EFFECTS OF *EURYCOMA LONGIFOLIA* STANDARDIZED EXTRACT ON FEMALE REPRODUCTIVE DISPOSITION AND PERTURBATION PATHWAYS IN FEMALE RATS

BY

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ABSTRACT

Eurycoma longifolia is a traditional plant that has long been used for enhancement of male reproductive functions by enhancement of male testosterone levels thus improving the spermatogenesis process. However, effects of *E. longifolia* on the female reproductive functions are lacking and have yet to be elucidated. The present work was performed to investigate the potential and beneficial effects of *E. longifolia* standardized extract, TAF 273, on female reproductive functions and abnormalities. The effects of TAF 273 were investigated in normal female rats and estradiol valerate-induced polycystic ovarian condition rats as well as foetal development and teratogenicity conditions in female rats. Effects of TAF 273 on normal female rats were assessed in female Sprague-Dawley rats, aged between 8 to 10 weeks old. Animals were orally treated with 2.5, 5, 10, 25, 50 and 100 mg/kg of TAF 273 over 5 and 14 days. Oestrous cycle and levels of reproductive hormones were then evaluated together with the histomorphological changes in the ovaries. Similar sets of animals were used for induction of polycystic ovarian syndrome (PCOS) by a single subcutaneous dose of 2 mg estradiol valerate per rat. Rats with vaginal cornification at the end of 60 days were taken to be successfully induced. Treatments with Clomiphene citrate and TAF 273 were administered orally daily for 28 days. The hormonal levels were then measured followed by assessment of histomorphology of the ovary. Steroidogenesis-related genes and proteins (CYP11A1, CYP17A1 and CYP19A1) were also examined in histological paraffin sections using HRP-DAB method and quantitative RT-PCR. Evaluation of foetal development and teratogenicity was done by administration of a single subcutaneous injection of 0.15 mg/kg of estradiol valerate to dams on day 12 of gestation and concurrent treatment with TAF 273 (25, 50 and 100 mg/kg) from day 12 to day 19 of gestation. Foetal assessment was conducted following termination of pregnancy at day 21. The administration of TAF 273 extract did not significantly alter the oestrous cycle of the rats during the 5 and 14 days of treatment which was supported by normal levels of the reproductive hormones as well as normal morphology of the ovaries. TAF 273 extract at a dose of 100 mg/kg also possess beneficial effects in improvement of PCO by improving the ovarian morphology as evident by the presence of more abundant healthy follicles as opposed to cystic follicles and the occurrence of corpora lutea as an indication of ovulation. Hormonal imbalances and expressions of steroidogenic genes were also found to be attenuated probably through its action on oestradiol hormones and CYP19A1 gene. A higher potency of abortion and foetus showing abnormalities in external morphology was observed in female dams exposed to estradiol valerate on GD12. The number of live foetuses in dams treated with 100 mg/kg TAF 273 extract increased but with decreased body weight and crown-rump length of the foetuses. Foetuses with malformations in the form of kyphosis, scoliosis and kinky tail were more pronounced in EV group compared to the control and TAF 273-treatment groups. In conclusion, results obtained showed that the *E. longifolia* standardized extract, TAF 273, has the potential in maintaining normal functions of the female reproductive system and it may have economic value in the management of female disorders and reproductive health.

خلاصة البحث

إن يوريكوما لونجفوليا (*Eurycoma longifolia*) هو نبات تقليدي يستخدم منذ القدم لتعزيز وظائف الإنجاب للذكور من خلال تعزيز مستويات هرمون التستوستيرون الذكوري وبالتالي تحسين عملية انتاج الحيوانات المنوية. ومع ذلك، فإن آثار يوريكوما لونجفوليا على وظائف الإنجاب الأنثوية غير موجودة ولم يتم توضيحها بعد. وقد تم الاضطلاع بهذا العمل لمعرفة الآثار المحتملة والمفيدة من مستخلص يوريكوما لونجفوليا ، TAF 273، على وظائف الإنجاب للإناث. تم التحقق من آثار TAF 273 علي إناث الفئران العادية وفئران معالجة باستراديول فاليرات التي تسبب حالة تكيس في المبايض وكذلك تؤثر على تطور الجنين والظروف المسخية في إناث الفئران. تم تقييم تأثير TAF 273 على إناث الفئران العادية سبراغ-داولي، الذين تتراوح أعمارهم بين 8 إلى 10 أسابيع من العمر. تم علاج الحيوانات عن طريق الفم مع 2.5، 5، 10، 25، 50 و 100 ملغ / كغ من TAF 273 على مدى 5 و 14 يوما. ثم تم تقييم دورة المهبل ومستويات الهرمونات الإنجابية جنبا إلى جنب مع التغيرات الهيستومورفولوجية في المبايض. تم استخدام مجموعات مماثلة من الحيوانات لتحريض متلازمة المبياض المتعدد الأكياس مع حقن واحد تحت الجلد من استراديول فاليرات (2 ملغ / فأر). وقد أخذت الفئران كورنيفيكاتيون بالمهبل بعد نهاية ستين يوما لتكون عملية الحقن ناجحة. العلاجات مع كلوميفين سترات و TAF 273 كانت تدار عن طريق الفم يوميا لمدة 28 يوما. ثم تم قياس مستويات الهرمونات تليها تقييم هيستومورفولوجيا المبيض. كما تم فحص الجينات والبروتينات ذات الصلة بالستيرويد CYP11A1، CYP17A1 و CYP19A1 في أقسام البارافين النسيجية باستخدام طريقة HRP-DAB والكمية RT-PCR. وقد تم تقييم تطور الجنين والمسخ عن طريق إعطاء حقن تحت الجلد واحد من 0.15 ملغ / كغ من فاليرات استراديول في يوم 12 من الحمل والعلاج المتزامن مع TAF 273 (25، 50 و 100 ملغ / كلغ) من يوم 12 إلى يوم 19 من الحمل. أجري تقييم الجنين بعد إنهاء الحمل في اليوم 21. بعد استخراج TAF 273 لم يغير كثيرا علي الدورة الشهرية للفئران خلال 5 و 14 يوما من العلاج الذي كان مدعوما من المستويات العادية للهرمونات التناسلية وكذلك بطبيعة مورفولوجيا المبيض.اعطاء مستخلص TAF 273 بجرعة 100 ملغ / كغ التي لها أيضا آثارا مفيدة في تحسين تكيس المبايض من خلال تحسين مورفولوجيا المبيض كما يتضح من وجود بصيلات صحية أكثر وفرة بدلا من بصيلات الكيس المبيضي وتكون الجسم الأصفر كمؤشر للإباضة. كما وجد أن الاختلالات الهرمونية وتعبيرات الجينات الستيرويدية قد قلت على الأرجح من خلال عملها على هرمونات الاستراديول وجين CYP19A1وقد أظهر التعرض لاستراديول فاليرات لإناث الفئران فعالية عالية من الإجهاض مع العيوب الخارجية لتشكل الجنين. أظهرت الأجنة في المجموعة المعالجة ب 100 ملغم / كغم من مستخلص TAF 273 عددا كبيرا من الأجنة الحية ولكن مع انخفاض وزن وطول الجسم. وكانت الملاحظات من تشوهات في شكل محدب ومقوس للذيل في مجموعة EV أكثر وضوحا مما كانت عليه في مجموعة المقارنة والعلاجات. في الختام، أظهرت النتائج التي تم الحصول عليها أن مستخلص يوريكوما لونجفوليا موحد، TAF 273، لديه القدرة على الحفاظ على الوظائف العادية للجهاز التناسلي الأنثوي وقد يكون لها قيمة اقتصادية في إدارة اضطرابات الإناث والصحة الإنجابية.

APPROVAL PAGE

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DECLARATION

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LIST OF ABBREVIATIONS

AES	Androgen Excess Society
AFP	Anti-estrogen Alpha-fetoprotein
AMT	Amitriptyline
ASRM	American Society of Reproductive Medicine
β2AR	β2-adrenergic receptor
BPA	Bisphenol A
BSA	Bovine Serum Albumin
CC	Clomiphene citrate
cDNA	Complementary Dioxyribonucleic Acid
CL	Corpus Luteum
CREM	cAMP Responsive Element Modulator
CRL	Crown-rump Length
CVM	Congenital Vertebral Malformations
CYP11A1	Cholesterol Side-chain Cleavage
CYP17A1	17α-hydroxylase/17, 20-lyase
CYP19A1	Aromatase
D	Dioestrus
DAB	3, 3'- diamino benzidine
DDT	Diphenyl-dichloro-trichloroethane
DES	Diethylstilbestrol
DF	Developing Follicle
DHEA	Dehydroepiandrosterone
DHEAS	3-sulfoconjugate dehydroepiandrosterone
DHT	Dihydrotestosterone
EDCs	Endocrine Disruptor Chemicals
E1	Estrone
E2	Oestradiol
E3	Estriol
EE2	17alpha-ethynilestradiol
ELISA	Enzyme-linked Immunosorbent Assay
ED	13α, 21-dihydroeurycomanone
EL	Eurycomanol
EN	Eurycomanone
EP	13α (21)-epoxyeurycomanone
ER	Oestrogen Receptor
ESHRE	European Society of Human Reproduction and Embryology
EV	Estradiol Valerate
FSH	Follicle Stimulating Hormone
gDNA	Genomic DNA
GD	Gestation Day
GF	Graafian Follicle
GnRH	Gonadotropin-releasing Hormone
H&E	Hematoxylin and Eosin Stains
HIER	Heat Induced Epitope-Retrieval

HPG	Hypothalamus-Pituitary-Gonadal Axis
HPLC	High Performance Liquid Chromatography
HRP	Horse Radish Peroxidase
HRT	Hormone Replacement Therapy
HSD17B	17b-hydroxysteroid Dehydrogenase
HSD3B1	3β-hydroxysteroid Dehydrogenase
ICRACU	Integrated Centre for Research Animal Care and Use
IHC	Immunohistochemistry
IGFBP1	Insulin-like growth factor-binding protein 1
I.P	Intraperitoneal
IQR	Interquartile Range
IUGR	Intrauterine Growth Restriction
IVF	In Vitro Fertilization
LD_{50}	Median Lethal Dose
LH	Luteinizing Hormone
M	Metoestrus
mRNA	Messenger Ribonucleic Acid
NaCl	Sodium Chloride
NIH	National Institute of Health
NKEA	National Key Economic Areas
NOAEL	No-observed Adverse Effect Level
OE	Oestrus
OECD	Organisation for Economic Co-operation and Development
P	Pro-oestrus
P450scc	Cholesterol Side-chain Cleavage Cytochrome P450 Enzyme
PAPP-A	Pregnancy-associated Plasma Protein-A
PBS	Phosphate Buffer Saline
PCB	Polychlorinated Biphenyl
PCO	Polycystic Ovarian Condition
PCOS	Polycystic Ovarian Syndrome
PCR	Polymerase Chain Reaction
PE	Persistent Oestrus
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
SD	Standard Deviation
SEM	Standard Error Mean
SGA	Small-age for Gestation
SPSS	Statistical Package for Social Sciences
StAR	Steroid Acute Regulatory Protein
TAF 273	Quassinoids-rich, Standardized Extract
TAG	Triacylglycerol
VLBW	Very Low Birth Weight
WHO	World Health Organisation

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

The female reproductive system is strictly regulated by sex steroids and the gonadotropins which are under the control of the hypothalamus-pituitary-axis (HPG) axis through feedback mechanism (Alexander & Cotanch, 1980). The balance of sex steroids and the gonadotropins are vital for normal reproductive function and sexual development as well as for successful pregnancy (Bates & Bowling, 2013; Carreau, Wolczynski, & Galeraud-Denis, 2010). Any disruptions in the delicate balance of these hormones may not only affect fertility and complicate pregnancy but cause adverse effects on the developmental of the offspring (Alexander & Cotanch, 1980). Infertility is one of the most common reproductive disorders in both men and women of reproductive age associated with impairment in hormonal balance. Around 80 to 90% couples worldwide are affected by infertility and unable to get pregnant after 1 year of unprotected sexual intercourse. Secondary infertility may also affect couples who failed to have successful second pregnancy or subsequent child (WHO, 1983).

Oestrogen is the main female sex hormone which is produced primarily in the ovaries. The oestrogen levels in female are under the influence of endogenous and exogenous factors. Fluctuation in oestrogen levels is normal especially during menstrual cycle and in premenopausal and menopause women (Morrison, Brinton, Schmidt, & Gore, 2006; Somerville, 1971) However, high levels of oestrogen beyond the normal range in women at reproductive age may result in reduced sexual functions and capabilities for reproduction as well as development of diseases, reflecting a

disruption in the endocrine function (Hoyer & Keating, 2014; Kamphuis, Bhattacharya, Van Der Veen, Mol, & Templeton, 2014; Ptak, Hoffmann, & Rak, 2017). There has been a serious concern regarding the exposure of oestrogenic compounds environmentally or clinically that can interfere with endocrine system in females of reproductive age as well as during pregnancy (Hemminki, Gissler, & Toukomaa, 1999; Joffe, 2003; Nielsen, Ostergaard, & Larsen, 2008). Oestrogenic compounds also known as endocrine disruptor chemicals (EDCs) was defined by U.S. Environmental Protection Agency (EPA) as a group of naturally occurring or synthetic compounds that can mimic the effects of endogenous oestrogen by interfering with the oestrogen signalling pathway from the synthesis to specific binding to the oestrogen receptors (Kavlock et al., 1996; Pelekanou & Leclercq, 2011). Examples of oestrogenic compounds that have been documented in relation to increased oestrogen levels include pesticides (e.g diphenyl-dichloro-trichloroethane, DDT), plastic contaminants (e.g. bisphenol A, BPA) and pharmaceuticals (e.g. diethylstilbestrol, DES; 17alpha-ethynilestradiol, EE2). Phytoestrogens in dietary components have also been listed as EDCs (Frye et al., 2012).

Exposure to excess exogenous oestrogen during a hormone-sensitive period in prenatal environment and neonatal as well as during childhood, puberty and adulthood can result in abnormalities and perturbation in the female reproductive system and function which may lead to infertility (Cui, Shen, & Li, 2013; Diamanti-Kandarakis et al., 2009; Hilakivi-Clarke, de Assis, & Warri, 2013; Shirwalkar, Modi, & Maitra, 2007). The high levels of oestrogen in women of reproductive age may lead to polycystic ovarian syndrome (PCOS) and endometrial cancer (Barrett & Sobolewski, 2014; Benjamin, Toles, Seltzer, & Deutsch, 1993; Kim & Chapman-Davis, 2010; Shafiee, Chapman, Barrett, Abu, & Atiomo, 2013). Meanwhile, exposure to oestrogen

during early pregnancy has since been known to cause teratogenic effects and may also be a risk factor for the development of cancer during adulthood (Abbott, Padmanabhan, & Dumesic, 2006; Behnam-Rasouli & Nikravesh, 1997; Hemminki et al., 1999).

Plants have been used worldwide as an alternative remedy for treatment of various diseases owing to their therapeutic potential which is believed to have fewer side effects compared to the modern drug (George, 2011). Research in plants with beneficial effects especially in female reproductive aspects has garnered much attention to ensure their safety and efficacy for the purpose of development and production of plant-based medicines (Saxena, 2001). In Malaysia, plants such as *Labisia pumila (Kacip Fatimah), Ficus deltoidea (Mas cotek), Quercus infectoria* (*Manjakani*) and *Eurycoma longifolia* have long been consumed as traditional medicine to help restore hormonal balance in females and have also been used during postpartum period (Abdullah, Chermahini, Suan, & Sarmidi, 2013; Ramamurthy, Kumarappan, Dharmalingam, & Sangeh, 2014). However, *E. longifolia* was the least described on the potential effects on female reproductive health.

Eurycoma longifolia Jack (Simaroubaceae), also known as 'Tongkat Ali' is a plant indigenous to South-East Asian countries especially Peninsular Malaysia and Indonesia but is also cultivated throughout the tropics. *E. longifolia* has since received accolades and recognition as one of the important natural products able to assist men in problems with fertility (Bhat & Karim, 2010). It is also a known remedy of a myriad of ailments ranging from blood pressure conditions, dysentery, malaria, wound ulcer, boils, fever and fatigue. The many uses of *E. longifolia* in traditional and modern treatments are attributed mainly to the presence of secondary metabolites possessed by the plant. Phytochemical studies on *E. longifolia*, particularly from the

roots, have led to the identification, isolation and characterization of a wide array of chemical compounds including quassinoids, squalene derivatives, biphenylneolignans, tirucallane-type triterpenes, canthine-6-one, l-carboline alkaloids (Gimlette & Thomson, 1977), eurycomaoside, eurycolactone, eurycomalactone, eurycomanone and pasakbumi-B (Bhat & Karim, 2010).

The effects of *E. longifolia* on male reproductive functions have been extensively studied and the most notable use of the plant is as an aphrodisiac for loss of sexual desires and impotence in men and enhancement of reproductive functions and parameters. Recent studies on fertility in male rats showed that eurycomanone from *E. longifolia* standardized, quassinoids-rich extract possessed the ability to enhance the production of testosterone thus leading in the improvement of spermatogenesis and enhanced fertility (Low, Das, & Chan, 2013). This was later elucidated by work on the Leydig cells that showed testosterone steroidogenesis was enhanced by aromatase and phosphodiesterase inhibition at varying concentrations of *E. longifolia* extract (Low, Choi, Wahab, Das, & Chan, 2013). Although *E. longifolia* is traditionally used by men to promote fertility and enhance sexual function, women during postpartum have also been known to consume the extract of the roots from the plant for energy restoration, vitality and enhancement of blood flow (Ismail, Ismail, & Lassa, 1999). It was also reported previously that two major compounds of the plant, eurcycomanone and 13α, 21-dihydroeurycomanone, show anti-oestrogenic properties against 17α-ethynylestradiol (EE)-induced uterotrophy of immature rats (Teh et al., 2011). A recent study by Abdulghani et al. (2012) has shown that the standardized extract of *E. longifolia*, TAF 273, have a potential ability in restoration of the oestrous cycle of testosterone-induced polycystic ovarian syndrome in immature female rats. Despite the extensive research of the standardized extract in male, the safety and

potency of the extract in female counterpart have not been fully investigated. Little is known on the effect of the extract on female reproductive system and functions as well as in female reproductive disorders and teratogenicity in relation to exogenous oestrogenic exposure thus suggesting an avenue for further research that might have an impact on the common society.

Therefore, the current study was undertaken in order to investigate the potential effects of *Eurycoma longifolia* standardized extract (TAF 273) on female reproductive system and functions. In the first experiment (Chapter 4), investigations on possible effects of the plants on female reproductive system and functions was done by evaluation of the oestrous cycle, reproductive hormones and the histology of the ovary during 5- and 14-day treatment. The second experiment (Chapter 5) involved the investigation on potential effects of TAF 273 extract in the treatment of polycystic ovarian condition in estradiol valerate-induced rats by evaluation of the oestrous cycle, reproductive hormones, the histology and immunohistochemical staining of the ovary and mRNA expression of steroidogenic genes. The third experiment was done to explore the protective role of TAF 273 extract on estradiol valerate-induced teratogenicity in female rats by evaluation of maternal and foetal development. A better understanding on the possible effects of plant extracts on female reproductive system and functions will provide baseline information on the safety and efficacy of the plants for maintenance of female reproductive functions, improvement of interventions to preserve fertility and to assist in the development of achieving favorable reproductive outcomes with the use of plants as potential alternative treatment.

1.2 RESEARCH QUESTIONS

- How is the oestrous cycle and reproductive functions of female rats affected by the application of *Eurycoma longifolia* standardized extract?
- What are the underlying molecular mechanisms of the ameliorative effects of *Eurycoma longifolia* standardized extract on female reproductive diorders?
- Can *Eurycoma longifolia* standardized extract provide protective effects on foetus exposed to teratogens?

1.3 RESEARCH HYPOTHESES

Eurycoma longifolia standardized extract did not produce any adverse effects towards female reproductive functions with respect to the oestrous cycle and reproductive hormones. *E. longifolia* standardized extract also has restorative effects on the ovary through counteraction of perturbation on pathways in female rats with polycystic ovarian condition. *E. longifolia* also show protective effect on the foetus against teratogenic effects of estradiol valerate as a possible endocrine reproductive disruptor.

1.4 RESEARCH OBJECTIVES

The study was designed to evaluate the effects of the standardized extract, TAF 273, on female reproductive functions and disorders as well as teratogenicity in experimental female rats.

The study aimed to achieve the following objectives:

- To assess the effects of *Eurycoma longifolia* standardized extract on oestrous cycle and reproductive functions in nulliparous female rats.