THE EFFECTS OF STANDARDISED EXTRACT OF *EURYCOMA LONGIFOLIA* JACK (TAF 273) ON THE FEMALE RAT REPRODUCTIVE SYSTEM

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by

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
%	Percentage
μ**g**	Microgram
2BP	2-bromopropane
ACE	Angiotensin converting enzyme
AChE	Acetylcholine esterase enzyme
ACN	Acetonitrile
AIs	Aromatase enzyme inhibitor
Ang II	Angiotensin II
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
APS	Alkaline Picrate Solution
ARs	Androgen receptors
B0	Maximum binding
BMI	Body mass index
BW	Body weight
$^{\circ}$**C**	Degree of Celsius
cAMP	Cyclic adenosine monophosphate
CIA	The Central Intelligence Agency in US
CL	Corpus luteum
cm	Centimetre
CNS	Central nervous system
COX-2	Cyclooxygenase-II enzyme
CV	Coefficient of variation
D	Dioestrus stage of oestrus cycle
D0	Day zero of pregnancy
DHEA	Dehydroepiandrosterone

DHT Dihydrotestosterone

DPX Mountant for histology is a mixture of distyrene, a plasticizer, and xylene

Di 13α,21-dihydroeurycomanone

DSM-IV The Diagnostic and Statistical Manual of Mental Disorders-IV

DW Distilled water

E Oestrus stage of oestrus cycle

ED_{50} The dose of EE that is pharmacologically effective for 50 % effective dose

EDSTAC Endocrine Disruptor Screening and Testing Advisory Committee's

EE Ethynyl estradiol

EGF Epidermal growth factor

EIA Enzyme ImmunoAssay

Ep 13α(21)-epoxyeurycomanone

Est.R Oestrogen receptor

EV Estradiol valerate

Eu Eurycomanone

Fabs Final absorbance

FDA US Food and Drug Administration

FSH Follicle stimulating hormone

FSAD Female sexual arousal disorder

FSD Female sexual disorder

FRS Female reproductive system

FSR Female sexual response

GnRH Gonadotropin-releasing hormone

GM-CSF Granulocyte-macrophage colony stimulating factor

h Hour

hCG Human chorionic gonadotrophin

HPLC-UV High performance liquid chromatography- ultraviolet detector

HPG Hypothalamic-pituitary-gonadal axis

ICH International Conference on Harmonization

Iabs Initial absorbance

IL-6 Interleukin-6

IP Intraperitoneal injection

IS Number of implantation site

IUA Inner uterus adhesion

Kg kilogram

KΩ kilo-ohms

LH Luteinizing hormone

L-ovary Left ovary

LOD Limits of detection

LOQ Limits of quantification

LQ Lordosis quotient

LUT Lower urinary tract

M Metoestrus stage of oestrus cycle

MCF7 Breast cancer cell line

mg Milligram

mL Milliliter

MP Mobile phase

MPOA Medial preoptic area

MTs Microscopical techniques

n Sample size of animal

NGF Nerve growth factor

NHP Neonatal handling procedure

NO Nitric oxide

NSB Non-Specific binding

NSAIDs Non steroidal anti-inflammatory drugs

NOS Nitric oxide synthase enzyme

NS Normal saline

OC Oestrus cycle

OECD Organization for Economic and Cooperation and Development

ovx Ovariectomised

P Proestrus stage of oestrus cycle

P450scc P450 side-chain cleavage

PAF platelet-activating factor

pc Post-coitus

PCO Polycystic ovary

PCOS Polycystic ovarian syndrome

PDEs Phosphodiesterases

PGs Prostaglandins

PMSG Pregnant mare serum gonadotropin

Prog Progesterone

PRL Prolactin

R-ovary Right ovary

Prog.Rs Progesterone receptors

RT Retention time

SC Subcutaneous injection

SD Standard deviation

ss Stock solution

StAR steriodogenic acute regulatory

T Technique

TA Total activity

TAF 273 Methanolic standardised extract of *E. longifolia* Jack

Tmx Tamoxifen

T/CM Traditional and complementary medicine

TGF-β Transforming growth factor-*Beta*

TGF-α Transforming growth factor-*Alpha*

TP Testosterone propionate

USA United States

USM Universiti Sains Malaysia

WHO Woulrd Health Organization

w/w Weight per weight

VEC Vascular endothelial cells

VEGF Vascular endothelial growth factors

VO Vaginal opening

VS Vaginal smear

KESAN EKSTRAK TERPIAWAI *EURYCOMA LONGIFOLIA* JACK (TAF 273) PADA SISTEM REPRODUKTIF TIKUS BETINA

ABSTRAK

Eurycoma longifolia Jack telah menunjukkan kesan farmakologi yang berpotensi pada sistem reproduktif jantan. Suatu fraksi ekstrak methanol, TAF 273 menunjukkan penambahbaikan dalam fertiliti tikus jantan serta peningkatan dalam jumlah sperma yang signifikan. Objektif-objektif kajian ini ialah menguji hipotesis bahawa TAF 273 mungkin boleh menambah baik ovulasi serta fungsi-fungsi reproduktif lain seperti kehamilan dan perlakuan seks pada tikus betina. TAF273 telah diberikan kepada tikus betina pada keadaan fisiologikal dan patologikal tertentu dan beberapa parameter telah dinilai untuk melihat kesan biologinya.

Keputusan kajian menunjukkan bahawa TAF 273 secara signifikannya (p<0.01) meningkatkan pengovulan tikus normal yang subur (7.4 $\pm$ 1.4) dan hiposubur (11.0 $\pm$ 4.6) berbanding tikus kawalan (masing-masing 5.6 $\pm$ 1.4 and 3.4 $\pm$ 1.5). Ia menyebabkan penambahbaikan yang signifikan (p<0.001) dalam perlakuan seks pada tikus hiposubur , dalam tikus yang dirawat dengan TAF 273, peratus "lordosis quotient" selepas rawatan ialah 86.9% berbanding 22.7% sebelum rawatan. TAF 273 turut menunjukkan perlindungan yang signifikan terhadap kesan mudarat estradiol valerat (2 mg/rat) pada tisu-tisu ovari dan uterun tikus ovari polisistik. Tambahan pula, TAF 273 telah meningkatkan secara signifikan (p<0.05) dalam keteraturan kitaran esterus (OC) serta indeks kehamilan dalam tikus ovari polisistik aruhan

testosteron; tikus dirawat dengan testosteron+TAF273 (62.5 %) dan tikus dirawat dengan testosterone (37.5%) menunjukkan OC normal. Indeks kehamilan tikus dirawat dengan testosteron+TAF 273 dan testosterone ialah masing-masing 80% dan 36% . TAF 273 juga menyebabkan peningkatan signifikan (p<0.001) dalam paras estrogen urin pada peringkat prooestrous OC dalm tikus normal (14.0 $\pm$ 1.9 ng/mg kreatinin) dan hiposubur (2.9 $\pm$ 0.6 ng/mg kreatinin); sebelum rawatan, paras hormon ialah masing-masing 5.4 $\pm$ 0.6 and 1.6 $\pm$ 0.3 ng/mg kreatinin .

Kesan-kesan biologi TAF 273 ini pada fungsi sistem reproduktif betina mungkin disumbang oleh sifat antiestrogennya. Menggunakan esei uterotropik, TAF 273 menyebabkan penurunan signifikan (p<0.05) berat uterus tikus betina tidak matang. Akhirnya, ekstrak terpiawai TAF 273 yang digunakan dalam kajian ini disahkan mengandungi amaun kuasinoid yang signifikan. Bahan aktif utamanya lalah eurikomanon (15.3%; w/w), 13α(21)-epoksieurikomanon (12.4%; w/w) dan 13α(21)-dihidroeurikomanon (2.8%; w/w).

Kesan-kesan *E. longifolia*, sepertimana yang ditunjukkan dalam kajian ini menunjukkan bahawa *E. longifolia* Jack dan kuasinoidnya mungkin menambahbaik fertiliti tikus betina. Penambahbaikan dalam fertiliti tikus betina mungkin disebabkan aktiviti antiestrogenik kuasinoid-kuasinoid yang terdapat dalam TAF 273.

THE EFFECTS OF STANDARDISED EXTRACT OF *EURYCOMA LONGIFOLIA* JACK (TAF 273) ON THE FEMALE RAT REPRODUCTIVE SYSTEM

ABSTRACT

Eurycoma longifolia Jack has shown promising pharmacological effects on the male reproductive system. A fraction of methanol extract, TAF 273 has shown improvement of male rat fertility and an increment in sperm numbers significantly. The objectives of the present studies are to test the hypothesis that TAF 273 could improve ovulation and other reproductive functions such as pregnancy outcome and sexual behaviour in female rats. TAF 273 was administered to female rats in various physiological and pathological states and several parameters were assessed for its biological effects.

The results showed that TAF 273 significantly (p<0.01) increased ovulation in normal fertile (7.4 $\pm$ 1.4) and hypofertile rats (11.0 $\pm$ 4.6) compared with the control (5.6 $\pm$ 1.4 and 3.4 $\pm$ 1.5, respectively). It caused significant (p<0.001) improvement in the sexual behaviour of hypofertile rats; in rats treated with TAF 273, the lordosis quotient percentage after treatment was 86.9% compared with 22.7% before treatment. TAF 273 showed also significant protection against the detrimental effects of estradiol valerate (2 mg/rat) on ovarian and uterine tissues in polycystic ovarian rats. Moreover, TAF 273 significantly improved (p<0.05) the regularity of the oestrous cycle (OC), as well as pregnancy indices in testosterone-induced polycystic ovaries in rats; the rats treated with testosterone+TAF 273 (62.5%) and testosterone-treated rats (37.5%) exhibit normal OC. The pregnancy indices of rats treated with

testosterone+TAF 273 and the testosterone-treated rats were 80% and 36.0% respectively. TAF 273 also caused a significant (p<0.001) increase in the level of urine oestrogen in the proestous stage of the OC in the normal (14.0 $\pm$ 1.9 ng/mg of creatinine) and hypofertile rats (2.9 $\pm$ 0.6 ng/mg of creatinine); before treatment, the hormone levels were 5.4 $\pm$ 0.6 and 1.6 $\pm$ 0.3 ng/mg of creatinine, respectively. These biological effects of TAF 273 on female reproductive system function may be attributed to its antioestrogenic property. Using uterotrophic assay, TAF 273 caused significant (p<0.05) uterine weight reduction in immature females. Finally, the standardised extract of TAF 273 used in this study was confirmed to contain significant amounts of quassinoids. The major active constituents were eurycomanone (15.3%; w/w), 13α(21)-dihydroeurycomanone (12.4%; w/w) and 13α(21)-epoxyeurycomanone (2.8%; w/w).

The effects of *E. longifolia*, as shown in the present study indicate that *E. longifolia* Jack and its quassinoids may improve female fertility. This improvement in female fertility may be due to the antioestrogenic activity of the quassinoids contained in TAF 273.

CHAPTER 1

INTRODUCTION

1.1 Function of the female reproductive system

The female reproduction system (FRS) has two main roles: reproduction and sexual functions. For reproduction, it should have (1) the ability to produce ova, (2) receive spermatozoa, (3) provide a suitable environment for the fertilization of the ova by spermatozoa, (4) provide an environment for the development of the foetus, and (5) expel the developed foetus to the external environment (Barrett et al., 2009). The main reproductive organs and the central nervous system (CNS) form an integrated network that exerts a control on the hormonal and nervous systems. The female reproductive system consists of the ovaries, uterine fallopian tubes (also called oviducts or uterine tubes), and vagina (Barrett et al., 2009).

1.2 Reproductive organs and their physiological functions

1.2.1 Vagina

The vagina is a musculomembranous canal extending from the vulva to the uterine cervix. The vagina has several physiological functions and responds to nerve impulses and hormone actions. It is the main female reproductive part involved in sexual activities. The vagina is very important for receiving the spermatozoa (Barrett et al., 2009). Disorders in vaginal functions may lead to a decrease in sexual response and may lead to sexual dysfunction (this will be discussed in Chapter 5).

1.2.2 Uterus

The uterus is a muscular organ and is triangular in shape. The uterus has many important functions for successful reproduction: (1) permitting the travel of the sperm from the male, and (2) developing offspring which is implanted in the endometrial lining of the uterus and continues its development during the term of pregnancy (Barrett et al., 2009). Any abnormality in physiological function of the uterus may lead to infertility (Chapter 2 and 6).

1.2.3 Ovary

The ovaries are considered the primary female sex organ (gonads). Each ovary is in contact with the uterus and the fallopian tube via ligaments. Its primary role is the release of a mature oocyte, during each menstrual cycle, that is fully capable of fertilization, embryonic development, and to prepare the accessory reproductive organs for the pregnancy and birth of an offspring by producing steroid hormones. The basic functional units in the ovaries are the follicles. The depletion of this pool leads to reproductive senescence, and the total number of ovarian follicles is determined early in life (Barrett et al., 2009; Latini et al., 2010). Ovulation disorder is one of the main ovarian dysfunctions leading to infertility.

1.2.4 Fallopian tube (oviduct)

The fallopian tubes (also called uterine tubes or oviducts) transfer fertilised ova from the site for fertilisation to uterus. The oviduct (fallopian tube) plays important roles in mammalian reproduction, namely (1) the ovum picked up after ovulation. (2) The ovum is moved into ampulla where fertilisation occurs. (3) Sperm travels from a reservoir near the uterotubal junction toward the ampulla by the oviduct. (4) The oviduct

also provides a desirable microenvironment for the capacitation of spermatozoa, fertilisation, preimplantation development, and transport of the preimplantation embryos to the uterus. The oviduct's functions can be affected by substances, for example tobacco, which may lead to reduced fertility. Several studies have shown that substance exposure has an effects on the pick-up of the ovum and the transport of the fertilised ovum (Talbot and Riveles, 2005).

Table 1.1: Female reproductive organs and their function

Organs	**Functions**
Ovaries	Produce ova and sex hormones
Fallopian tubes	Conduct ova; location of fertilization
Uterus	Implantation of fetus
Cervix	Serves as a canal for menstrual blood on the way out, and semen on the way in
Vagina	Serve as birth canal and as an exit for menstrual flow

The gonad functions in the female are correlated with age. Before puberty, the ovaries only produce sex hormones, and after puberty they start to produce ova. These functions of the female gonads partially decrease at menopause.

1.3 Female function of reproductive time window in human

The functions of the female reproductive system (FRS), unlike other system functions, have time windows. The functions of the FRS can begin at puberty and end at reproductive caducity/menopause (Christan, 2007). Hence, this window is also called the fertility window. Fertility, according to the Practice Committee of the American Society for Reproductive Medicine (PCASRM) in collaboration with the Society for Reproductive Endocrinology and Infertility (SREI) (PCASRM and SREI, 2008), has been defined as the capacity to produce offspring.

The window of fertility could refer to the ova production time period during the reproductive age. Generally, the reproductive age in women is between puberty and menopause. The likelihood of conception remains relatively stable from cycle to cycle within individuals. Fertility is relatively decreased by about half among women in their late 30's compared to women in their early 20's as reviewed by the PCASRM in collaboration with the SREI PCASRM and SREI (2008).

Puberty and menopause are contrasting time periods in the female. In the human, puberty is the time at which a female begins the process of sexual maturation such as the menstrual cycle, and leads to the achievement of fertility. Menopause is defined as the time when the menstrual cycle is absent for 12 consecutive months in a female, provided no other biological or physiological cause can be identified. It is the end of fertility and the end of the childbearing years. In humans, the timing of natural menopause is variable and menopause usually occurs between the ages of 45 and 55 years (McGee and Hsueh, 2000). Another study reported that natural menopause can also occur in a woman at ages between 30 and 60 years because there are endogenous and exogenous factors influencing the time of menopause (Garai et al., 2004). The reduction or disturbance in function of the FRS window may lead to sub-fertility or infertility and sterility. Moreover, many internal factors play an important role in female fertility such as age, puberty onset age, menopause onset age, and ovum viability.

1.3.1 Female function of reproductive time window in rat

The rat's fertility window during the reproductive age is like that of the human between puberty and menopause. In rats, as a continuously ovulating animal, the signs of puberty are visible at the vaginal opening around 35–40 days after birth, and

menopause starts when the oestrus cycle becomes irregular, normally around 10–12 months of age (Knobil and Neill, 2006). In rats, natural menopause causes a change in the oestrous cycle, which ranges between irregular and persistent dioestrus or the oestrous stage which usually occurs at the age of 10–14 months (Knobil and Neill, 2006).

1.4 Sub-fertility, infertility and sterility

Infertility is one of the serious problems affecting both male and female. Reviews of recent studies from developed countries have found neither consistency nor consensus on the definition of infertility. In general, female infertility can be divided into unexplained and explained. Infertility with explained cause is sometimes called sub-fertility, and infertility due to unexplained reasons may be called infertility or sterility, as confirmed by an evaluation of the female reproductive system (Bretveld et al., 2006).

Explained infertility is further classified as primary or secondary and by pathologic type because the etiologic factors for each may differ. Primary infertility is said to occur when pregnancy is absent. Primary infertility describes a couple who has attempted, but never achieved, conception. Secondary infertility arises after having conceived at least once, regardless of the outcome, but being unable to conceive subsequently. Unexplained infertility describes a couple who has no abnormalities, but who are unable to conceive.

Sub-fertility describes stillbirth after a first successful birth of a child, despite being married, with no contraceptive use or breastfeeding, and a minimum of a three-year period of waiting (Sundby et al., 1998; Bolumar et al., 2000). Other researchers define sub-fertility as the inability to conceive a second time after conception had occurred.

The term ‘unexplained subfertility’ applies to the condition in which a couple, despite serious attempts, does not achieve pregnancy, while according to current knowledge no physiological or anatomical abnormalities can be found (Batstra et al., 2002). Infertility is clinically defined according to WHO as the inability to conceive after one or/and two years of regular, unprotected intercourse (WHO, 1993). Infertility in epidemiologic research is frequently defined as the inability to conceive after twelve months of unprotected regular sexual intercourse (Marchbanks et al., 1989; PCASRM and SREI, 2008). Twelve months is derived from the biological and clinical observations that about 90% of couples of normal fertility without using any form of contraception will conceive within a year (Cramer et al., 1979).

Infertility is different from sterility, which is the absolute and irreversible inability to conceive. Several epidemiological studies have reported that infertility can occur in a female during reproductive age (de Kretser, 1997; Adamson and Baker, 2003). Infertility appears in the female (57%) more than in the male (26%) (de Kretser, 1997; Adamson and Baker, 2003), because of the physiological nature of the female reproductive system. Demographic impaired fertility is often defined indirectly as the frequency of married women who fail to conceive a live born child after a suitable period of ‘time of exposure’, often five or even seven years according to The Central Intelligence Agency (CIA) in USA (CIA-US, 2010).

1.5 Causes of female infertility

Different epidemiological studies have documented causes of female infertility. They may be due to one or more of the following causes:

1. tubule factors
2. ovulation disorders
3. endometriosis
4. unknown causes

As shown in Fig. 1.1, the percentage for each of the causes listed above as 1, 2, and 4 is approximately around 27%–31%. Another minor cause of female infertility is endometriosis which has been found to be around 5.5% (de Kretser, 1997; Adamson and Baker, 2003).

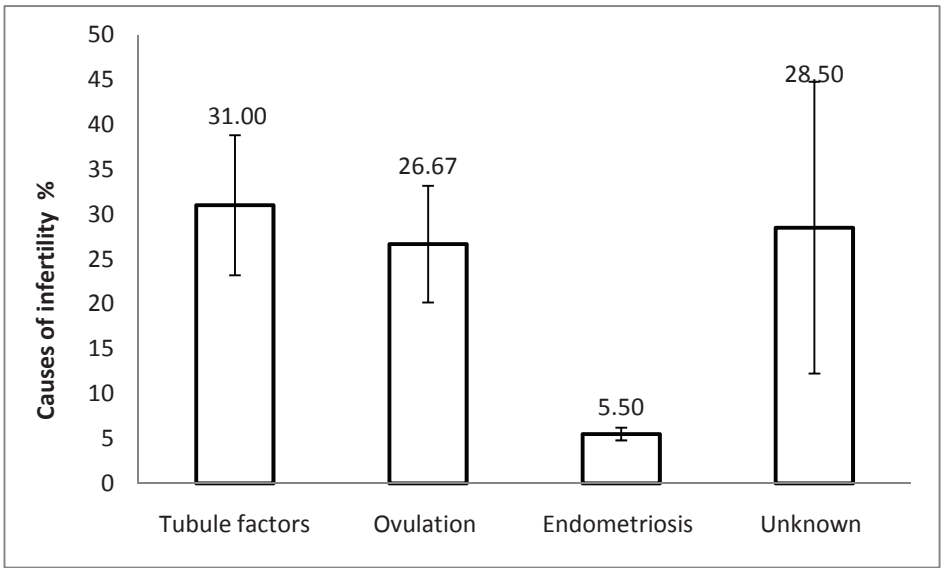

Figure 1.1: Causes of female infertility (de Kretser, 1997; Adamson and Baker, 2003)

1.5.1 Ovulation disorders as an important cause of female infertility

Polycystic ovarian syndrome (PCOS), an ovulation disorder, is the main cause of infertility in the female. It is characterised by a stop in ovulation and has been documented by several studies (see Fig. 1.2). Other causes are hypothalamic dysfunction, hyperprolactinaemia, and premature ovarian failure.

PCOS is a syndrome of unknown aetiology and is characterised by ovaries that are studded with fluid-filled cysts. Ovarian cysts develop either from follicles that fail to rupture completely (follicular cysts) or from corpora lutea that fail to degenerate (luteal cysts) (Knochenhauer et al., 1998).

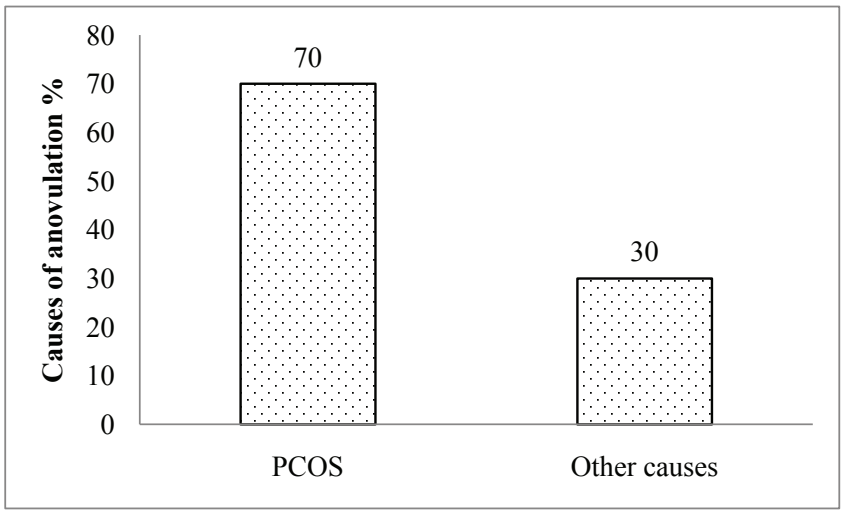

Figure 1.2: Factors affecting anovulation in female infertility (Knochenhauer et al., 1998)
(PCOS = polycystic ovarian syndrome)

1.6 Age as a factor in female infertility

During reproductive age, the older the female, the least fertile. In other words, the reproductive age is directly proportional to infertility (Fig. 1.3). Miscarriages are more common in older pregnant women. Hence, an age-related decline in female fertility begins many years prior to the onset of menopause, despite continued regular ovulatory cycles. This drop in fertility is associated with diminished ovarian reserve which is due to the depletion of the ova and to a gradual decline in average ovum quality (Adamson and Baker, 2003). Ovarian reserve is a term frequently used to describe a woman's reproductive potential with respect to ovarian follicle number and ovum quality (Adamson and Baker, 2003).

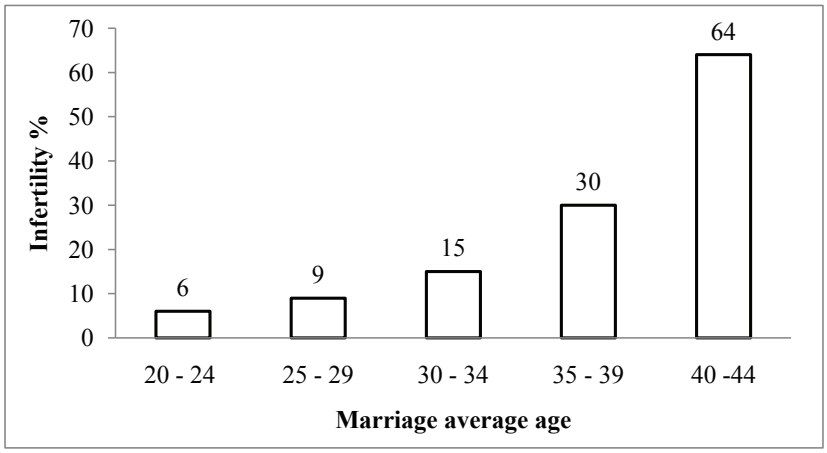

Figure 1.3: Age as a factor in female infertility (de Kretser, 1997; Adamson and Baker, 2003)

1.7 Miscellaneous factors affecting female infertility

Several environmental factors can increase infertility in the female, such as car exhaust. Benzo(a)pyrene, which is a common car exhaust compound, causes a significant reduction in fertility in test animals (Kristensen et al., 1995). A more than fourfold increase in spontaneous abortions has been documented in women workers

associated with electronic manufacturing units because of their exposure to a number of organic solvents such as xylene, acetone, etc. Alcohol reduces fertilisation success, a 50% reduction in conception having been found in experiments on test animals given intoxicating doses of alcohol 24 hours prior to mating (Sinclair and Pressinger, 2010).

1.7.1 Unhealthy lifestyle

Several negative lifestyle variables have been identified to have negative effects on female fertility and these factors are summarised in Table 1.2. A significant and progressive reduction in fertility was associated with an increase in the number of negative lifestyle variables (Hassan and Killick, 2004). Several epidemiological studies investigating negative factors on fertility showed the cumulative reduction due to negative life style factors on fertility, shown in Table 1.2. Time to pregnancy and conception probabilities are progressively longer, respectively, continually lower, with an increased number of negative lifestyle variables. Couples who had five or more negative variables were more likely to be sub-fertile compared with those without any of these variables (Hassan and Killick, 2004).

1.8 Female infertility treatments and their limitations

Despite the development of various assisted reproductive technologies in anovulation infertility treatment, the number of infertility cases are still increasing. In the last decade it has been reported that infertility has gradually increased by 66% in women since 1960 (Pusalkar et al., 2009). There are few classes of ovulation inducers such as nonsteroidal oestrogen agonist and antagonist like clomiphene citrate, antioestrogenic drug like tamoxifen, and a new class of drugs known as aromatase enzyme inhibitor (AIs), e.g., letrozole. However, these drugs are known to

Table 1.2: Lifestyle factors that may cause female infertility

Study No	Factors	Effect on reproductive function	References
1	Obesity (BMI>35)	Time to conception increases twofold	(Hassan and Killick, 2004)
2	Underweight (BMI<19)	Time to conception increases fourfold	(Hassan and Killick, 2004)
3	Heavy smoking (>15 cigarettes per day)	Significant increment in infertility	(Hassan and Killick, 2004)
4	Alcohol(>2drinks/day)	Relative risk of infertility increases to 60%	(Eggert et al., 2004)
5	Caffeine (>250 mg/day or 7 cups/day)	Fertility decreases 45%	(Wilcox et al., 1988; Hassan and Killick, 2004)
6	Illicit drugs	Relative risk of infertility increases 70%	(Mueller et al., 1990)
7	Toxins, solvents	Relative risk of infertility increases 40%	(Hruska et al., 2000)
BMI = body mass index			

be associated with 10% to 20% increased risk of multiple births (Mitwally et al., 2005; Holzer et al., 2006), endometrial cancer (Hughes et al., 2000; Marttunen et al., 2001), the development of drug tolerance to induced-ovulation, and a low rate of pregnancy. In women treated with clomiphene citrate, a discrepancy has been observed between ovulation and pregnancy rates (Goldfarb et al., 1968). Moreover, it has been reported that after clomiphene citrate treatment, the incidence of miscarriage is higher than expected in conception cycles (Goldfarb et al., 1968). Thus the side effects of ovulating agents mentioned previously indicate an enhanced need for new drugs for infertility treatment. Medicinal plants were considered one of the main sources of new drugs (Gurib-Fakim, 2006).

1.9 Other resources for drug to improve fertility and reproductive system performance

Currently, many experimental and clinical trial studies in the literature report positive effects of acupuncture in the treatment of female infertility (Stener-Victorin et al., 2000; Westergaard et al., 2006), but scientists have raised the concern that the outcome of acupuncture on fertility may be due to the placebo effect. Several reasons pointing to this include: (1) lack of standardization, (2) there is no repeated existing positive protocol of study; (3) in order to be accepted as a conventional therapy, acupuncture should have a beneficial effect on any medical condition and the outcome is due to a specific effect of the needle or to a placebo effect (Stener-Victorin and Humaidan, 2006). Out of 250,000 species only 6% have been investigated for biological activities and 15% for their chemical constituents; it looks increasingly likely that we have only succeeded in scratching the surface of this wonderful resource (Gurib-Fakim, 2006).

1.9.1 Herbal medicines used for the female reproductive system disorders

Several plants have been used for the enhancement of fertility. For most of them, the findings have been based on ethno-botanical claims. These plants include red clover (*Trifolium pretense*), Siberian ginseng (*Eleutherococcus senticosus* root) and Evening Primrose Oil (Dove and Johnson, 1999). Unfortunately, no scientific studies have been conducted but there are some case studies using mixture of herbs. The effect of plants and herbal products in the treatment of PCOS are shown in Table 1.3. The areas of biological action of the herbs on FRS have seen very few scientific studies and currently there is no clinical data on these herbs. These plants are also part of folklore indicating their use as female and male fertility promoters. They have been claimed to be a uterine tonic. They may possibly also contain a progesterone-like constituent, since they are also useful to help to prevent miscarriage, delay periods, and alleviate

painful periods. Korean Ginseng has been known to help increase sperm counts, testosterone levels, and sex drive in animal studies. It also has a traditional use in helping female fertility as well. The pharmaceutical industry has used *Dioscorea villosa* for decades in the production of steroids and hormones such as progesterone and cortisone. In its natural form, *Dioscorea villosa* helps prevent habitual miscarriage due to hormonal imbalance.

1.10 *Eurycoma longifolia* Jack

Eurycoma longifolia Jack from the Simaroubaceae family, locally known as Tongkat Ali, grows wildly in the jungle slopes of Malaysia. Their roots are popularly sought after as an essential ingredient in Malay herbal medicine. *Eurycoma longifolia* is also known as 'Tongkat Ali' or 'Penawar pahit' (bitter medicine for poison and pain) in Malaysia. It is known as 'Pasakbumi' in Indonesia, 'Iao-don' in Thailand and 'Cay ba binh' in Vietnam (Chan et al., 1998). These roots have been used as a male aphrodisiac following traditional claims of increase in virility and sexual prowess (Gimlette and Thomson, 1977). Recently, the male aphrodisiac activity of the plant has also been reported in animals (Table 1.4), but no similar report in women has been documented, except its use in folk medicine (Ab Rahman et al., 2007) after childbirth for the improvement of health (Kuan et al., 2007).

Table 1.3: Plant and herbal products used for treatment of polycystic ovarian syndrome

No	**Name of plant or herbal product**	**Type of study**	**Subjects**	**Outcome**	**Reference**
1	Shakuyaku-kanzo-to	Clinical	20 infertile Japanese with PCOS	Reduced testosterone level 5 (25%)	(Takahashi and Kitao, 1994)
2	Jinqi Jiang-tang Tablet	Clinical	24 obese women with PCOS	Improvement of carbohydrate metabolism and reduction in androgen biosynthesis in women with PCOS	(Hou et al., 2006)
3	Nourishing Yin	Clinical	43 women with PCOS	Significantly reduced the serum level of insulin in PCOS women	(Jia and Wang, 2006)
4	Korean Red Ginseng Extract	Experimental	Polycystic ovary-induced female rats using estradiol valerate (EV)	Lowered ovarian nerve growth factor (NGF) protein and NGF mRNA expression in EV-treated animal	(Pak et al., 2009)
5	Korean Red Ginseng Extract	Experimental	Polycystic ovary rat model induced by EV	Korean red ginseng extract showed regulation of NGF expression in female rats with Polycystic ovary	(Pak et al., 2005)
6	Korean Red Ginseng Extract	Experimental	Immature rats with pregnant mare serum gonadotropin (PMSG)	Improved oocyte quality, decreased androgens and increased 17β-estradiol serum levels preovulatory & postovulatory serum progesterone level.	(Yu et al., 2003)
7	Unkei-to	Clinical	100 patients	In the PCOS group 50.0% and 60.0% in non-PCOS group, successful ovulation has occurred	(Ushiroyama et al., 2001)
8	Tiangui Fang	Clinical	PCOS patients with clomiphene-resistant & high androgen and insulin	Induced ovulation, reduced high concentration of insulin. Restoration of menstrual cyclicity. Serum testosterone and body mass index were lowered	(Hou et al., 2000)
9	Sairei-to	Clinical	PCOS patients	Ovulatory rate was 70.6%. Serum LH, LH/FSH ratio were significantly decreased and with normal serum testosterone levels	(Sakai et al., 1999)
10	Bu Shen Hui Yu	Clinical	PCOS patients	Reduced the symptoms of PCOS	(Huang, 2008)
11	Bushen Huayu Qutan	Clinical	PCOS patients	Significantly lowered obesity and serum level of testosterone	(Wu et al., 2007)
12	Ganshao	Clinical	Clomiphene-resistant PCOS patients	Seven subjects were pregnant with ovulation rate being 89.5%	(Yang and Zhang, 2005)

Table 1.4: Aphrodisiac activities of *E. longifolia* roots in rodent

	***E. longifolia* extracts used**	**Animals**	**Duration of treatment**	**Aphrodisiac effect**	**Reference**
1	Crude root powder	Sexually sluggish and impotent male rats	acute (one day) and sub-chronic (12 days)	Reduced ejaculation latencies increased mounting% and ejaculating%	Zanoli et al. (2009)
2	Chloroform, methanol, butanol, and water extracts	Sluggish old male rats	10 days, twice daily	Increased sexual arousal of yawning and stretching	Ang et al. (2004)
3	Chloroform, methanol, butanol, and water	Middle-aged male rats	daily for 12 weeks	Enhanced accompanied by a decrease in animal hesitation time	Ang et al. (2003a)
4	Chloroform, methanol, butanol, and water extracts	Middle-aged mice and retired breeders	After 2 weeks of treatment	Enhanced sexual qualities by decreasing animal hesitation time for mounting	Ang et al. (2003b)
5	Chloroform, methanol, butanol, and water extracts	middle-aged, old-aged and retired breeders male rats	10 days, twice daily	Increased orientation activities towards receptive females	Ang et al. (2002)
6	Chloroform, methanol, butanol, and water extracts	Non-copulator male rats	3 and 8 months	Decreased the hesitation time of animals for coupling	Ang and Cheang (2001)
7	Chloroform, methanol, water and butanol extracts	Inexperienced castrated male rats	10 days	Improved sexual performance in mounting, intromission and ejaculation	Ang et al. (2000)
8	Chloroform, methanol, water and butanol extracts	Coupling experienced male rat	10 days, twice daily	Increased mounting frequency	Ang et al. (1997)

1.10.1 Scientific studies on *E. longifolia* Jack

Several biological, phytochemical and pharmacokinetic studies of *E. longifolia* Jack have been conducted. This literature review of *E. longifolia* was done by using SciFinder® version. A total of 123 titles of articles on *E. longifolia* were found. Articles on *E. longifolia* can be classified into patent studies or published studies in reviewed journals. Out of 123 (100%) studies, 67 (54.47%) were carried out to observe the effect of *E. longifolia* extract on animals. Fifty six (45.53%) from the total 123 were performed on phytochemistry and pharmacokinetic related work since 1962 until now. Different biological activities of *E. longifolia* have been tested. Approximately 12 activities have been documented for *E. longifolia*. These biological activities are the enhancement of sexual behaviour and libido, cytotoxicity, antimalaria, improvement of male fertility, effects on testosterone level in men, anticancer, antitumor, toxicity, weight loss remedy and reduction of fat mass in the body, anxiolytic, antiparasitic, antihypertensive, antiulcer, and antibacterial effects. The percentages of studies which have been done from the total 67 (100%) of biological studies are shown in Fig. 1.4.

The biological effect of *E. longifolia* related to the reproductive system will be elaborated in the next section because no studies have been carried out on the female reproductive system. However, several studies have been done on the biological activity of *E. longifolia* on the reproductive system of male sexual behaviour and the enhancement of fertility in fertile and infertile male rats.

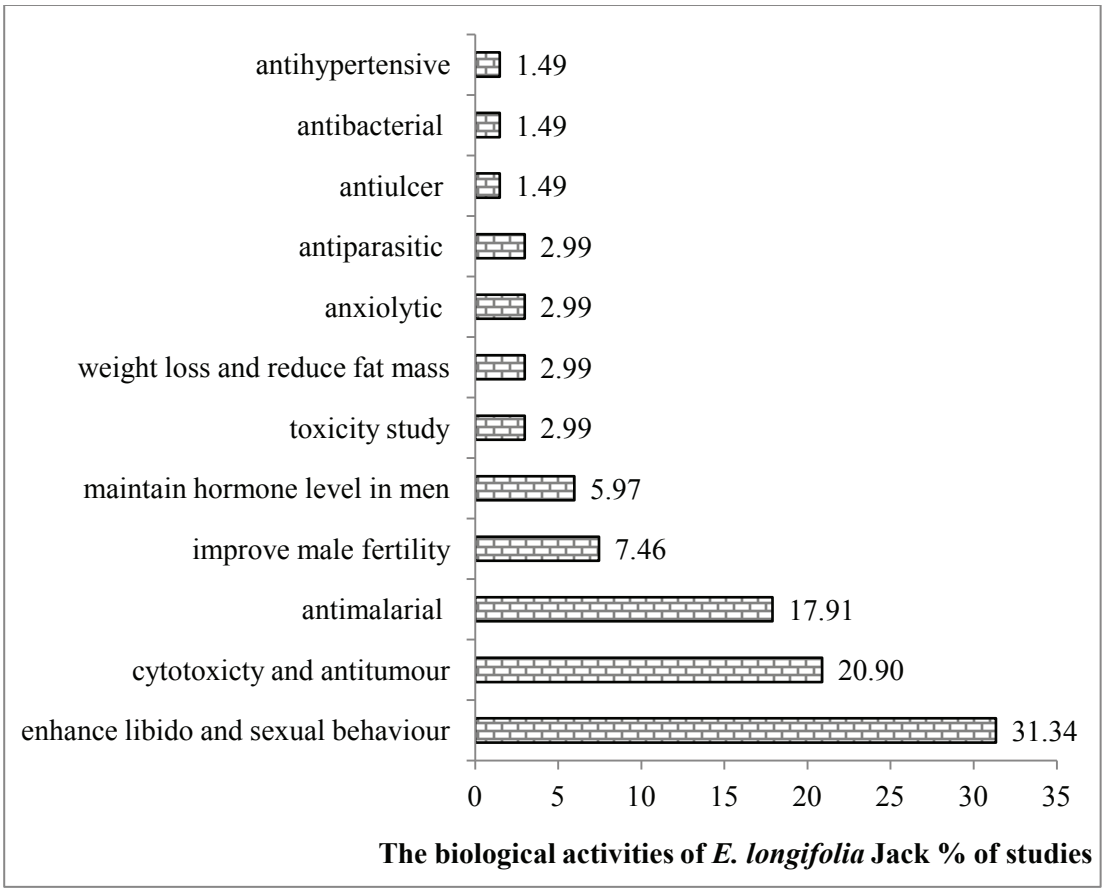

Figure 1.4: Percentage of biological activity studies that have been conducted on *E. longifolia* Jack

1.10.2 Biological activity of *E. longifolia* Jack on the reproductive system

1.10.2(a) Effect of *E longifolia* Jack on spermatogenesis

There are recent studies available on the effects on the male reproductive system of *E. longifolia* root, wherein it has been administered to cause an enhancement of male reproduction. Wahab et al. (2010) evaluated the effect of an aqueous extract of *E. longifolia* Jack on spermatogenesis in oestrogen-treated male rats treated with dose 8 mg/kg body weight once daily for fourteen consecutive days. Their results showed an increase in sperm counts and motility in the treated animals as compared to the control. Chan et al. (2009) investigated the effect of standardised methanolic extract *E. longifolia* Jack on spermatogenesis in *Andrographis paniculata*-treated male rats treated with various doses of *E. longifolia* (50, 100 and 200 mg/kg body weight once daily for

forty-eight consecutive days). Their results showed the sperm counts of rats were increased by 78.9%, 94.3%, and 99.2%, after given the extract alone of *E. longifolia* at doses of 50, 100 and 200 mg/kg respectively when compared with that of the control. The standardised methanolic extract of *E. longifolia* reversed low sperm count, poor motility, and abnormal morphology of the spermatozoa induced by the *A. paniculata* fraction.

Tambi and Imran (2010) in their clinical study investigated the effect of *E. longifolia* Jack aqueous extract on idiopathic male infertility in 350 patients treated with a dose of 200 mg. Their results showed improvement in all semen parameters in treated patients.

An *in vitro* study of the crude ethanolic extract of the root of *E. longifolia* showed decrease of the basal release in testosterone by rat Leydig cells, but increased production of testosterone by rat Leydig cells treated with human chorionic gonadotropin (Lin et al., 2001). There are numerous literature reports available on the aphrodisiac activities of *E. longifolia* roots, wherein it has been administered to increase male virility and sexual ability (Table 1.4).

1.11 Experimental study approaches

Infertility in the female is usually associated with biological processes in the body which are generally due to oestrogen regulation (McDonnell and Miranda, 2003). Potential clinical applications of antioestrogens and selective oestrogen receptor (ER) modulators are used in treatment of infertility.

i) Hence, the oestrogenic property of methanolic extract of *E. longifolia* Jack (TAF 273) would be evaluated by performing validated bioassays which are uterotrophic, and induced vaginal cornification (kindly see Chapter 2 & 3).

ii) Subsequently, the effect of TAF 273 would be tested on reproductive performance parameters such as number of offspring, pups, and ova number in female rats when given before mating (see Chapter 4).

iii) Infertile rats would be used to determine the effects of TAF 273 on PCOS. Infertility in rats would be induced by administration of testosterone or estradiol valerate. The effect of TAF 273 on ovulation would be investigated on ovulated normal female rats and non-ovulated female rats (see Chapter 4).

iv) In experimental animals as well in humans, sexual behaviour is associated with infertility. So TAF 273 would be evaluated on sexual behaviour in normal and hyposexual animal models as well as sex hormones which will be measured in urine at proestrus stage of oestrus cycle of rats (Chapter 5).

v) Adhesion is one of the causes of infertility in the female. The effect of TAF 273 on uterine adhesion would be investigated and adhesion would be induced by EV + coitus in female rats (Chapter 6).

vi) High performance liquid chromatography-ultraviolet detector (HPLC-UV) analysis would be developed and validated for simultaneous determination of three quassinoids and it would be used for standardisation of the methanolic extract of

E. longifolia Jack (TAF 273) (Chapter 7).

1.12 General objective

The general objective is to investigate the pharmacological effects of standardised methanolic extract of *E. longifolia* Jack (TAF 273) on the female reproductive system of rats. For more clarification, see the flow chart of the study in Fig. 1.5.

1.12.1 Specific objectives

1. To evaluate the oestrogenic effect of methanolic extract of *E. longifolia* Jack (TAF 273) using *in vivo* assays which include uterotrophic and vaginal cornification assays in female rats.

2. To investigate the pharmacological effects of TAF 273 on reproductive performance including sexual behaviour, ovulation, and ovarian hormones level in the urine, in fertile and infertile female rats.

3. To investigate the pharmacological effects of TAF 273 on polycystic ovary and uterine adhesion induced by sex hormone administration in rats.

4. To develop and validate HPLC method for standardising TAF 273.

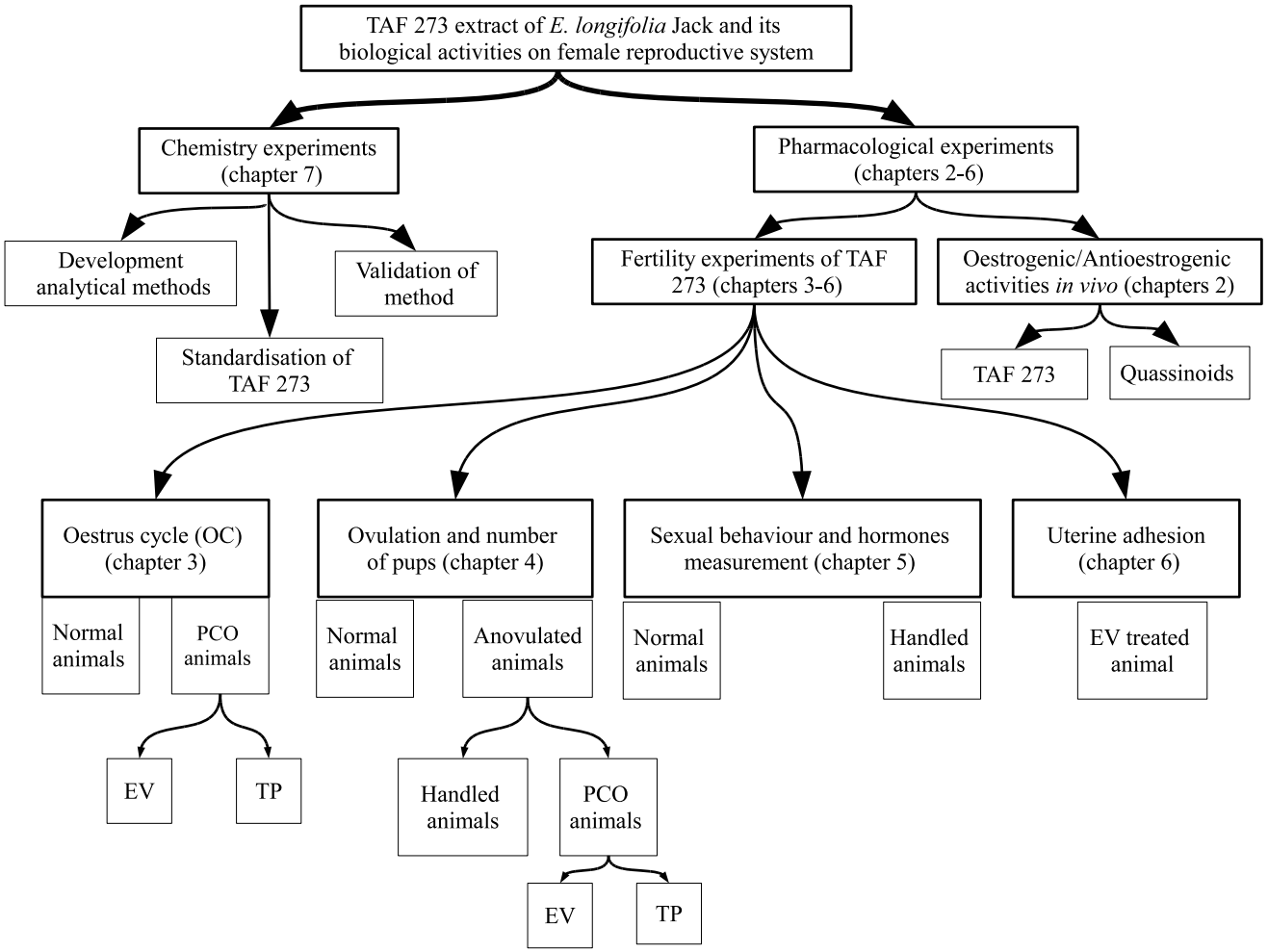

Figure 1.5: Flow chart of experimental studies on standardised extract of TAF 273 in the female reproductive system
EV= estradiol valerate; PCO= Polycystic ovarian; TP= Testosterone propionate

CHAPTER 2

OESTROGENIC ACTIVITY OF TAF 273 USING UTEROTROPHIC ASSAY

2.1 Background

2.1.1 Response of uterus to hormone changes

The physiological functions of the uterus start during puberty (Bridges et al., 1996). The uterus undergoes changes during menstrual phases. It prepares in advance for implantation of the fertilised ovum at the required time. The uterus receives and implants the fertilised ova where it develops into a foetus. Endometrial changes in the uterus associated with the monthly cyclical production of oestrogens and progesterone (Walter et al., 2010) by the ovaries operate through the following stages: (1) proliferation of the uterine endometrium; (2) development of secretory changes in the endometrium; and (3) the shedding of the outer layer of the endometrium, which is known as menstruation if no fertilisation of ovum has taken place.

Water content of the endometrial tissues ranges from 78% to 84% of the dry weight of the uterine tissue (Marshall and Senior, 1986), and it increases especially during both the proliferative and the luteal phase. Glucose is one of the most important metabolites in the endometrium which undergoes phosphorylation in the plasma membrane of the gland cells, and utilisation by the uterus is increased. These uterine responses are controlled by ovarian hormones, mainly oestrogen and progesterone (see Plate 2.1).

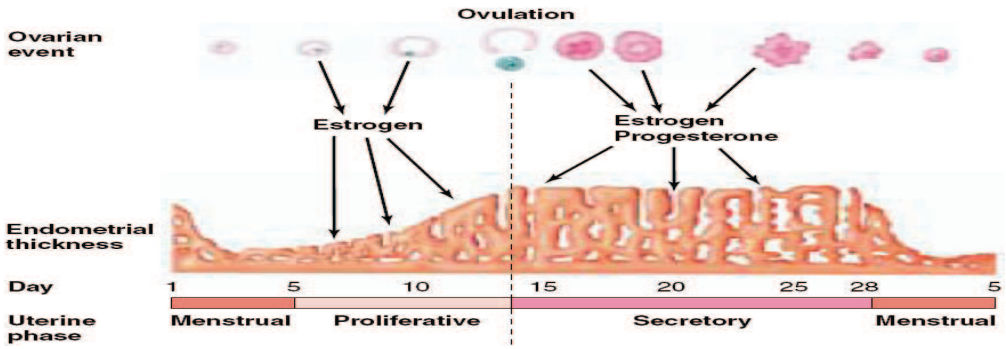

Plate 2.1: Uterine changes during the menstrual cycle in human (Vander et al., 2001)

2.1.2 Biphasic Uterine Response to Estradiol Changes in Physiology

Oestrogen stimulation is a complex process of epithelial proliferation and differentiation of the uterus that leads to the formation of a multilayered secretory endometrium. This uterine response can be induced by exogenous oestrogen and oestrogen mediator administration in human and experimental animals (Andrade et al., 2002; Zhang, 2010). Early studies in neonatal and prepubertal rodents have found that the uterine tissue proliferates in response to oestrogen (Katzenellenbogen and Greger, 1974).

Oestrogen has two types of receptors: Est.R-α and Est.R-β, which have been identified in different tissues (Brandenberger et al., 1999). The presence of oestrogen receptors (Est.R-α) in the tissue of the uterus is very important for uterine response. In Est.R-α-null uteri animals there is a lack of oestrogen-induced uterine epithelial proliferation (O'Brien et al., 2006). Treatment of ovariectomised or immature rodent animals with oestrogens (e.g., estradiol or diethylstilbestrol) has long served as an experimental model to mimic the uterine events that occur during the oestrous phase of the rodent cycle or immediately after the preovulatory estradiol surge (Kang et al., 2000). The morphological and biochemical changes that occur in the rodent uterus after oestrogen stimulation are known as the biphasic response and

underlie various changes according to their temporal pattern (Table 2.1) (Clark and Markaverich, 1988 cited in Knobil and Neill, 1993).

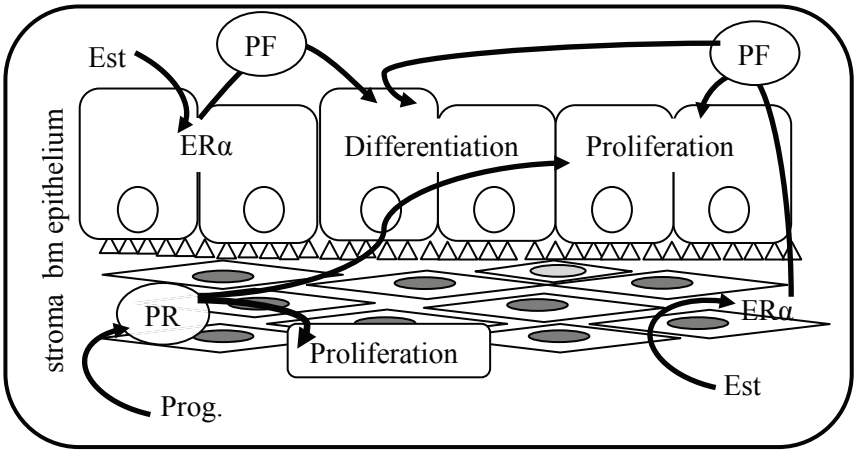

Plate 2.2: Local factors integrate sex hormone action on uterus (Knobil and Neill, 2006). Key words: Est = oestrogen Prog=Progesterone; PF= Paracrine Factors; Est.R= oestrogen receptor; Prog.R= progesterone receptors.

Table 2.1: Biphasic response of the rodent uterus to oestrogens

Early uterotrophic responses (within 6 hours)
Nuclear localization of oestrogen receptor
Activation of receptor-tyrosine kinase pathways
Changes in gene expression (induction/repression of 'early' genes)
Increased protein synthesis
Water imbibition (increase water absorption by uterus)
Hyperemia
Eosinophil infiltration
Albumin accumulation
Increased electrolytes
Lysozyme labilization
Increased cyclic nucleotides, prostaglandins and associated enzyme activity
Increased glucose metabolism and associated enzyme activity
Calcium influx
Increased lipid synthesis
Late uterotropic responses (within 24 hours)
Second peak of nuclear localization of oestrogen receptor
Changes in gene expression (induction/repression of 'late' genes)
Increased protein synthesis
Increased DNA synthesis
Epithelial proliferation in 'waves'
Cellular hypertrophy
Overall increase in uterine dry weight

Ref: (Clark and Markaverich, 1988 cited in Knobil and Neill, 1993)

2.1.3 Role of other important hormones and their receptors on uterine response

Progesterone plays a central role in the normal function of the uterus. The physiological effects of progesterone are mediated by interaction with specific intracellular progesterone receptors (Prog.Rs). Prog.Rs are expressed as two isoforms of

receptors, Prog.R-A and Prog.R-B (Conneely et al., 2002). Recombination experiments using uterine tissues from PR-null mice demonstrate that the antiproliferative functions of progestins in the uterine epithelium are paracrine mediated and require Prog.R-A in the uterine stroma (Ismail et al., 2003). It has been suggested that Prog.R-A contributes to the antioestrogenic activities of progesterone observed in the uterus. Prog.R-A has shown the ability to inhibit responses induced by both Prog.R-B and Est.Rs (Conneely et al., 2002). Prog.R-A is necessary for normal uterine function and female fertility, while Prog.R-B is important for the function of other tissues in the reproductive organs, such as the tissues of the mammary gland (Conneely et al., 2002).

Androgen receptors (ARs) are present in uterine tissues of many species (Pelletier, 2000; Pelletier et al., 2004), but the function of androgen signalling in the uterus remains unclear. In rodents, ARs are present in all uterine cell types but most highly expressed in the myometrium, where expression may be positively regulated by oestrogens (Weihua et al., 2000; Pelletier et al., 2004). In humans, ARs are also detected in the myometrial and endometrial uterine tissues and their levels increase during the proliferative phase (Mertens et al., 2001). Treatment of hypophysectomised rats with dihydrotestosterone (DHT) is known to cause increased uterine weight in rodents, indicating that ligand-dependent AR action can affect uterine phenotypes (Mobini Far et al., 2007). Elevated androgens are also postulated to provide for some uterine maintenance in ER-α-null females (Couse and Korach, 1999). More recent

studies have demonstrated that AR agonists cause increased myometrial thickness in the uteri of ovariectomised rats as well as inhibit oestrogen-induced epithelial proliferation (Nantermet et al., 2005).

Glucocorticoid receptors (GRs) are present throughout the cell types of the rodent uterus (Rhen et al., 2003). The uterus is not considered a 'classic' target tissue of glucocorticoid action, although limited experimental data indicate an increment in uterine weight GR-mediated glucocorticoid action is present in the uterus (Rhen et al., 2003). Surprisingly, stimulation of the GR signaling pathway by dexamethasone treatment in ovariectomised rats elicits a pattern of gene expression that is remarkably similar to oestrogen (Rhen et al., 2003).

2.1.4 Local factors role in integration of oestrogen uterine response

Several autocrine and paracrine actions of polypeptide growth factors have been reported to be an integral component of the uterine response to oestrogens (Plate 2.2). These factors included epidermal growth factor (EGF) (Huet-Hudson et al., 1990), insulin growth factor (IGF-1) (Andrade et al., 2002) and transforming growth factor-α (TGF-α) (Jovanovic and Kramer, 2010).

2.1.5 Oestrogenic biological assays

From the above explanation, it can be seen that oestrogen and their receptors are primary initiators of the biological processes in the uterus. So ER mediators can affect the uterus as well as fertility in females either negatively or positively depending on the net action on the reproductive system and the time taken. There are many tests and assays for the evaluation of the biological effects of ER mediators out of which many have been modified and some of them have even been validated by the

Organization for Economic and Cooperation and Development (OECD, 2007) and by the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) (Charles et al., 2005).

The assays for oestrogenic effects can be divided into two main types of assays, either *in vitro* or *in vivo*. *In vitro* assays can be sub-classified into two types of assays either cell-based assay or drug molecule receptor interaction assay. *In vivo* biological assays for oestrogenic activity can be classified into two types of assays: invasive and non-invasive. The *in vivo* assays will be elaborated here.

2.1.5(a) *In vivo* assay overviews

The measurements of oestrogenicity of compounds can be divided into two types as primary and secondary. The primary measurements which include uterine weight, vaginal cornification and female sexual receptivity which have been used for last 70 years were reviewed by Gray, (1998) and Baker, (2001). The secondary measurements which include age of puberty, and vaginal opening were reviewed by Parker, (1966 cited in Ema et al., 2010).

The cornification of vagina (non-invasive assay) depends on efficacy of oestrogenic property of the tested compound. In some cases weak oestrogen compounds may not induce cornification at all. So the endpoint of this assay is not sufficiently sensitive. For example, Dodds et al., (1938 cited in Papaconstantinou et al., 2000) found that diethystilbestrol produced full oestrus in ovariectomised rats but could not produce any vaginal cornification.

The uterotrophic assay (invasive assay) in which the uterine weight measured is generally more sensitive than considering the age of vaginal opening or vaginal

cornification; however, this is not always the case. Uterine histology and biochemical measures appear to be at least as sensitive to oestrogens as uterine weight (An et al., 2003).

2.1.6 Uterotrophic assay

Uterotrophic assay originally was used as a pharmacological screen to identify oestrogenic agents (Christian et al., 1998). The uterotrophic assay has been used since 1930. The first standardisation for uterotrophic assay was done in 1962. The validation has been done for this biological assay by OECD (Owens and Ashby, 2002) and EDSTAC (Charles et al., 2005). According to OECD and EDSTAC, rodent uterotrophic bioassay is one of the tests for the screening and testing of potential endocrine interfering compounds. The uterotrophic bioassay has undergone an extensive validation programme by OECD (2007) and EDSTAC (Charles et al., 2005). These validation studies showed the relevance and reproducibility of the bioassay. An increase in uterine weight is commonly considered to be one of the sensitive indicators of oestrogenicity when measured in the ovariectomised (ovx) or immature female rat or mouse after 1–3 days of treatment (Kang et al., 2000).

2.1.6(a) Principle of uterotrophic assay

This is based on the principle that the phase of uterine growth is under the control of oestrogens in the natural oestrous cycle. Uterine growth during the natural oestrous cycle is rapid and easily measurable within two days. When the natural source of oestrogen is not available, either because the animal is immature or because it has been ovariectomised, the growth of the uterus becomes sensitive to external sources of oestrogen. When exposed to such a source, the immature or ovx animal's uterus

will increase in weight due to the imbibition of fluid and cell proliferation initiated by oestrogen. Therefore, the main endpoint in this assay is uterine weight, measured using blotted/dry and wet weight of uterus. Chemicals that act as oestrogen agonists would be expected to cause a statistically significant increase in uterine weight, while oestrogen antagonists, when co-administered with a potent reference oestrogen, would be expected to decrease uterine growth. Uterotrophic assay evaluates the ability of a chemical to elicit biological activities consistent with agonists or antagonists of natural oestrogens (e.g. $17\beta - estradiol$). However, its use for agonist detection is more common than for antagonists (OECD, 2007).

2.1.6(b) Animal models used for uterotrophic assay

The type of animal used is important for the uterotrophic bioassay. Sensitivity relies on an animal test system in which the hypothalamic-pituitary-gonadal (HPG) axis is not functional, leading to low endogenous levels of circulating oestrogen. This ensures a low baseline uterine weight and a maximum range of response to administered oestrogens. Immature females after weaning and prior to puberty and young adult females after ovariectomy with adequate time for uterine tissues to regress are two oestrogen sensitive states in the female rodent that meet this requirement (OECD, 2007).

According to OECD (2007) and (EDSTAC cited in Charles et al., 2005), currently two animal models have been used in uterotrophic assay. These animal models of rats are ovariectomised adult female (ovx-adult) and the immature non-ovariectomised (immature). Both animal models have shown comparable sensitivity and reproducibility in studies done by the OECD validation test program. However, the immature animal model has an intact HPG axis, which causes a somewhat less specific but covers a larger scope of investigation than the ovariectomised animal.

Uterotrophic assay in an immature animal has the ability to respond to stimulation from the presence of substances that interact with the HPG axis rather than just the oestrogen receptor (ER). Moreover, the method using immature rats has been given preference due to animal welfare and ethics where surgical pre-treatment of the animals are prevented and also a possible reduction of those animals entering oestrus stage.

2.1.6(c) Criteria used in uterotrophic assay

Before running the uterotrophic assay, some points must be taken into consideration in order to reduce the variation. According to OECD (2007) and EDSTAC (Charles et al., 2005), these points are animal preparation, age of animal, route of administration of tested compound or mixtures, and removal and preparation of the uterine tissues for weight measurement. In the intact (i.e., ovaries present versus castrated or ovariectomised) juvenile female rat, the age at which treatment is initiated (typically 19–21 days) and the duration of treatment are critical variables that affect uterine weight. Exposure duration longer than 3 or 4 days or the use of juvenile females 24–25 days of age at the start of the study is not recommended because of the potential confounding of the results of the treatment effect with the onset of natural estrous cyclicity and its concurrent fluctuations in uterine weight and histology.

2.1.6(d) Modified uterotrophic assay (intraperitoneal)

In uterotrophic assay, the recommended routes of administration of the testing agents are two: oral, and subcutaneous. In the modified uterotrophic assay, the recommended route of administration is intraperitoneal according to the EDSTAC guideline (Charles et al., 2005). The rats are injected intraperitoneally with the test agent daily

for three days. The intraperitoneal injection method may enhance the sensitivity of the assay and is capable of detecting the oestrogenic potential of a compound that has been cited as an example of a compound not detectable by the recommended routes such as the subcutaneous route, e.g., methoxychlor (Laws et al., 2000).

2.1.6(e) Sample size

According to several guidelines such as OECD (2007) and EDSTAC guidelines (Charles et al., 2005), six animals per group has been used to carry out the analysis in uterotrophic assay. In these experiments, six or more animals have been used after external factors such as room temperature, number of pups per mother, and date of birth have been adjusted.

Several plants and their compounds have been tested on the uterus of rats to determine the oestrogenic activity using uterotrophic assay (Umberger, 1963; Turnbull et al., 1999; Breinholt et al., 2004; Kretzschmar et al., 2005; Circosta et al., 2006; Fazliana et al., 2009; Arbo et al., 2009; Möller et al., 2010).

TAF 273 standardised extract of *E. longifolia* Jack would be investigated for possible oestrogenic or antioestrogenic activity by using uterotrophic assay. Previous *in vitro* studies on *E. longifolia* extracts and pure compounds have shown antioestrogenic activity on breast cell line (MCF7) (Tee et al., 2007). In Malay traditional medicine, *E. longifolia* has been used by women after delivery (Ab Rahman et al., 2007; Kuan et al., 2007). The confirmation of oestrogenic or antioestrogenic activity will give an indication whether the extract induces fertility or antifertility effects. Further experiments would be designed based on the results of these studies.

2.1.7 Objectives

The objective of this section was to investigate the oestrogenic/antioestrogenic affect of standardised methanolic extract of *E. longifolia* Jack (TAF 273) and two pure compounds isolated from the extract.

2.2 Materials and Methods

2.2.1 Chemical used

	Chemicals	Sources
1	Ethynyl estradiol (EE)	Sigma Chemical Co., USA
2	Tamoxifen	Sigma Chemical Co., USA
3	Sesame oil	Sigma-Aldrich Chemie GmbH, Netherlands
4	Tween 20	Sigma Chemical Co., USA
5	Sodium chloride	Ajax Chemicals, Australia

2.2.2 List of Equipment

1	**Equipment**	**Sources**
2	Analytical balance (measures up to 210.0 g)	Ohaus, Switzerland
3	Animal balance (measures up to 400.0 g)	Harvard, England
4	Analytical microbalance	Sartorius, Germany
5	Micropipettes (10–100 µl and 100–1000 µl)	Eppendorf, Germany
7	Stirrer/Heater	IKA-WERKE GMBH & Co., Germany
9	Sonicator	UL-10, Lab Companion^{TM}, Korea
10	Vortex	Certomat® MV, Germany

2.2.3 Plant extract and pure compounds

The TAF 273 methanolic extract of *E. longifolia* Jack and its isolated compounds, eurycomanone (Eu), and 13α,21-dihydroeurycomanone (Di) (Appendix A) were provided by Professor Chan Kit Lam of the Pharmaceutical Chemistry Laboratory, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Penang.

2.2.4 Preparation of chemicals and *E. longifolia* Jack extract

2.2.4(a) TAF 273 Preparation

The TAF 273 standardised extract (100 mg) was mixed with 20 mL of normal saline in a conical flask. The mixture was then sonicated (UL-10, Korea). The TAF 273 solution (final concentration of 5 mg/mL) was used as stock solution for 0.5 mg/kg, 5 mg/kg and 10 mg/kg dosages. The TAF 273 stock solution was stored at 4 °C during the experiment.

2.2.4(b) EE dosage preparation

Ethynyl estradiol (EE) (0.09 mg) was mixed with 3 mL of 20% Tween 20 and the volume made up to 5 ml with normal saline. The mixture was mechanically mixed using vortex mixer. The final concentration was 18 µg/mL and used as stock solution (ss). EE at concentration 1.3 µg/mL was prepared from stock solution by withdrawing 108 µL of stock solution and addition of normal saline to make up of 1.5 mL solution. EE solution was stored at 4 °C.

2.2.4(c) Tamoxifen (Tmx)

Tamoxifen (1 mg) was weighed and dissolved in 0.5 mL of 20% Tween 20 and the volume made up to 1 mL with normal saline solution giving a final concentration of 1 mg/mL. The Tmx solution was mixed by vortex and then stored at 4 °C.

2.2.4(d) Eurycomanone (Eu)

Eurycomanone (1 mg) was weighed and dissolved into 1 mL of normal saline solution giving a final concentration of (1 mg/mL). Finally, the eurycomanone solution was vortexed and stored at 4 °C.

2.2.4(e) 13α,21-dihydroeurycomanone (Di)

13α,21-dihydroeurycomanone (1 mg) was weighed and dissolved into 1 mL of normal saline solution giving a final concentration of 1 mg/ml. Finally, the solution was vortexed and then stored at 4 °C.

2.2.5 Animals

Sprague Dawley adult rats 10–12 weeks old and weighing 180–220 g were obtained from the Animal Research and Service Centre of Universiti Sains Malaysia (USM), Penang and housed singly in cages. The initial body weight of each animal was recorded. The protocol was approved by the Animal Ethics Committee of USM under approval number USM/PPSF/50(092)jid.2 (Appendix G).

2.2.5(a) Immature female rats with exact date of birth

Each female with defined oestrous cycle with a proven fertile male were kept together overnight starting either from the evening of their proestrous stage or morning of oestrous stage. The following morning, the vaginal smear from each female was inspected for mating signs. A sperm positive vaginal smear on post-coitus (pc) confirmed that mating had transpired and was designated as day zero (D0) of pc. The mated female and male were separated and returned to their original cages. Three to four mated females were housed in one cage.

Approximately 7 days before delivery, all female rats were housed individually, and daily between 9–10 am the delivery and the presence of pups were checked regularly. On first day of birth, the pups were gathered from eight to ten per litter by randomly selecting them. Neonatal female rats at day 21 of their postnatal were separated, their body weights were recorded and randomly allocated to different experimental groups.

2.2.5(b) Animal housing and identification

All animals were housed in an air-conditioned room ($22 \pm 2\ ^{\circ}C$), under a 12:12 h light–dark cycle (lights off at 7:00 PM) either in the Animal Research and Service Centre, Universiti Sains Malaysia (USM), Penang or at the Pharmacology Department Laboratory, School of Pharmaceutical Sciences, USM. Food and water were provided *ad libitum*. Permanent markers with different colour were used for identification of animals. In addition, cage labels were also used to identify the groups.

2.2.6 Uterotrophic assay

Uterotrophic assays were followed according to the previously described method of Odum et al. (1997) and Owens and Ashby (2002) with slight modification. Immature Sprague Dawley female rats, aged 21 days (body weight 35–45 g) were randomly allocated into the different groups of treatment. The animals were weighed daily and sacrificed 24 h after the final treatment. The body of the uterus was excised just above its junction with the cervix and at the junction of the uterine horns with the ovaries (Odum et al., 1997; OECD, 2007). The excised uterus was trimmed free of fat. Uterine wet and blotted weights were recorded. The wet weight includes the uterus and the luminal fluid contents. The blotted weight is measured after the luminal contents of the uterus have been removed. Each uterus was individually processed to remove the luminal fluid. Both uterine horns were pierced. The uterus was placed on gauze and gently pressed with a second piece of gauze to completely remove the luminal fluid. The uterine weight was expressed as a percentage of the corresponding control.

2.2.7 Experimental design

A preliminary study was conducted to evaluate oral, subcutaneous and intraperitoneal administration effects of TAF 273 on uterine weight of rats. The oral and subcutaneous routes of administration showed no significant difference on uterine weight (result shown in Appendix B). Intraperitoneal route showed significant difference between rat treated groups. Hence the study was performed using the intraperitoneal route since the latter route showed a significant response. EE was used as positive control and it was administered intraperitoneally. A dose response study of EE was performed. EE was chosen for of the following reasons: (1) EE is recommended by OECD (Gelbke et al., 2004) and EDSTAC (Clode, 2006). (2) EE

is more potent than oestrogen (Mukherjee et al., 2003). (3) EE has been used as positive control in many studies (Kang et al., 2000; Kanno et al., 2001; Kamata et al., 2005).

2.2.7(a) Determination of ED_{50} of uterotrophic response of EE in immature rats

Different doses of EE (0.02, 0.2, 2, and 4 µg/kg) were administered intraperitoneally to different groups of immature (21-day-old) rats weighing 35–45 g (n = 6 for each group) for three consecutive days. On the fourth day, assays similar to that described in Section 2.2.6 were performed. Uterine weight was normalised as percentage increase to calculate the ED_{50} of EE. ED_{50} was calculated using the regression equation from the dose response curve of EE.

2.2.7(b) *In vivo* oestrogenic effect of TAF 273 on uterus of female rats

Immature (21-day-old) Sprague Dawley female rats weighing 35–45 g were randomly divided into five groups (n = 6 for each group). They were treated intraperitoneally with vehicle (group 1), 4 µg/kg/d EE (group 2), 10 mg/kg/d TAF 273 (group 3), 5 mg/kg/d TAF 273 (group 4), and 0.5 mg/kg/d TAF 273 (group 5) for 3 consecutive days. The same uterotrophic assay procedure described in Section 2.2.6 was followed. Results were expressed as a percentage of the control relative uterine weight to the body weight.

2.2.7(c) *In vivo* antioestrogenic effect of TAF 273 (10 mg/kg) on uterus of female rats

Immature (21-day-old) rats weighing 35-45 g were randomly divided into three groups (n = 6 for each group). They were treated intraperitoneally with vehicle (Group 1), 1.3 µg/kg/d EE (group 2) and 10 mg/kg/d TAF 273 co-administered with 1.3 µg/kg/d EE (group 3) for three consecutive days. At 24 h after the final dose, measurements

were taken in the same manner as described in Section 2.2.6. The results were expressed as a percentage of the control relative uterine weight to body weight.

2.2.7(d) *In vivo* antioestrogenic effect of quassinoids on uterus of female rats

The 21-day-old animals weighing 35-45 g were randomly divided into five groups (n = 6 for each group). Groups 1 and 2 were treated intraperitoneally with vehicle and 1.3 µg/kg/d ethynyl estradiol (EE), respectively. Groups 3, 4, and 5 were treated intraperitoneally with combinations of 1 mg/kg/d 13α,21-dihydroeurycomanone (Di) and 1.3 µg/kg/d EE, 1 mg/kg/d eurycomanone (Eu) and 1.3 µg/kg/d EE, and 1 mg/kg/d tamoxifen as positive control and 1.3 µg /kg/d EE, respectively. All the treatments were performed for three consecutive days. The animals were weighed daily and sacrificed 24 h after the final treatment. The uterus was excised; similar procedures as previously described in Section 2.2.6 were performed. The results were expressed as a percentage of the control relative uterine weight to body weight.

2.2.8 Statistical analysis

The data were expressed as mean ± SD. Tests of normality (Kolmogorov-Smirnov) and homogeneity (Levene's test) were done. Parametric data involved statistical comparisons between more than two groups were analysed with one-way analysis of variance (ANOVA) followed by *post hoc* Tukey HSD multiple comparison tests. Nonparametric data were analysed with the Kruskal-Wallis test followed by Mann-Whitney test and Wilcoxon test analyses. Differences were considered to be statistically significant at a probability level of 5%. SPSS® ver. 15 which is subscribed to by the Universiti Sains Malaysia was used for data analysis.

2.3 Results

2.3.1 Effective dose of EE (ED_{50})

The dose of EE which increased wet uterine weight by 50% (ED_{50}) is 1.3 µg/kg. The ED_{50} was obtained from the following equation which was generated from the dose response curve of EE (Fig. 2.1).

$$y = 38.37X$$

y = the increment of uterine weight, X = dose of EE in µg/kg) (Fig. 2.1).

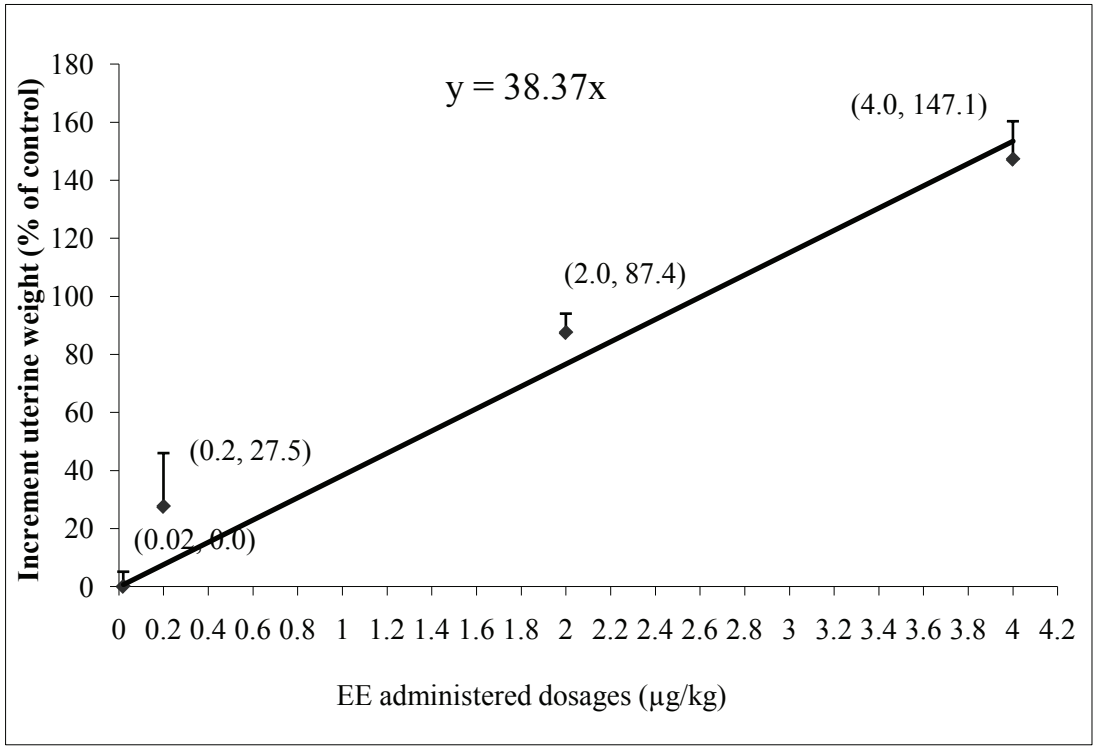

Figure 2.1: Dose response curve of EE on uterotrophic activity in immature rats
EE = ethynyl estradiol

2.3.2 Oestrogenic effect of TAF 273 on uterus of rats

Fig. 2.2 illustrates the intraperitoneal (IP) administration effects of TAF 273 at different doses on the uterine wet weight percentage of the immature female rat groups. The TAF 273 doses (0.5, 5.0, and 10 mg/kg/d) decreased the uterine wet weight to 94.7%, 92.9%, and 74.1%, respectively, when compared with the control treated (100.0%). However, only the highest TAF 273 dose (10 mg/kg/d) showed significant (p<0.05) difference compared with the control. In contrast, the standard drug EE at 4 µg/kg/d significantly (p<0.01) increased uterine wet weight (292.0%) in immature rats compared with the control.

Fig. 2.3 illustrates the effects of the different TAF 273 doses on the uterine blotted weight percentage of the immature female rat groups. The 0.5, 5.0, and 10 mg/kg/d TAF 273 doses decreased the uterine blotted weight to 84.9%, 92.3%, and 59.7%, respectively, compared with the control (100.0%). Only the highest TAF 273 dose (10 mg/kg/day) produced significant (p<0.05) reduction in the uterine blotted weight (59.7%) when compared to control. A dose of 4 µg/kg/d EE significantly (p<0.01) increased the uterine blotted weight by 257.9% compared with the control.

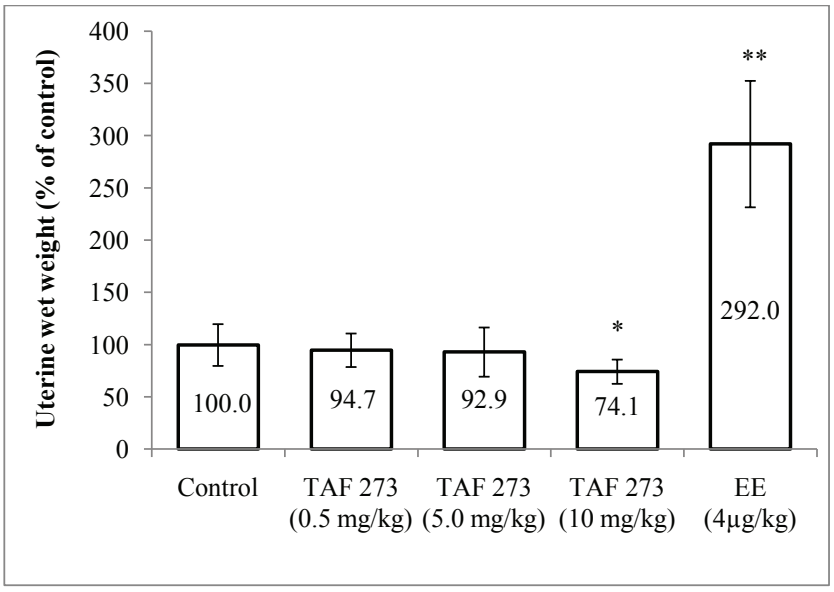

Figure 2.2: Effect of IP administration TAF 273 on uterine wet weight in immature female rats
Values represent the mean ± SD for 6 uteri per group. *p<0.05 and **p<0.01 are significantly different compared to control (treated with normal saline).
EE = ethynyl estradiol

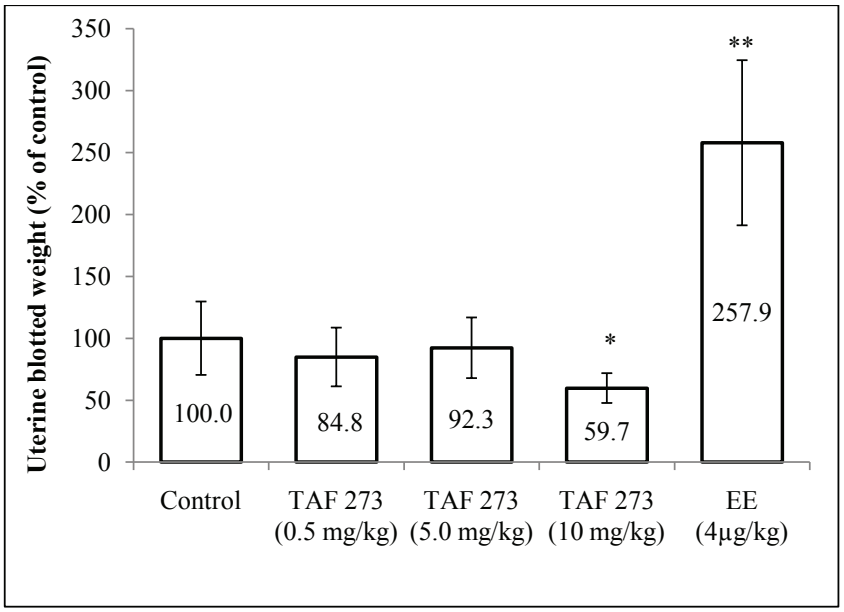

Figure 2.3: Effect of IP administration TAF 273 on uterine blotted weight in immature female rats
Values represent the mean ± SD for 6 uteri per group. *p<0.05 and **p<0.01 are significantly different compared to control treated with normal saline.
EE = ethynyl estradiol

2.3.3 Antioestrogenic effect of TAF 273 on uterus of rats

In the previous experiment (Section 2.3.2) 10 mg/kg/d TAF 273 significantly (p<0.05) decreased both uterine wet and blotted weights in immature female rats.

Fig. 2.4 illustrates the IP administration effect of the TAF 273 (10 mg/kg/d) and EE (1.3 µg/kg/d) combination on the uterine wet weight percentage of the immature rats. The combination of TAF 273 and EE significantly (p<0.01) increased the uterine wet weight of immature rats (181.9%) compared with the control (vehicle), but less than EE alone. The uterine wet weight in EE group increased significantly (p<0.001) by 231.0%, (Fig. 2.4). No significant difference was observed on the uterine wet weight of the immature rats in the TAF 273 (10 mg/kg/d) and EE (1.3 µg/kg/d) combination compared with EE alone.

The effects of the TAF 273 and EE combination on the uterine blotted weight percentage in the different groups of immature rats treated with EE (1.3 µg/kg/d) alone or the TAF 273 (10 mg/kg/d) and EE combination are shown in Fig. 2.5. The EE+TAF 273 combination significantly (p<0.01) increased the uterine blotted weight of immature rats (183.1%) compared with the control. EE alone significantly (p<0.001) increased the uterine blotted weights in immature rats (224.5%) compared with the control (vehicle treated). No significant difference was observed on the uterine blotted weight percentage of EE and TAF 273+EE treated groups.

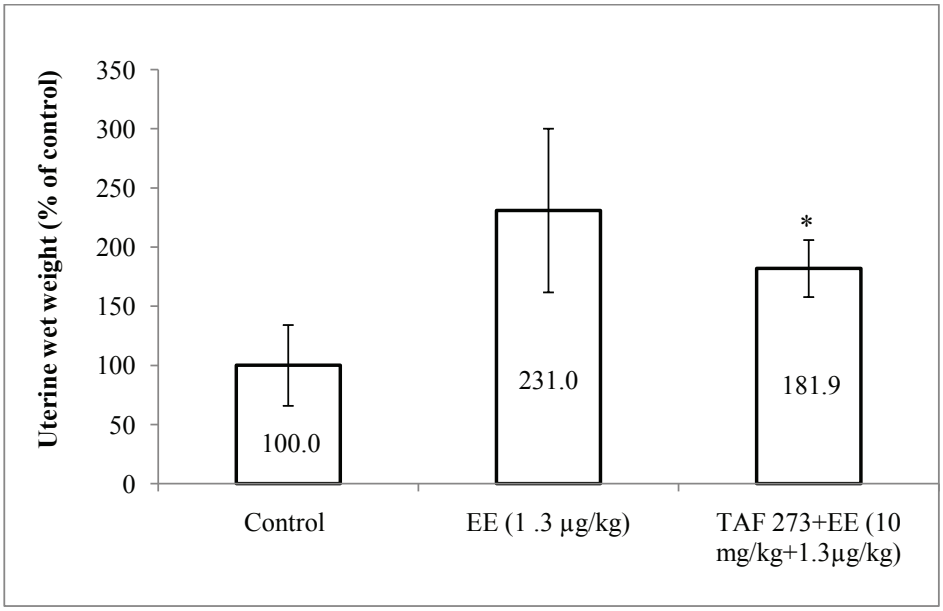

Figure 2.4: Effect of IP administration TAF 273 (10 mg/kg/day) on EE-uterotrophic in uterine wet weight of immature female rats
Values represent the mean ± SD for 6 uteri per group. $^{**}p<0.01$ and $^{***}p<0.001$ significantly different compared to control.
EE= ethynyl estradiol.

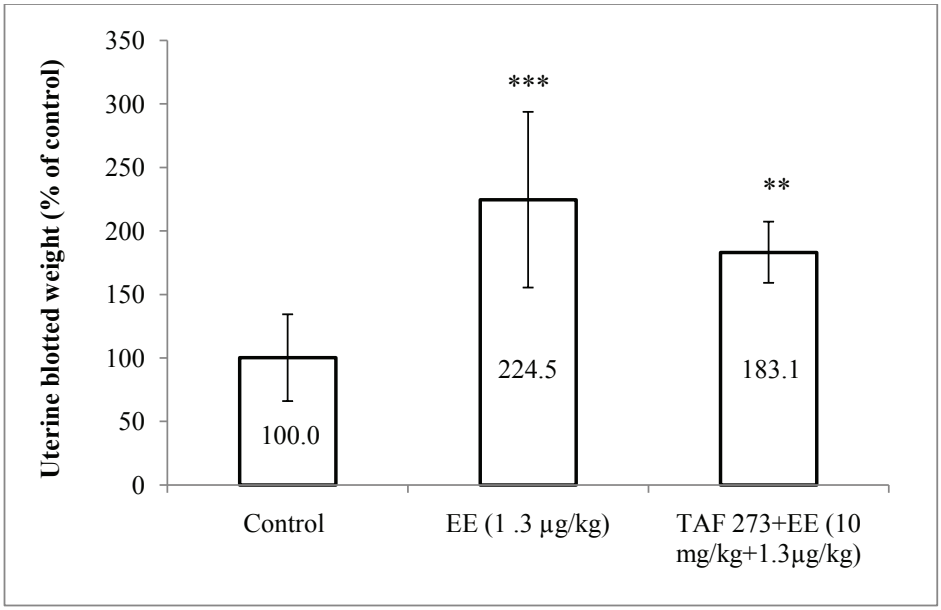

Figure 2.5: Effect of IP administration TAF 273 (10 mg/kg/day) on EE-uterotrophic in uterine blotted weight of immature female rats
Values represent the mean ± SD for 6 uteri per group. $^{**}p<0.01$; $^{***}p<0.001$; significantly different compared from control.
EE= ethynyl estradiol.

2.3.4 Antioestrogenic effect of quassinoids on uterus of rats

The intraperitoneal administration effects of the 13α,21-dihydroeurycomanone (Di) and EE, eurycomanone (Eu) and EE, and tamoxifen (Tmx) and EE combinations on the uterine wet weight percentage in immature rat groups are illustrated in Fig. 2.6. EE significantly (p<0.001) increased uterine wet to 320.3% compared with the control animals administered with vehicle (100.0%). Di+EE, Eu+EE and Tmx+EE significantly (p<0.001) increased the uterine wet weight to 270.7%, 207.8%, and 200.2%, respectively, compared with the control animals. The Di+EE, Eu+EE and Tmx+EE decreased in rat uterine wet weight compared with the animals treated with EE alone. Eu+EE and Tmx+EE significantly (p<0.001) decreased in uterine wet weight (207.8%, and 200.2%, respectively) compared with the EE treatment alone. No significant difference on the uterine wet weights was found between the Eu+EE and Tmx+EE groups.

The effects of Di+EE, Eu+EE, and Tmx+EE on uterine blotted weight percentage of the immature rats are shown in Fig. 2.7. EE significantly (p<0.001) increased the uterine blotted weight to 242.2% compared with the control animals. Di+EE, Eu+EE and Tmx+EE significantly (p<0.001) increased the uterine blotted weight to 219.6%, 176.7%, and 178.9%, respectively, compared with the control animals. Di+EE, QE+EE and Tmx+EE significantly (p<0.001) increased the uterine blotted weight, which is similarly to the uterine wet weight results. Eu+EE and Tmx+EE significantly (p<0.001) reduced the uterine blotted weight, to 176.7% and 178.9%, respectively, compared with the animals treated with EE alone. No significant difference was observed on the uterine blotted weight percentages of the Eu+EE and Tmx+EE treated groups.

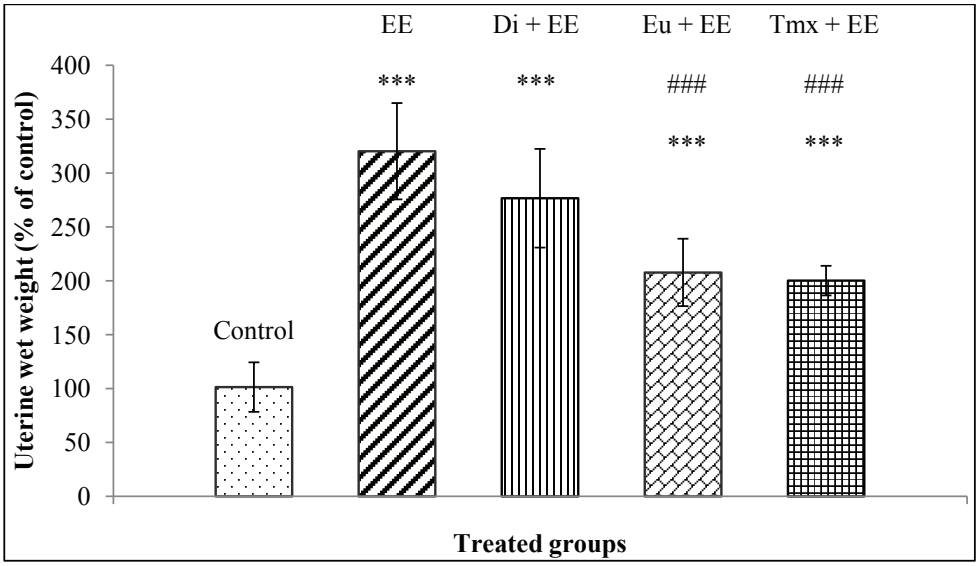

Figure 2.6: Effect of IP administration quassinoids on EE-uterotrophic in uterine wet weight of immature female rats

Data are given in mean ± SD; n= 6 each group. ###p<0.001 indicates significant difference compared to that of EE-treated animals, whereas ***p<0.001; significantly different compared to control.

EE= ethynyl estradiol (1.3 µg/kg/d); Tmx= tamoxifen (1 mg/kg/d); Di= 13α,21-dihydroeurycomanone (1 mg/kg/d); Eu= eurycomanone (1 mg/kg/d).

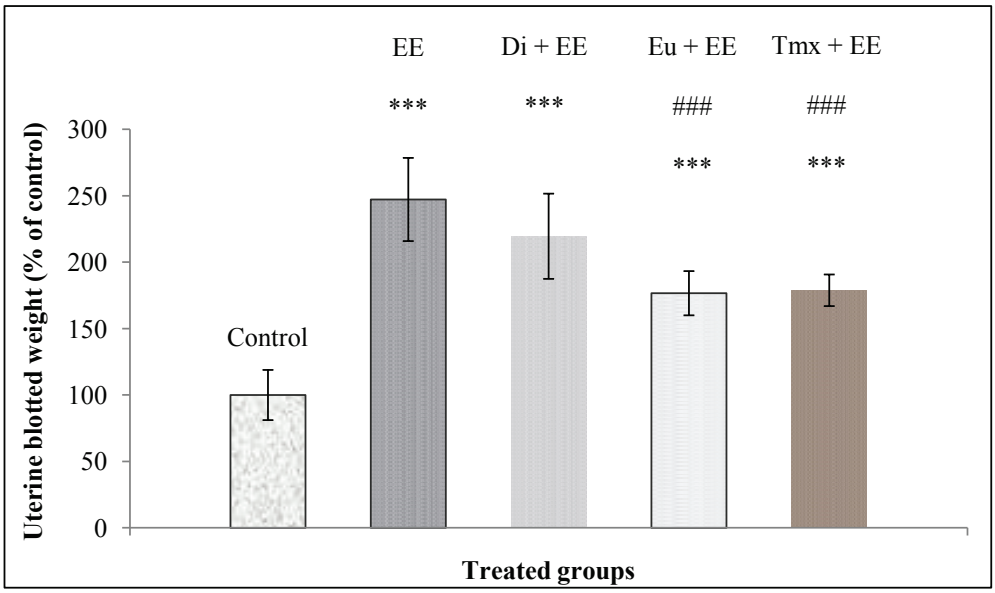

Figure 2.7: Effect of IP administration quassinoids on EE-uterotrophic in uterine blotted weight of immature female rats

Data are given in mean ± SD: n= 6 each group. ###p<0.001 indicates significant difference compared to that of EE-treated animals, whereas ***p<0.001; significantly different compared to control.

EE= ethynyl estradiol (1.3 µg/kg/d); Tmx= tamoxifen (1 mg/kg/d); Di= 13α,21-dihydroeurycomanone (1 mg/kg/d); Eu= eurycomanone (1 mg/kg/d).

2.4 Discussion

Uterotrophic assay was utilised to evaluate the effect of oestrogen compounds on the reproductive system. Chemicals like oestrogens increase the growth of the uterus of rodents by binding to the oestrogen receptors. Uterotrophic assay for oestrogenic activity has several advantages, which include:

1. it accounts for pharmacokinetics property of tested compounds or extract of herb because uterotrophic assay is one of the *in vivo* assays;
2. it is a well-defined, acceptable method used for decades;
3. it evaluates a broader range of mechanisms; and
4. it provides a comprehensive evaluation of the whole endocrine system as a unit.

Other tests of oestrogenic activity such as the *in vitro* have disadvantages; *in vitro* evaluations can provide both false positive and false negative results. *In vitro* false positives (i.e., active *in vitro* but not *in vivo*) arise when a chemical is not absorbed or distributed to the target tissue, is rapidly metabolically inactivated and excreted, and/or when some other forms of toxicity predominate *in vivo*. False negatives are considered to be of greater concern if *in vitro* tests were used for the exclusion of *in vivo* methods. *In vitro* evaluations can result in false negatives due to their inability, or unknown capacity, to metabolically activate toxicants.

Oestrogen effect on uterus causing an initial response which increases weight due to water imbibition (Reel et al., 1996) may be taken as wet uterus weight. This response is followed by a weight gain due to tissue growth (O'Brien et al., 2006). The oestrogenic receptors (Est.Rs) are Est.R-α and Est.R-β. Est.R-α is present in the female reproductive tract of rodents throughout late fetal and neonatal development, puberty, and adulthood (Kuiper et al., 1997). These effects of oestrogen occur through

Est.Rs. The early phase effects of water imbibition and hyperaemia as well as the late-phase effects of increased DNA synthesis and epithelial proliferation are absent in Est.R-α-null uteri (Couse et al., 1995; Korach et al., 1996).

In uterotrophic assay, different routes of administration have been commonly used and recommended by OECD Guideline (OECD, 2007) and these include oral and subcutaneous mode of administration. Although these routes of administration are recommended to perform uterotrophic assay study, other routes of administration such as intraperitoneal have been alternatively recommended to improve uterotrophic assay EDSTAC Guideline (Charles et al., 2005). The intraperitoneal route was selected in the present study to maximise the sensitivity (Cook et al., 1997), acknowledging that subsequent studies will be required to determine whether a sufficient dose can be delivered via the most reliable route. The intraperitoneal route of administration was used in experiments to enhance the sensitivity of the assay (Sekihashi et al., 2001) and to reduce false negative responses and also to facilitate potency comparisons by eliminating absorption (Cook et al., 1997).

Standardised extract of *E. longifolia* TAF 273 may have antioestrogenic activity because it significantly reduced the uterine wet and blotted weight of immature female rats given a high dose (10 mg/kg/d) of TAF 273 (Figs. 2.2 and 2.3). The uterotrophic effect was reduced by approximately 20% when immature female rats were co-administered with TAF 273 and 1.3 µg/kg/d of EE (Figs. 2.4 and 2.5). Antioestrogen-like effects occurred when phytochemicals were co-administered with potent oestrogens to immature female animals. The phytochemicals reduced the uterotrophic effect of the potent oestrogen (Boettger-Tong et al., 1998). This effect may have been caused by aromatase enzyme inhibition (Sumitani et al., 2000) which leads to a reduced intracrine estradiol synthesis (Makela et al., 1995).

Presently, *E. longifolia* may have an antioestrogenic effect at a high concentration of 10 mg/kg/d. The result in this study concurs with a recent study by Wahab et al. (2010) where *E. longifolia* can reverse the effect of oestrogen by increasing spermatogenesis and sperm counts in rats after 14 consecutive days of treatment. Most exogenous oestrogens like phytoestrogen have been reported to show weak antioestrogenic activity (Witorsch, 2002). The tested extract of TAF 273 contained mainly quassinoids and the the pharmacological effect may be attributed to these chemical components. Moreover, studies on the male reproductive system have suggested that quassinoids were responsible for the pharmacological action (Ang et al., 2004; Low et al., 2005). However, the endocrine system is highly complex and there are many ways in which a compound may interfere with it. Other experiments were done on pure compounds to support previous results.

Several previous studies on antioestrogenic activity have used the intraperitoneal route of administration (Landau, 1976; Himeno, 1987; Al-Bekairi et al., 1990; O'Connor et al., 1996). Although OECD Guidelines recommend the administration of test compounds via the oral or subcutaneous routes, there is also a recommendation to choose a particular route of administration to increase the efficacy and avoid any problems due to metabolic or pharmacokinetics of the test compound. Hence, the IP route of administration has also been recommended to be used as an alternative route of administration in uterotrophic assay by the Endocrine Disruptor Screening and Testing Advisory Committee's (EDSTAC) (Cook et al., 1997; Clode, 2006).

The quassinoids, 13α,21-dihydroeurycomanone (Di) and eurycomanone (Eu) showed poor oral bioavailability (Low et al., 2005; Chan et al., 2009) and would therefore require higher doses to be used for oral administration (Zhu et al., 2004). The substances were intraperitoneally administered because

quassinoids had been previously reported to have poor oral bioavailability (Low et al., 2005; Chan et al., 2009). In general, the oral route of administration requires higher quantities of compounds than intraperitoneal route (Zhu et al., 2004). Hitherto, there is no report of any quassinoid in the Simaroubaceae family possessing antioestrogenic activity, except indirectly that eurycomanone from *E. longifolia* which was found to show anti-proliferative effect on oestrogen-sensitive MCF7 cell line (Tee et al., 2007).

In the current study, the antioestrogenic activity of 13α,21-dihydroeurycomanone (Di), eurycomanone (Eu) and tamoxifen (Tmx) were compared with the ethynyl estradiol (EE)-induced uterotrophic activity of the rat uterus (Figs. 2.6 and 2.7). The Eu+EE and Tmx+EE combinations administered to the immature rat groups significantly (p<0.001) decreased both uterine wet and uterine blotted weights compared with immature rat group treated with EE alone. However, the same combinations significantly (p<0.001) increased both the uterine wet and uterine blotted weights compared with the control group of the immature rat which treated with vehicle. No significant difference on the uterine wet and blotted weights was observed between the Eu+EE and Tmx+EE groups.

Eurycomanone (Eu) may have antioestrogenic activity similar to that of the drug tamoxifen. The reduction in uterine weights by the eurycomanone when coadministered with EE was not reduced to the level of uterine weight values of the control animals. This observation may be due to the partial agonist property of Eu, or the dose used in the study may not be sufficient to block the oestrogenic effect of EE completely. Further studies involving investigating the uterine weight upon treatment of the rats with the quassinoids alone should be conducted. No previous study on eurycomanone possessing antioestrogenic activity has been reported.

However, a previous study has reported that eurycomanone from *E. longifolia* shows antiproliferative effects on the oestrogen-sensitive MCF7 cell line (Tee et al., 2007).

Despite the dual actions of tamoxifen, many studies have used the drug as a positive control to study antioestrogenic activity (Kim et al., 2002; Yamasaki et al., 2004; Arbo et al., 2009). According to EDSTAC, 'oestrogens' refers to compounds whose

effects are mediated through the oestrogen receptor (ER), initiating a cascade of cell/tissue specific effects similar to those initiated by estradiol, as opposed to 'oestrogen-like', a term used for those chemicals resembling oestrogen which are not or have not been shown to be mediated through the ER. In contrast, antioestrogenic activity are not specifically limited to ER-mediated interactions. Antihormones can act via: (1) the steroid hormone receptor; (2) steroid hormone synthesis inhibition; (3) reduction of bioavailability by reducing the amount of free hormone in the serum; (4) increased hormone metabolism leading to reduced serum hormone levels; and (5) other mechanisms.

In conclusion, the present study demonstrates that TAF 273 and the its pure compounds 13α,21-dihydroeurycomanone (Di) and eurycomanone (Eu) have antioestrogenic property in uterotrophic assay.

CHAPTER 3

EFFECT OF TAF 273 ON THE OESTROUS CYCLE OF THE RAT

3.1 Background

3.1.1 Vaginal tissue

The vagina in humans and experimental animals has similar functions and is controlled by similar biological factors in the body. It is well vascularised but does not contain glands (Michailova et al., 2005), and is lubricated by cervical mucus, a fluid transudate from the rich vascular network. It has an acidic pH 3.5–4.5. This acidity is due to conversion of the glycogen into lactic acid which is stored in the epithelial cells by vaginal commensal bacteria (Merk et al., 2005). Histologically, there are three cell types present in the vaginal epithelium: parabasal cells, the intermediate cell layer, and superficial cells (Michailova et al., 2005). The parabasal cells and the intermediate cell layer are under the influence of oestrogen during the reproductive years, while the superficial cells are under the influence of oestrogen after menopause (Michailova et al., 2005).

3.1.2 Biological effects of hormones on vaginal tissue

The vagina consists of three layers: the mucous membrane epithelium, the muscular layer, and the adventitial connective tissue. The mucosa is composed of a non-keratinised epithelium. The epithelium layer undergoes changes under the influence of oestrogen. Oestrogen induces proliferation and maturation of the

epithelium, enhances glycogen storage in the vaginal cells, and increases the blood flow to the vaginal tissue (Miyagawa et al., 2010). Progesterone and testosterone also stimulate the formation of glycogen in the vaginal cells same like oestrogen. In contrast, only oestrogens have the ability to induce vaginal cornification, which completes the differentiation process in the superficial layer. Progesterone exerts a strong shedding effect on the superficial cells; less shedding is evident following oestrogen stimulation (Cornet et al., 2002; Knobil and Neill, 2006). Under experimental conditions, where the combined activity of several hormones has been studied, progesterone is synergistic with oestrogen during the phases of proliferation and differentiation of the intermediate layer, whereas progesterone is antagonistic to oestrogen in the differentiation of the superficial layer. Due to the marked desquamative and shedding properties of progesterone, the vaginal epithelial cells are shed before they are able to undergo complete differentiation. Testosterone is synergistic with oestrogen in the process of proliferation and differentiation of the intermediate layer. Testosterone never causes cornification when acting alone, and its differentiating effect is less than that of oestrogen. The desquamating effect of testosterone is less than that of progesterone (Cornet et al., 2002; Knobil and Neill, 2006).

3.1.3 Menstrual cycle in humans

The menstrual cycle is a cycle of physiological changes in the reproductive system that occurs in the fertile female. It may be divided into three distinct phases: menstruation, the follicular phase, and the luteal phase. Menstruation is defined as the monthly discharge of blood, mucous and endometrial tissues from the uterus of a non-pregnant woman during the reproductive life (from the period of puberty to menopause) (Mbilu, 2002). Under the physiological condition of the reproductive

system, cessation of menstrual flow is a major indication of conception. It occurs in reproductive-age females of certain mammalian species. Overt menstruation is a process where bleeding from the vagina occurs which is seen primarily in humans and their close evolutionary relatives such as chimpanzees and other primates (Strassmann, 1996). Regularity of menstrual cycles in human is a good indicator of reproductive performance and health. If there are functional problems of the reproductive organs, an irregular (or absent) menstrual periods will be present. For example in the human if the ovary is not able to ovulate due to conditions such as a polycystic ovary, the menstrual cycle is either absent or displays irregularity.

The cycle is the result of a balanced coordination between several hormones. The normal flow of menstruation comes for 3–5 days where bleeding occurs from the uterus and the normal cycle pattern ranges from 24–32 days. The most fertile time covers the period from some 5 days before ovulation to 1–2 days after ovulation (Guerrero, 1975). The fertile period is the time of the highest likelihood of pregnancy's resulting from sexual intercourse. However in a few, ovulation times are varied, and a variety of methods have been developed to help women estimate the fertile and the relatively infertile days in the cycle. These fertility awareness methods include the calendar-based, and the observation of one or more of the two fertility signs (basal body temperature, cervical mucus). The former method relies on the cycle length (Wilcox et al., 2000) and latter relies on observations of fertility signs (Billings, 1992).

3.1.4 Oestrous cycle (OC) in rats

According to Westwood (2008), in the 19th century the term oestrus was initially used by British physiologist Walter Heape (1923) as a Latin adaptation of the Greek word 'oistros' to explain the sexual desire of the female period time. Heape also utilised

anestrus, the season of non-breeding when reproductive organ are inactive and attempts of mating are resisted; proestrus, the animal's coming into heat; oestrus, the female is willing to copulate with the male; metoestrus, in the absence of conception, when oestral changes in the reproductive tract subside; and dioestrus, the reproductive tract prepares for receipt of the ovum (Westwood, 2008).

The OC can be defined as the rhythmic changes in the appearance of the epithelial cells. Research previously carried out in characterising the oestrous cycle in rats by Stockard and Papanicolaou, (1917), and also by Long and Evans, (1922) cited by Knobil and Neill (2006). In 1923, Allen and Doisy use ovariectomised mice to assess the vaginal response following administration of oestrogen (Goldman et al., 2007).

The laboratory rat is a spontaneously ovulating, polyestrous mammal. Westwood (2008) reported that several groups of researchers have investigated the normal length of OC and duration of the individual stage of OC in rats since 1922. They found that the average OC was 4.8 days. The duration of the individual stage of OC based on vaginal smear pattern for rats with a 4- or 5-day cycle were found to be as follow: proestrus, 12 to 14 hours; oestrus, 25 to 27 hours; metoestrus, 6 to 8 hours; and dioestrus, 55 to 57 hours. The 5-day OC generally shows either an extra day of oestrous or an extra day of diestrous (Knobil and Neill, 2006). The vaginal epithelial cell structure assessment has long been used in reproductive system study (Westwood, 2008). Oestrus cyclicity only ceases during pseudo-pregnancy, pregnancy, and lactation. In rats as well as in humans, the oestrous cycle (OC) displays irregularity when PCO is induced.

Oestrous cycle in rats will be found immediately after the vaginal orifice opens, which tends to appear between postnatal days 32 and 36 Dohler et al., 1977;

daSilvaFaria et al., 2004. A repeated pattern of ovulatory cycles, each lasting 4 or 5 days, will continue until approximately 10–12 months of age (Westwood, 2008). In rats, the fertile period is indicated by the female willingness to mate with male or the oestrous phase determined by checking the vaginal smear. However, the extensive use of OC in reproductive system in female rats, and the various methods of assessment and techniques of OC have been developed along with using OC.

Several external factors can affect OC in rats. In photoperiodic animals such as rats, light is an important regulator of OC. The rat is unique in that the cycle is characterised by a brief luteal phase. The events of the cycle are largely under photoperiodic control. So lighting periodicity plays a dominant role in the incidence and duration of the stages of the OC. Indeed, shifting of the light phase results in a coincidental shift of the proestrous surge (Blake, 1976). When placed in constant light, rats become acyclic (Hoffman and Cullin, 1975).

Continuous light can induce a persistent estrous phase in female rats (Singh, 1969), when exposed to constant light for 25 days, rats exhibit normal oestrous cycles, however, they developed irregular cycles (Singh, 1969). Temperature, food (Wade and Jones, 2004), and cool ambience (Reiter, 1968) are importance factors for the regularity of OC in rats. That is, the ovarian cycle continues throughout the year and it is not restricted to one season as in sheep.

3.1.5 Determination of stages of oestrous cycle

From a literature review, all techniques which were used for determination of stages of OC are non-invasive techniques. These techniques are microscopical and electrical techniques. In microscopical techniques (MTs), staining and non-staining techniques

have been used. MTs are most common techniques for assigning the oestrous phases of rats. The MTs can be easily determined and the stages of OC established by monitoring the cell types that appear in the vaginal smear under low magnification (10x or 40x objective lenses).

3.1.5(a) Microscopical techniques for determination of stages of oestrous cycle

Various staining techniques have been used in MTs, including Papanicolaou stain using multi-dyes developed by George Papanikolaou, the father of cytopathology, and simple staining techniques using only one dye (methylene blue), (Bancroft and Stevens, 1996). In non-staining methods, no dye is used (Marcondes et al., 2002). In the non-staining technique which is the direct examination method, unstained smear samples can be observed using the microscope. Both techniques, staining and non-staining, are reliable (Yener et al., 2007).

3.1.5(b) Electronic techniques for determination of stages of oestrous cycle

Electronic techniques of determining the stages of the oestrous cycle include simply inserting a small probe fitted with a pair of recording electrodes into the vagina, which may be an impedance checker and an oestrous cycle monitor. These two probes are far more convenient for monitoring individual cycles than more laborious methods in which vaginal smears are inspected for changes in numbers of cornified, nucleated, and leukocytic cells. However, they are also expensive and thus their use has essentially remained selective (Ramos et al., 2001). Another simple, inexpensive electrical meter (with resistance-measuring capacity), as commonly used by professional electricians, has been utilised for determination of stages of OC. This method can identify between different phases the electronic resistance in units of

kΩ: 180 ± 40 at proestrus, 480 ± 130 at oestrous phase and 123 ± 23 at diestrous phase (Ramos et al., 2001). This method may be useful in assessing the status of oestrous cycle in female rats but is rarely cited in the literature.

3.1.6 Various techniques of vaginal smear sample collection

Vaginal smear sample collection techniques can be divided into two, old and recent techniques. The old techniques used eyedroppers with a small volume of normal saline (NS)/distilled water (DW) (0.2–0.25 ml) or cotton swabs moistened with NS. The recent technique used micropipette with tip which has been performed by Marcondes et al. (2002) and his colleagues. Eyedropper tips should have a smooth surface like glass and should be tapered.

Smear evaluation can be done immediately, fresh and unstained, or be fixed, dried, and stained subsequent examination and preserved. In fresh evaluation, the use of 12 position raised ring slides have been found to be preferable. Evaluation of OC is very important in order to standardise observations in different studies and to make easy comparison and validation.

3.1.7 Evaluation of OC

The evaluation scale for assessing the OC in rats relies on the length of the OC and the cell ratio contained in the vaginal smear. Cycle length has been defined as either the number of days from one proestrus to the next proestrus or from one dioestrus to the next dioestrus. The regular OC has been defined as a 4- or 5-day cycle with 1 or 2 days of oestrus or one with a single day of oestrus and 2 or 3 consecutive days of dioestrus. An irregular OC has been also defined as a 6- or 7- day cycle with a persistent oestrus or dioestrus (Westwood, 2008). The irregular OC has been