthaman *et al*., 2002). However, geographical factors caused the inconsistency on the production of protodioscin (Adimoelja, 2000). Ferulic acid, as an effective constituent commonly found in various medicinal herbs increased sperm viability and motility in asthenozoospermic infertile human spermatozoa *in vitro* (Zheng and Zhang, 1997), however no *in vivo* study was available. Besides, pychnogenol (French maritime tree bark extract), L-carnitine, minerals such as zinc, selenium and supplements including vitamin B12, C, E and coenzyme Q10 showed improvement in male fertility (Gutmann and Covington, 2006). Flavonoids from plants that increased the catalase and superoxide dismutase (SOD) activities but decreased the lipid peroxidation level was believed for the semen quality improvement of oligozoospermic infertile men (Lara et al., 2008). In addition, some plants such as *Spilanthes acmella* (Sharma et al., 2011), *Bryonia laciniosa* (Chauhan and Dixit, 2010), *Anacyclus pyrethrum* DC (Sharma et al., 2013), *Lepidium meyenii* (Zheng et al., 2000), *Hibiscus macranthus* and *Basella alba* (Moundipa et al., 1999) showed androgenic, aphrodisiac and

spermatogenic activities. In Malaysia, traditional medicinal plants reported to increase male virility included *Coleus parviflorus* (Ubi Kemili), *Smilax myositiflora* (Ubi Jaga), *Polyalthia bullata* (Tongkat Ali Hitam) and *Eurycoma longifolia* (Zakaria and Ali Mohd., 1994). Amongst, *E. longifolia* is popularly used as a traditional ethno-remedy for the improvement of male libido, sexual prowess and fertility (Gimlette, 1971; Ang and Sim, 1998; Noor et al., 2004; Chan et al., 2009; Zanoli, et al., 2009; Wahab et al., 2010). Most of the traditional *E. longifolia* remedies are prepared by boiling the powdered roots with water, and the dry powdered extract is presently incorporated into over 200 canned beverages of carbonated drink, instant premixed tea and coffee for sale in the domestic market (Cyranoski, 2005).

1.3 Literature review

1.3.1 *Eurycoma longifolia* Jack: a traditional approach towards male infertility

Malaysia is a tropical country enriched with millions of flora and fauna and this biodiversity has contributed a reservoir of herbal sources yet to be discovered. The climate with high humidity and rainfall in Southeast Asia provides the best ecosystem for the growth of plants. Amongst, *Eurycoma longifolia* Jack, a small tree belonging to Simaroubaceae family, locally known as 'Pasak bumi' in Indonesia, 'Cay ba binh' in Vietnam and 'Tongkat Ali' in Malaysia (Gimlette, 1971). The plant thrives very well in the tropical climatic conditions, commonly found along the hilly jungle slopes and widely distributed in the primary, secondary, evergreen and mixed deciduous forests in Malaysia, Indochina, Sumatra, Borneo and the Philippines (Kuo et al., 2003).

Plate 1.1 *Eurycoma longifolia* Jack

1.3.1(a) Monograph and physiology of *Eurycoma longifolia* Jack

E. longifolia is a medium size slender shrub or tree reaching up to 10 m in height. The plant is often unbranched with reddish brown petioles (Malaysian Monograph Committee, 1999) or with a few upright branches and each of them is crowned by an umbrella-like rosette of leaves (Corner, 1951). The leaves of the plant reach a length of about 1 m, even pinnate, consist of 30-40 leaflets and dark green in colour. Each leaflet is about 5-20 cm long, 1.5-6.0 cm wide and much paler on the ventral side (Malaysian Monograph Committee, 1999). The flowers are wide, about 0.5 cm, hairy and purplish-crimson in colour (Corner, 1951). The petals of the flowers are small and have very fine pubescent (Malaysian Monograph Committee, 1999). The male and bisexual (hermaphrodite) flowers appear on the different trees and are slightly fetid (Corner, 1951). The fruits are about 1.0-1.5 cm in length, hard, ovoid in shape, yellowish brown in colour when young and will turn brownish red when ripe (Malaysian Monograph Committee, 1999).

1.3.1(b) Taxonomy of *E. longifolia* (Hsuan, 1978)

Kingdom:	Plantae
Division:	Spermatophyta
Subdivision:	Angiospermae
Class:	Dicotyledoneae
Order:	Geraniales
Family:	Simaroubaceae
Genus:	*Eurycoma*
Species:	*longifolia* Jack

1.3.1(c) Chemical constituents of *E. longifolia*

For 30 years since the first scientific documentation on *E. longifolia* in 1982, more than 60 chemical constituents have been isolated and characterized. *E. longifolia* contains different types of chemical constituents which can be generally classified into six major groups including quassinoids, alkaloids, anthraquinones and their glucosides, tirucallane-type triterpene, squalenes and biphenylneolignans. Table 1.1 showed the list of chemical constituents discovered from the plant following the year of its documentation.

Table 1.1 List of novel chemical constituents isolated from *E. longifolia*.

Name of chemical constituents	References
Laurycolactone A, laurycolactone B, eurycomalactone	Suong et al. (1982)
Eurycomanone **(1)**, eurycomanol **(2)**	Darise et al. (1983)
3,4-Dehydroeurycomalactone, 5,6-dehydroeurycomalactone, 6-hydroxy-5,6-dehydroeurycomalactone	Bates et al. (1984)
10-Hydroxycanthin-6-one	Chan et al. (1986)
Eurycomanol-2-*O*-*β*-D-glycopyranoside	Chan et al. (1989)

Table 1.1 Continued.

Name of chemical constituents	References
Longilactone, 13,21-dihydroeurycomanone **(3)** , 13β,21-dihydroxyeurycomanone, 14,15β-dihydroxyklaineanone	Morita et al. (1990)
13β,18-Dihydroxyeurycomanol	Chan et al. (1991)
Eurylene, teurilene	Itokawa et al. (1991a)
Longilene peroxide	Itokawa et al. (1991b)
Biphenylneolignans	Morita et al. (1992)
6α-Hydroxyeurycomalactone	Chan et al. (1992)
11-Dehydroklaineanone, niloticin, dihydroniloticin, piscidinol A, bourjotinolone A, 3-episapelin A, melianone, hispidone	Itokawa et al. (1992)
Eurylactone A, eurylactone B	Itokawa et al. (1993)
14-Deacetyl eurylene	Morita et al. (1993a)
6-Dehydroxylongilactone, 7α-Hydroxyeurycomalactone, 13α(21)-epoxyeurycomanone **(4)**, 15-acetyl-13α(21)-epoxyeurycomanone, 12,15-diacetyl-13α(21)-epoxyeurycomanone, 12-acetyl-13,21-dihydroeurycomanone, 15β-acetyl-14-hydroxyklaineanone, 6α-acetoxy-14,15β-dihydroxyklaineanone, 6α-acetoxy-15β-hydroxyklaineanone	Morita et al. (1993b)
9,10-Dimethoxycanthin-6-one, 10-hydroxy-9-methoxycantin-6-one, 11-hydroxy-10-methoxycantin-6-one, 5,9-dimethoxycanthin-6-one, 9-methoxy-3-methylcanthin-5,6-dione, canthin-6-one, canthin-6-one-3N-oxide, 9-methoxycanthin-6-one, 1-methoxymethyl-β-carboline, β-carboline-1-propionic acid, 11-hydroxycanthin-6-one, 9-methoxycanthin-6-one-3N-oxide, 3-methylcanthin-5,6-dione	Mitsunaga et al. (1994)
Eurycolactone A, Eurycolactone B, Eurycolactone C	Ang et al. (2000)
9-Hydroxycanthin-6-one, 9-hydroxycanthin-6-one-N-oxide, chrysophanol, parietin, emodin, emodin-8-*O*-β-glucoside, aloeemodin-8-*O*-β-glucoside, pulmatin, chrysophanein, parietin-8-*O*-β-glucoside	Lin et al. (2001)
Eurycolactone D, Eurycolactone E, Eurycolactone F	Ang et al. (2002)
12-*Epi*-11-dehydroklaineanone, 15β-hydroxyklaineanone	Jiwajinda et al. (2002)

Table 1.1 Continued.

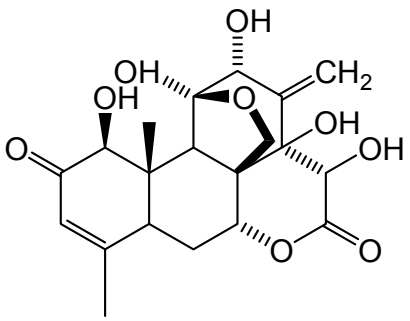
Name of chemical constituents	References
n-Pentyl β-carboline-1-propionic acid, 5-hydroxymethyl-9-methoxycantin-6-one, 1-hydroxy-9-methoxycantin-6-one, methyl β-carboline-1-carboxylate, 4,5-dimethoxycantin-6-one, 10-methoxycantin-6-one, 8-hydroxy-9-methoxycantin-6-one, 5-methoxycantin-6-one, 5-hydroxymethylcantin-6-one, picrasidine Q, 7-methoxymethyl-β-carboline-1-propionic acid, canthin-6-one 9-O-β-glucopyranoside, 1-hydroxycanthin-6-one	Kuo et al. (2003)
Eurycomaoside	Bedir et al. (2003)
Eurycomalide A, eurycomalide B, 13β,21-dihydroxyeurycomanol, 5α, 14β,15β-trihydroxyklaineanone	Kuo et al. (2004)
14-*Epi*-13,21-dihydroeurycomanone, 12,15-*O,O*-diacetyl-13,21-dihydroeurycomanone, 6α, 14β,15β-trihydroxyklaineanone, ailanquassin A, 3α,4α-epoxyeurycomalide B, 3ξ,4ξ-epoxy-5,6-dehydroeurycomalactone, 5α-hydroxyeurycomalactone, 5-dehydro-3-hydro-7β-hydroxy-6-oxoeurycolactone E, Δ4(18)-eurycolactone E isomer, 6α-hydroxyeurycolactone E	Miyake at al. (2009)
2,3-Dehydro-4α-hydroxylongilactone, scopolin	Teh et al. (2010)

1.3.1(d) Pharmacological aspect of *E. longifolia*

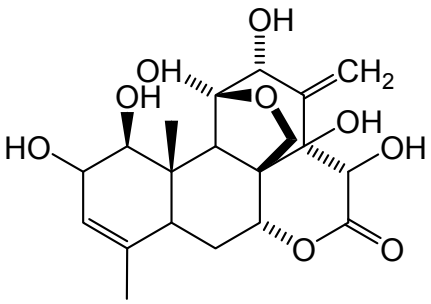
E. longifolia is popularly sought after as herbal remedies to treat various diseases. The root of the plant, in particular, has long been used for the treatment of fever, headache, intestinal worms, ulcers, pain (Perry and Metzger, 1980) and consumed as sexual tonic (Gimlette, 1971).

Scientific interest in *E. longifolia* took impetus late in the 20th century following observation of its potent activity against malaria and cytotoxicity on cancer cell lines. Chan *et al.* (1986; 2004) reported that the quassinoids, eurycomanone **(1)** possessed anti-plasmodial effect against the multi-drug resistant Thailand K1, chloroquine-resistant Gombak A and chloroquine-sensitive D10 strain of *Plasmodium falciparum* parasite. Moreover, **1** was also found to be cytotoxic against human breast cancer cell lines MCF-7

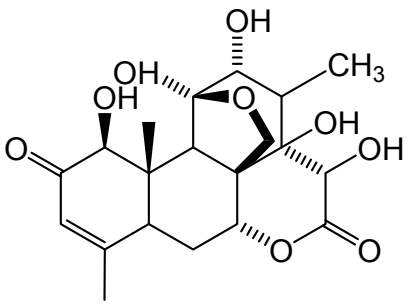
(Kuo et al., 2004) and P388 leukaemia cells (Morita et al., 1990). Besides, a eurycomanone-enriched fraction was also reported to induce apoptosis via the p53 pathway in HepG2 liver cancer cells (Zakaria et al. 2009). Eurycomanone **(1)** was also reported to suppress the protein expression of prohibitin, annexin I and endoplasmic reticulum protein 28 that involve in the cell cycle regulation in A-549 lung cancer cells (Wong et al., 2012). Apoptosis activation via caspase 9-independent pathway has been reported for one of the crude fractions of *E. longifolia* in MCF-7 breast cancer cells (Tee et al. 2007). However the bioactive chemical constituents in the fraction were not clearly defined. In addition, one of the alkaloids 9-methoxycanthine-6-one, isolated from the root of *E. longifolia* showed cytotoxic effects against A-549 and MCF-7 cell lines (Kuo et al., 2004). Beside antimalarial and cytotoxic properties, **1** also possessed antipyretic properties (Chan et al., 1995).



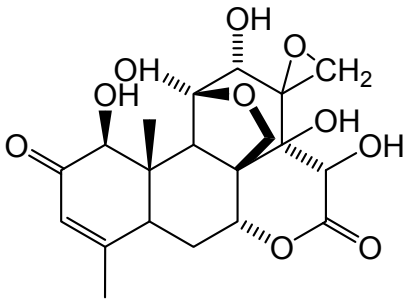
Eurycomanone **(1)**



Eurycomanol **(2)**



13,21-Dihydroeurycomanone **(3)**



13α(21)-Epoxyeurycomanone **(4)**

Figure 1.1. Chemical structure of the four major quassinoids: eurycomanone **(1)**, eurycomanol **(2)**, 13,21-dihydroeurycomanone **(3)** and 13α(21)-epoxyeurycomanone **(4)**.

Beginning from the late 1990, huge attention has been focused on the aphrodisiac properties of *E. longifolia* (Ang and Sim, 1997). Since then, more than twenty scientific reports and proceedings on the *E. longifolia* aphrodisiac enhancement in experimental animals have been published and presented (Bhat and Karim, 2010), however there are no clinical evidence to support the animal findings. Furthermore, possible mechanistic actions of the bioactive chemical constituents that repeatedly showed the aphrodisiac activity have not been clearly elucidated. A recent study demonstrated that *E. longifolia* had no significant enhancement effect on the sexual performance of the sluggish rodents but concluded that the improvement of the sexual performance could be primarily attributed to the elevation of serum testosterone level (Zanoli et al*.,* 2009).

Renewed interest in *E. longifolia* on the fertility improvement effect began in 2001 when Lin et al. (2001) reported that the ethanolic extract of *E. longifolia* concomitant with the human chorionic gonadotropin (hCG) increased the testosterone production of the Leydig cells. Therefore, the involvement of the hCG and the testosterone following the *E. longifolia* treatment may hypothesize that the plant may improve the male fertility. Three years later, Noor et al. (2004) showed that aqueous extract of *E. longifolia* increased the sexual behaviour and sperm quality of the experimental animals. However, the bioactive chemical constituents and the possible mechanism of actions were still not clearly defined. According to Noor at al. (2004), the aqueous extract significantly increased the male sexual behavior and increased the sperm quality, reversed the adverse effect of estrogen in male rats (Wahab et al., 2010), improved the sperm quality of idiopathic infertility clinically (Tambi and Imran, 2010), enhanced sexual well-being (Ismail et al., 2012) and increased the testosterone level of the late-onset hypogonadism males (Tambi et al., 2011). In fact, Asiah et al. (2007) proposed that a glycopeptide of 4.3 kDa, known as eurypeptide in the *E. longifolia* aqueous extract was the aphrodisiac marker. However, the glycopeptide was not indigenously found in *E. longifolia*, but also detected in *Smilax myosotiflora*, *Rafflesia* sp. and *Labisia pumila* (Asiah et al., 2007). Hitherto, the absolute structural configuration was not documented

(Asiah, et al., 2007). Beside the glycoproteins, the aqueous extract also contained the low concentration of the quassinoids especially **1** as reported by Tambi et al. (2011) and Shuid et al. (2011). In contrast, the crude polar organic extract of *E. longifolia* containing greater than 2 % of quassinoids including **1**, improved the sperm concentration of the normal and oligospermic animals (Chan et al., 2009). Thus, **1** and the quassinoids which co-exist in both aqueous and organic polar extracts may play an important role in the male reproductive system. Beside the male, **1** was also involved in the improvement of the female reproductive disorder (Abdulghani et al., 2012).

In view of the quassinoid **1** showing potential improvement of spermatogenesis, a bioactivity-guided fractionation of *E. longifolia* was performed to selectively yield an extract that contained high concentrations (> 10 % w/w) of **1** and its analogues, eurycomanol (**2**), 13,21-dihydroeurycomanone (**3**) and 13α(21)-epoxyeurycomanone (**4**) (Low et al., 2011; Chan et al., 2012). Therefore, the detailed pharmacological properties of the quassinoid-rich *E. longifolia* extract including its efficacy on the spermatogenesis cycle, sperm production rate and male fertility improvement effects were investigated.

1.3.1(e) Bioavailability and pharmacokinetics studies of *E. longifolia*

Bioavailability is a measure of the degree in which a dose of a substance becomes physiologically available to the body tissue. A pharmacokinetic profile relates to the measurement or modeling of the absorption, distribution, metabolism and excretion of drugs or chemicals in a biological system as a function of time (Abou-Donia, 1995). The pharmacokinetic profile of a drug or chemical substances is clinically important as its pharmacological and toxicity effects virtually depends on the dosage and bioavailability. By knowing the absolute bioavailability, various factors that affect the absorption of the chemicals or drugs can then be further investigated to optimize the disposition in the systemic circulation (Tan, 2004).

Following the administration of a chemical or drug in an oral dosage form, disintegration and dissolution of the compound should occur rapidly to ensure satisfactory absorption. The physico-chemical properties such as lipophilicity and pH stability of the compounds have been considered as the major factors prior to absorption of the compounds as such cellular membrane of the epithelium cells lining up on the surface of intestinal tract, facilitating the absorption of any chemical constituent, can be considered as a combination of a physico-chemical and biological barrier to drug transport (Van de Waterbeemd, 2003). Beside the physico-chemical aspects of the substances, pre-systemic metabolism also known as high first pass effect may also influence the bioavailability of a drug or chemical substances during absorption when given orally (Tan, 2004).

Plant-based chemical substances often face the common dilemma of low absorption or poor oral bioavailability. Flavonoids from the *Gingko biloba* extract (Pietta et al., 1995), magnesium lithospermate B (MLB) isolated from *Salvia miltiorrhiza* (Zhang et al., 2004b), ginsenosides from *Panax notoginseng* (Xu et al., 2003), quaternary ammonium protoberberine from *Corydalis saxicola* (Li et al., 2006), lignans from *Phyllanthus niruri* (Murugaiyah and Chan, 2007) and curcumin from *Curcuma longa* (Yang et al., 2007) were reported to be poor in oral bioavailability. In addition, Tan et al. (2002) reported poor absorption of 9-methoxycanthine-6-one, an alkaloid isolated from the root of *E. longifolia* when administered orally to rats. Previous study reported that **1**, possessing poor lipophilicity with low $\log K_{ow}$ value, was poorly bioavailable in the circulation due mainly to the high polarity of the compound (Low et al., 2005). However, the bioavailability of the other major quassinoids, **2**, **3** and **4** in the quassinoid-rich *E. longifolia* extract remain obscure. Thus, the pharmacokinetic parameters of **2**, **3** and **4** were investigated and compared with **1** following oral and intravenous administration in rats. The physico-chemical properties of lipophilicity and pH stability of the quassinoids influencing their absorption in the gastro-intestinal tract were studied.

1.3.1(f) Toxicology aspect of *E. longifolia*

Toxicology is defined as a study of the adverse effects of chemicals or other agents on the living systems in order to predict the potential harmful effects to man and the environment (Conning, 1993). According to the Food and Drug Authority's regulations in Good Laboratory Practices, toxicology and pharmacokinetic properties of a drug must be evaluated and documented in animals prior to human study (The Merck Manual of Diagnosis and Therapy, 2005). The safety of the drug is determined by studying the acute, subchronic and chronic exposure of the drug in several animal species through various route of administration (The Merck Manual of Diagnosis and Therapy, 2005). Furthermore, any chemical substance that would be used on fertility improvement were required to be investigated for its possible toxic effect on the reproductive system and teratological defects using the animal model (OECD 421, 1995; OECD 416, 2001).

Formerly, acute toxicity test methods were designed to provide the dose-response curve by using several animals at each of three to five test doses of chemicals or drugs. By following this conventional method, the tolerance of the test population of laboratory animals towards potentially lethal doses was measured (Rispin et al., 2002). According to Rispin et al. (2002), the introduction of the new up-and-down procedure (UDP) on the median lethal dose (LD_{50}) was highly recommended and accepted. The efficiency of the new UDP dosing allowed the LD_{50} to be estimated using relatively fewer animals compared to that of the conventional acute toxicity test fixed-dose protocol (OECD 420, 2001). In addition, the present study also investigated the possible toxic effect of the bioactive *E. longifolia* quassinoid-rich fraction on the female reproductive system and prenatal development of the foetuses after oral administration of the extract. In fact, this information is essential for assessing risk-to-benefit ratio of the product, providing a reference to initiate clinical studies and to assess the potential toxicity.

A previous study reported that the LD_{50} of the 34 % alcoholic extract of *E. longifolia* was 1500-2000 mg/kg in mice (Satayavivad et al., 1998). However, the chemical profile of the extract was not clearly described. Nevertheless, one study showed that the LD_{50} of the **1** on the mice and brine shrimp was reported to be 50 mg/kg and 3.5 µg/mL, respectively (Chan and Choo, 2002). However, the toxicity signs, symptoms and necropsy of the dead animals were not reported. Recently, Shuib et al. (2011) reported that *E. longifolia* water extract should be used with caution especially in the elderly or patients with liver problems. In contrast, preliminarily sub-chronic 90-day toxicity study with a quassinoid-rich *E. longifolia* extract, no treatment-related adverse effects, significant changes of body weight, organ weight, haematology, serum biochemistry, food and water consumption of the treated animals were observed when compared to the control (Low, 2005). Furthermore, following the 180-day chronic oral administration of the same extract, the animals showed some changes in the blood haematological profiles regardless of genders (Low, 2005; Low et al., 2013). Changes on serum biochemical parameters were sporadically observed in both genders of the treatment groups but were without a dose-dependence relationship. Hence, a dosage of 50 mg/kg quassinoid-rich *E. longifolia* extract upon 90 and 180 days oral administration on rats was relatively safe (Low, 2005).

1.3.2 Male reproductive system

Organs in the male reproductive system comprise testes, a system of ducts, accessory sex glands such as prostate and several supporting structures including the scrotum and penis (Prakash, 2007) (Plate 1.2). Testes, located externally in the scrotum, are the male gonads that produce sperm and secrete hormones. The male reproductive system consists of the seminiferous tubules in testes, the vasa efferentia, the epididymis, the vas deferens and the urethra (Guyton and Hall, 1996). This system of ducts concerns with the production and maturation of spermatozoa in testes, storage of spermatozoa in epididymis and finally transporting the spermatozoa to the exterior during ejaculation. The accessory sex organs

including seminal vesicles and prostate glands produce the seminal fluid containing protein and vitamin to nourish the spermatozoa and their motility.

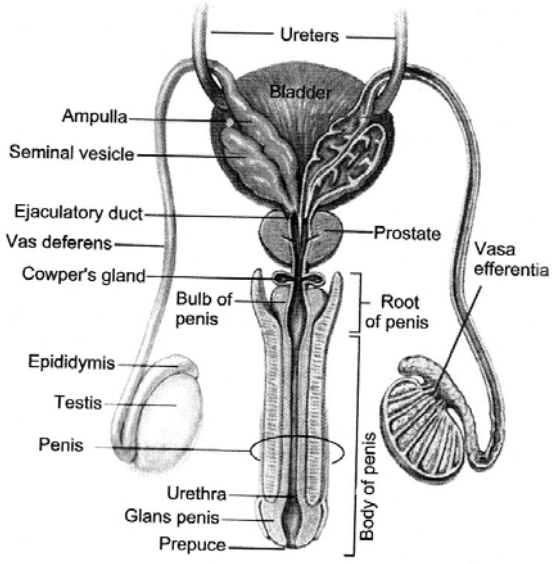


Plate 1.2. Human male reproductive system. Depicted from Prakash, (2007).

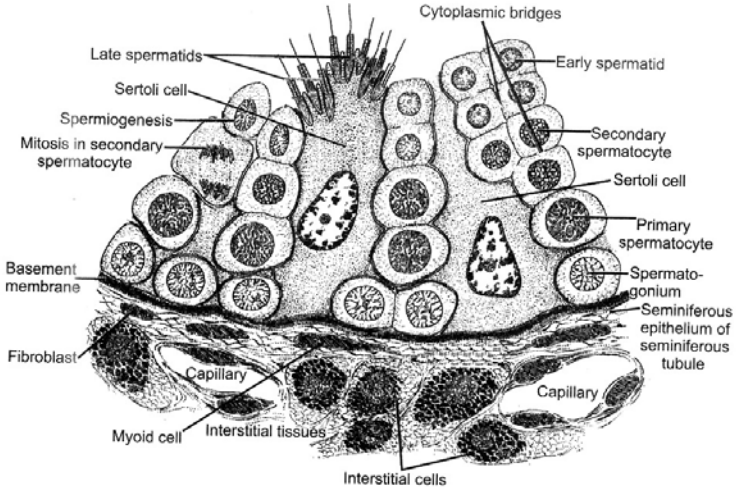


Plate 1.3. Arrangement of the germ cells or spermatocytes, Sertoli cells and interstitial or Leydig cells in seminiferous tubules. Depicted from Prakash (2007).

Testes are paired oval glands located externally in the scrotal sac. Testes contain sperm producing coiled tubules known as seminiferous tubules. Seminiferous tubules are long, convoluted and lined by highly specialized epithelium called spermatogenic cells and supporting cells known as Sertoli cells (Setchell, 1978). Spermatogenic cells including spermatogonia (stem), spermatocytes (germinal) and spermatids (undifferentiated spermatozoa) are the elements of germ cells development in the process known as spermatogenesis (Clermont, 1972) (Plate 1.3). Sertoli cells or also known as sustentacular cells form blood-testis barrier, provide support and nutrition to the developing spermatogenic cells, phagocytize the defected spermatocytes and synthesize androgen binding protein (ABP) and inhibin (Guyton and Hall, 1996). Sertoli cells are stimulated by follicle stimulating hormone (FSH) to produce androgen binding protein (ABP) and inhibin. ABP binds to testosterone secreted by the Leydig cells to facilitate the spermatogenesis process. On the other hand, inhibin in turn back regulates the secretion of FSH on pituitary gland. The intertubular or interstitial connective tissues are filled with blood vessels, macrophages, lymphocytes and a group of cells known as interstitial cells or interstitial endocrinocytes or Leydig cells (Setchell, 1978). Leydig cells are responsible for the production of androgen or male sex hormone testosterone following a process called steroidogenesis which is regulated by the luteinizing hormone (LH) secreted from pituitary gland. Hence, the spermatogenesis and steroidogenesis are intimately regulated by series of hormones that form an endocrinological regulatory system (Prakash, 2007).

1.3.2(a) Endocrinology regulation of male reproductive system

The endocrine system comprises a number of glands that release a product known as hormone into the adjacent tissues or into body fluids (Prakash, 2007). Hormone is defined as a chemical substance secreted by the glands of endocrine system into blood circulation system and carried to the target cells throughout the body. Hormones have powerful biological effect even at very low concentration. Chemically, hormones can be classified in the two major classes known as lipid soluble hormones such as steroid (testosterone and

estrogen) and thyroid hormones, distinguished from the water soluble hormones including peptides (insulin) and glycoprotein (FSH and LH).

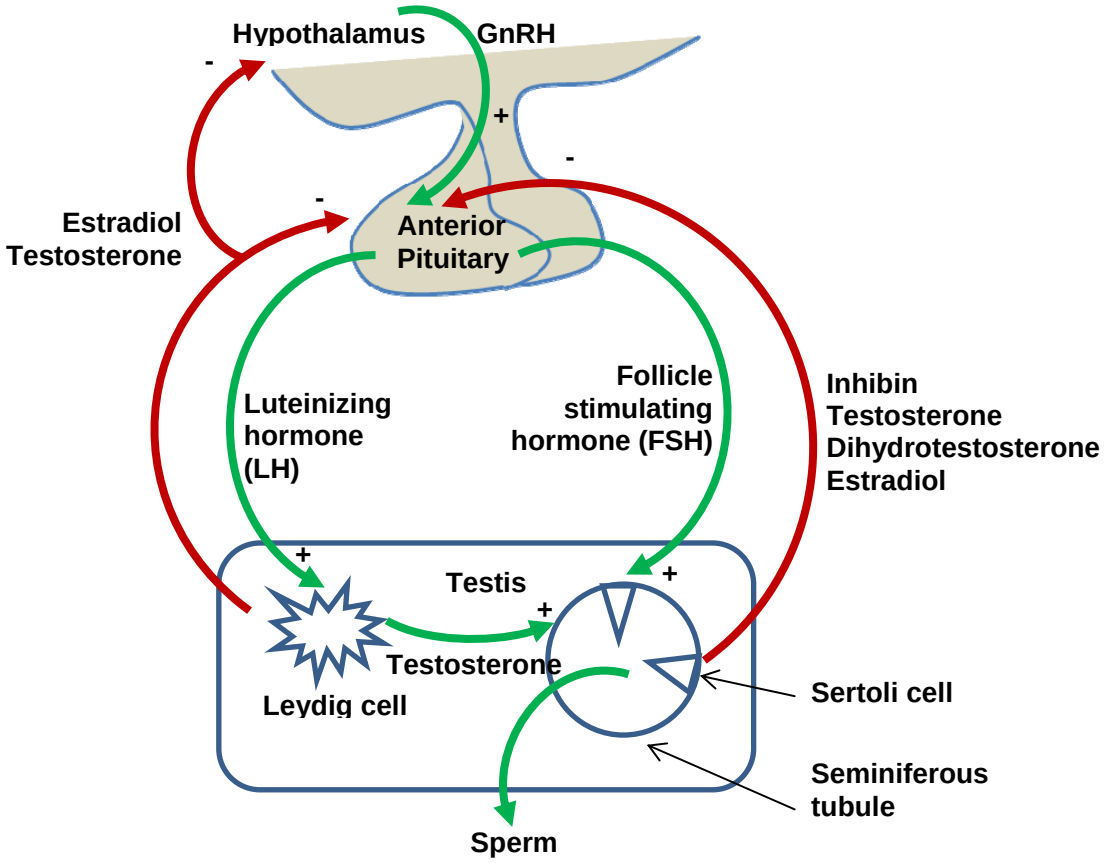


Figure 1.2. Hormonal regulation of testicular function by hypothalamus and pituitary known as hypothalamus-pituitary-gonadal axis. Modified from the figure depicted from Prakash (2007).

1.3.2(b) Hypothalamic-pituitary-gonadal axis

In general, the release of the gonadotropins, LH and FSH into the general circulation is stimulated by the GnRH from the hypothalamus. This allowed the depiction of a system known as hypothalamic-pituitary-gonadal (HPG) axis whereby the secretion of the gonadotropins modulates the secretion of androgens in the gonad and yet the elevated androgen level will inhibit back the secretion of the GnRH and gonadotropins (Prakash, 2007) (Fig. 1.2). In addition to inhibin, the major inhibitory effect of androgen on the

hypothalamus on gonadotropin release or known as negative feedback is mediated principally by estrogen and derives locally from the testosterone aromatization mainly in the germ cells and Sertoli cells (Carreau et al., 2011).

However, a recent study showed that GnRH releasing cells did not possess any sex steroid receptors including estrogen receptors but contained the kisspeptin receptor GPR54 and most of the kisspeptin producing cells possessed alpha estrogen receptor (α-ER) (Clarke, 2011). Therefore, estrogen may indirectly modulate the GnRH producing neurons by stimulating kisspeptin producing cells to secrete kisspeptin. The kisspeptin will in turn attenuate the GnRH production by acting on GnRH cells in hypothalamus (Clarke, 2011). Beside kisspeptin, a novel gonadotropin inhibitory hormone (GnIH) may also regulate the secretion of gonadotropins by modulating the GnRH cells (Clarke, 2011).

Development and maintenance of male reproductive system and secondary sex characteristics in males is mediated by testosterone (Prakash, 2007). The production of testosterone by Leydig cells in testes is influenced by LH or formerly known as interstitial cell stimulating hormone (ICSH) of the anterior pituitary. The secretion of LH in turn is regulated by gonadotropin-releasing hormone (GnRH), a decapeptide produced by hypothalamus. In fact, GnRH not only stimulates the release of LH, but also FSH in the anterior pituitary. By acting on the Sertoli cells in seminiferous tubules, FSH is mainly responsible for the initiation of spermatogenesis whilst full maturation of the spermatozoa from spermatids required both FSH and testosterone (Ruwanpura et al., 2010).

1.3.2(c) Spermatogenesis

Spermatogenesis is a process that produces the male gametes known as spermatozoa (Odell and Moyer, 1971). The process occurs in the coiled seminiferous tubules of the testis where the gonocytes produce germ cells that subsequently transform into the premature spermatozoa called spermatids (Prakash, 2007). Seminiferous epithelium is composed of

non-proliferating population of supporting cells known as Sertoli cells and a proliferating population of germ cells comprising spermatogonia, spermatocytes and spermatids (Setchell, 1978). The duration of cycle for the development from spermatogonia to mature spermatid is about 12.9 days and about 45-50 days to complete a spermatogenesis in rats (Clermont, 1972; Hisatomi et al., 1996).

Spermatogenesis can be divided into three distinct phases. The first phase is the mitotic multiplication and the growth of spermatogonia, which proliferates to spermatocytes and simultaneously maintains their number by renewal. In rat, spermatogonia undergo six mitotic divisions that subsequently become preleptotene spermatocytes. The second phase is the reduction phase involving primary and secondary spermatocytes, which have gone through the process of the meiotic division, produces genotypic haploid cells known as spermatids (Clermont, 1972). In the second phase, the spermatocytes in the meiotic division develop through leptotene, zygotene and pachytene to become secondary spermatocytes (Maeda et al*.,* 2000). The third phase involves the spermiogenesis of spermatids, which leads towards the differentiation and transformation of spermatids into a spermatozoon (Odell and Moyer, 1971; Clermont, 1972). Over the past four decades, major researches using innovative technologies such as immunocytochemistry, proteomic, genomic and genetically modified animal models have refined the roles of FSH and testosterone in the spermatogenesis. Earlier studies showed that FSH regulated the spermatogonial development whilst testosterone solely facilitated spermiogenesis (Ruwanpura et al., 2010).

In general, germ cell development involves the series of mitotic and meiotic divisions and it has been generally accepted that a stable population of germ cells is determined by the balance between the active division and death (apoptosis) of the cells (Ruwanpura et al., 2010). These processes are highly influenced by the FSH and testosterone at the Sertoli cells that regulate the survival of the germ cells as the germ cells do not contain hormone receptors (Ruwanpura et al., 2010). In fact, the biological effects of the germ cells

such as spermatogenesis are exerted through the receptors localize in/on the Sertoli cells that require junctional and functional communications between the germ and the Sertoli cells (Ruwanpura et al., 2010). Recent study has revealed that estrogen, which is derived from the irreversible aromatization of the testosterone in the Leydig cells, was controlling the apoptosis, maturation and differentiation of the haploid germ cells (Carreau et al., 2011). Hence, undoubtedly, testosterone is essential for spermatogenesis. The biosynthesis process of testosterone, commonly known as steroidogenesis, is virtually not associated with the spermatogenic cell apoptosis induced by estrogenic compounds (Alam et al., 2010).

1.3.2(d) Steroidogenesis

Steroidogenesis is defined as the biosynthesis of steroid hormones. Steroids synthesized in the sex organs, such as testes and ovaries are more commonly known as sex hormones. Cholesterol is the primary precursor of steroid hormone and the compound is synthesized from the precursor acetyl coenzyme A in the body (Solomons, 1996). In the Leydig cells, testosterone steroidogenesis can be virtually divided into two distinct upstream and downstream pathways. The upstream steroidogenesis pathway involves the initial binding of the agonist luteinizing hormone (LH), generation of cyclic adenosine monophosphate (cAMP), phosphorylation of protein kinase A (PKA) and transportation of cholesterol into inner mitochondria. In the downstream steroidogenesis pathway, cholesterol undergoes series of decarboxylation, oxidation and reduction processes involving a number of membrane bound cytochromic enzymes to produce testosterone (Fig. 1.3). Subsequently, the testosterone is transported into the Sertoli cell to the germ cells known as spermatocytes to facilitate the spermatogenesis process and also be further converted to estrogen by aromatase enzyme (CYP19) in seminiferous tubules.

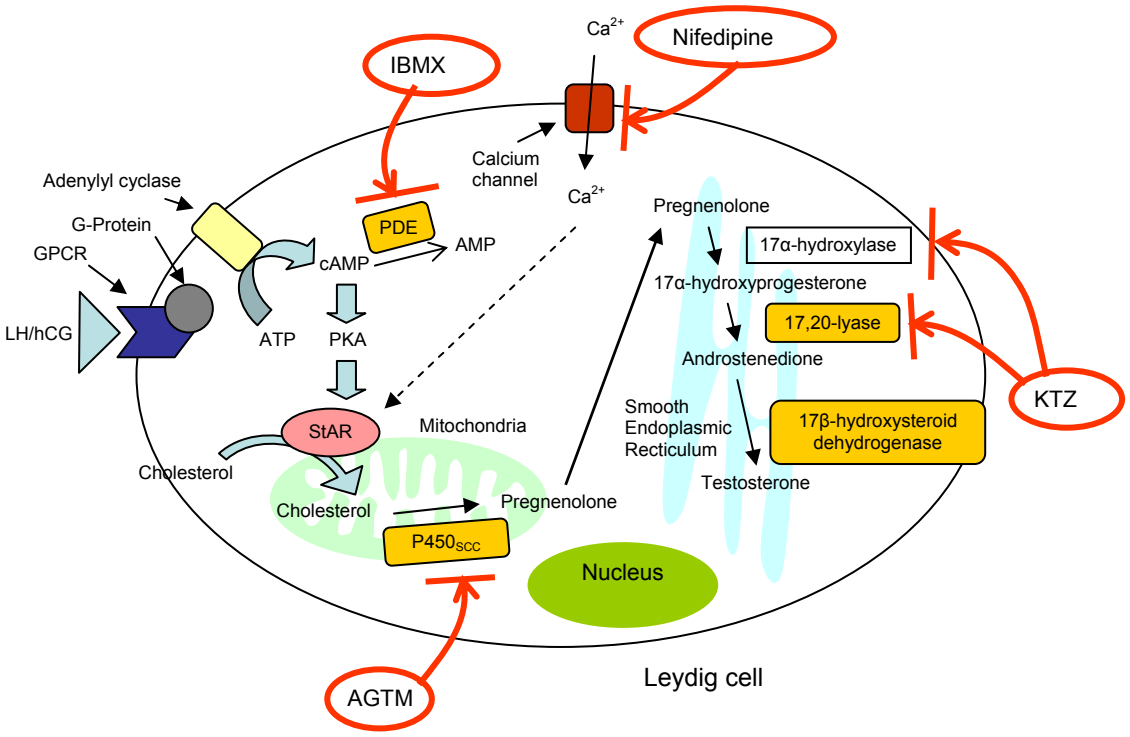


Figure 1.3 Steroidogenesis pathways in the Leydig cells

Luteinizing hormone (LH) is a type of gonadotropin, secreted from pituitary gland into blood stream, stimulated the Leydig cell to synthesize testosterone (Fig. 1.3). LH and human chorionic gonadotropin (hCG), a luteinizing-like hormone bind to the specific G Protein-coupled Receptor (GPCR), a seven-membered transmembrane receptor, activate the enzyme adenylyl cyclase to form secondary messenger cyclic adenosine monophosphate (cyclic AMP) (Browne and Bhalla, 1991). The cyclic AMP phosphorylates the protein kinase A (PKA) into the active form and the activated PKA in turn activates the steroidal acute regulatory protein (StAR) to deliver the cholesterol from cytoplasm (outer mitochondria) into the inner mitochondria (Stocco, 2000). In fact, the expression of the StAR genes was augmented with the elevated hCG level influenced by presence of calcium ion (Manna et al., 1999). Generally, cyclic AMP plays major role in the steroidogenesis and its concentration in the cell is maintained between the synthesis by adenylyl cyclase and the degradation by phosphodiesterases (PDEs) (Tsai and Beavo, 2011). Recent studies showed

that PDE4 and PDE8A modulated testosterone production in the mouse Leydig cells (Vasta et al., 2006; Tsai and Beavo, 2011). The inhibition of 3-isobutyl-1-methylxanthine (IBMX) on PDEs potentially elevates the cyclic AMP production (Vasta et al., 2006).

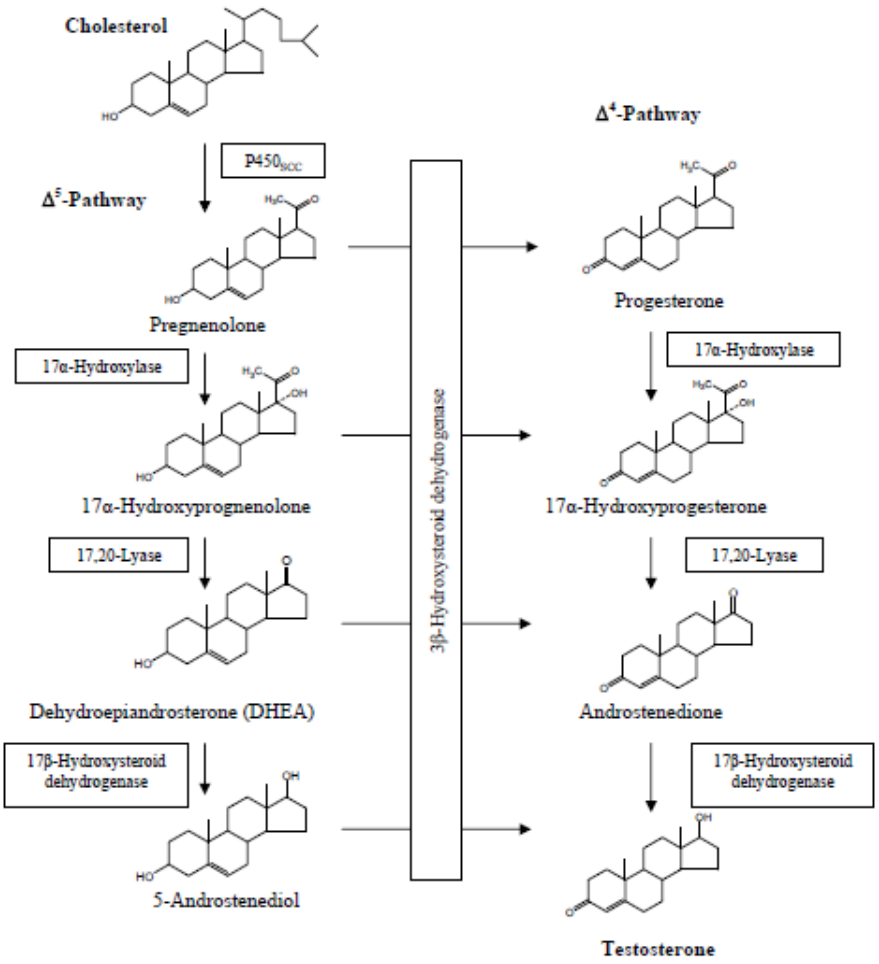


Figure 1.4 Steroidogenesis of testosterone from cholesterol.

In the downstream steroidogenesis, cholesterol is converted to pregnenolone through the series of complicated enzymatic decarboxylation and reduction process catalyzed by the cytochrome $P450_{SCC}$ (CYP11a), side chain cleavage enzyme in the inner mitochondria. The pregnenolone is then translocated to the smooth endoplasmic reticulum (SER) where testosterone is synthesized from either dehydroepiandrosterone (Δ^5) pathway or progesterone (Δ^4) pathway (Fig. 1.4). The conversion of pregnenolone to the testosterone involves a series

of cytochrome P450 enzymes such as 17α-hydroxylase (CYP17), 17,20-lyase (CYP17), 17β-hydroxysteroid dehydrogenase, 3β-hydroxysteroid dehydrogenase and $\Delta^{5,4}$-isomerase.

The inhibitory effects of the above enzymes have significantly led to the development of drugs in the steroidogenesis modulation. Aminoglutethimide (AGTM), a potent CYP11a and CYP19 inhibitor was initially developed as the first generation non-steroidal aromatase inhibitor but was not popularized due to the side effects (Chumsri et al., 2011). The rate limiting step in the production of androgenic steroids is CYP17, located in testicular Leydig cells, that catalyzed the 17α-hydroxylase reaction and the subsequent 17,20-lyase reaction to form the precursor for the steroidal hormone (Reid et al., 2008). Ketoconazole (KTZ), an imidazole antifungal agent, inhibited several cytochrome P450 enzymes, including CYP17, decreased levels of testosterone, androstenedione and DHEA (De Coster et al., 2008). However, the use of KTZ as a secondary hormonal therapy for prostate cancer was limited by its toxicity (Reid et al., 2008).

1.3.2(e) Cross-talk between testosterone, estrogen hormones and cyclic AMP

It is beyond doubt that testosterone is required for spermatogenesis. The production of testosterone is exerted by the cyclic AMP secondary messenger signaling pathway and influenced by the gonadotropins (Browne and Bhalla, 1991). Testosterone, the principal androgen and its metabolite mediate their effect mainly on the meiosis and spermiogenesis through the classical action by modulating the gene expression via the intracellular steroid binding androgen receptors and the non-classical actions that exert the rapid testosterone actions via membrane-associated androgen receptor (Rahman and Christian, 2007). The mitogenic effect of the androgen in the Sertoli cells was evidently proven following the activation of the two transcriptional factors, mitogen-activated protein kinase (MAPK) and cyclic AMP responsive element binding (CREB) protein by the testosterone (Fix et al., 2004). In order to ensure a stable germ cell population determined by the balance between the death (apoptotic) and proliferation of the cells (Ruwanpura et al., 2010), testosterone also acts co-

operatively with follicle stimulating hormone (FSH) as a survival factor of germ cells by regulating both the intrinsic and extrinsic apoptosis pathways (Meachem et al., 2005).

Beside the testosterone, estrogen, a steroid hormone also plays a role as the survival of the mammalian germ cells (Carreau et al., 2011). In the mammalian testes, estrogen was converted from the testosterone by the cytochrome P450 aromatase enzyme (CYP19) in the endoplasmic reticulum of the germ cells and the Leydig cells (Carreau et al., 2011). Thus, the inhibition on the CYP19 resulting in the reduction of the estrogen production has led to the development of the estrogenic-induced breast cancer chemotherapy drugs such as anastrozole (Arimidex®), exemestane (Aromasin®) and formestane (Lentaron®). Similar to the testosterone, estrogen acts via cyclic AMP signaling pathway with adenylyl cyclase stimulation effect (Aronica et al., 1994). However, estrogen showed the pro-apoptotic effect in addition to the anti-apoptosis effects on the different cell types. These divergent effects may probably correspond to the deviating effects possessed by the cyclic AMP where the molecule induced the apoptotic cell death in estrogen-responding MCF-7 breast cancer cell lines (Bøe et al., 1995) and human B-precursor cells (Myklebust et al., 1999) but prevented the $CD34^+$ cell from apoptosis (Negrotto et al., 2006).

Apparently, testosterone and estrogen are involved in the cell proliferation in spermatogenesis. Both steroidal hormones act towards the cyclic AMP secondary messenger signaling pathway to exert their biochemical effects. Testosterone binds to the androgen receptor to activate its biological effects and so does the estrogen. The effects of estrogen are mediated by two distinct receptors: estrogen receptor α (ERα) and estrogen receptor β (ERβ). Leydig cells express both ERα and ERβ (Akingbemi et al., 2003). The inactivation of ERα increased the gonadotropins and the testosterone (Akingbemi et al., 2003). Besides, the number of the Leydig cells was increased from the ERβ knockout mice (Gould et al., 2007). Thus, ERα may possess regulatory role in the steroidogenesis and ERβ may involve in the regulation of the Leydig cells proliferation.