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The potential of standardized quassinoid-rich extract of *Eurycoma longifolia* in the regulation of the oestrous cycle of rats

Suzanah Abdul Rahman[1*], Nur Amalina Ahmad[1], Nadia Hanis Abdul Samat[1], Syazana Zahri[1], Afif Raihan Abdullah[1], Kit-Lam Chan[2]

[1]*Department of Biomedical Science, Kulliyyah of Allied Health Sciences, International Islamic University Malaysia, 25200, Kuantan, Pahang, Malaysia*

[2]*School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800, Pulau Pinang, Malaysia*

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ABSTRACT

Objective: To evaluate the effects of *Eurycoma longifolia* (*E. longifolia*) standardized extract on the oestrous cycle, levels of reproductive hormones and histology of the ovaries of Sprague-Dawley rats.

Methods: Female rats were orally treated with *E. longifolia* standardized extract at the dose levels of 2.5, 5.0, 10.0, 25.0, 50.0 and 100.0 mg/kg of body weight over 5 days. Vaginal smears were monitored daily within the duration and after withdrawal of the treatment before being sacrificed. The body weights of the females were recorded before and after the 5 days treatment. At the end of the experiments, blood samples were collected for determination of testosterone, oestradiol and progesterone levels. Ovaries were removed, weighed and examined for histomorphological changes.

Results: The administration of *E. longifolia* standardized extract did not significantly alter the oestrous cycle of the rats during the 5 days treatment and after withdrawal of the treatments. This was supported by normal testosterone, oestradiol and progesterone levels as well as normal morphology of the ovaries.

Conclusions: The data obtained showed that *E. longifolia* standardized extract did not exhibit any toxic effect on reproductive activities of female rats suggesting potential use in the management of infertility.

1. Introduction

Traditional herbs and plants have long been used as treatment for various ailments since they are believed to produce fewer side effects when compared to the drugs which are available in the market [1]. However, some of these plants possess chemical compounds that have the ability to interfere with the normal female reproductive cycle [2–4] which may lead to infertility. Therefore, investigation on the probable side effects of natural products from plants on female reproductive organs, particularly in ovarian toxicity would be of significance.

Eurycoma longifolia Jack (*E. longifolia*) (genus: Eurycoma; family: Simaroubaceae), popularly known as 'Tongkat Ali', is indigenous to South-East Asian countries especially Peninsular Malaysia and Indonesia but it is also cultivated throughout the tropics [5]. Various parts of the plant are widely used as traditional remedy for the treatment of variety of ailments. The multiple applications of the roots include as treatment for malaria, aches, persistent fever, sexual insufficiency, dysentery, glandular swelling as well as health supplements [6–8]. The leaves have been traditionally used for ulcers, gum diseases and treatment of sexually transmitted diseases such as syphilis and gonorrhoea [5]. 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

*Corresponding author: Assoc. Prof. Dr. Suzanah Abdul Rahman, Department of Biomedical Science, Kulliyyah of Allied Health Sciences, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia.
Tel: +60 9 5705255
Fax: +60 9 5716776
E-mail: arsuzanah@iium.edu.my

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men. Nevertheless, women at postpartum have also been known to consume the extract of the roots from the plant for energy restoration and vitality and enhancement of blood flow [9]. 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. This was later elucidated by work on the Leydig cells that showed testosterone steroidogenesis was enhanced by aromatase and phosphodiesterase inhibition at varying concentrations [10,11]. Review on the effects of *E. longifolia* on the female reproductive functions and disorders are not as extensive as for the males. However, a recent study on testosterone-induced amenorrhoea and polycystic ovary-like conditions in rats showed the potential of *E. longifolia* in ameliorating chronic effects of androgens and in sustaining female fertility [12]. The study in this paper was undertaken to further explore the possible effects of the standardized extract of *E. longifolia* on female reproductive functions using rats as a model.

2. Materials and methods

2.1. Extract preparation

The standardized quassinoid-rich extract (TAF 273) was obtained by courtesy from the School of Pharmaceutical Sciences of Universiti Sains Malaysia in Penang, Malaysia. The extraction and fractionation of the prototypes were specifically done by chromatographic separation following the methods previously described by Low *et al.* [13].

2.2. Experimental animals

The experiment was carried out in nulliparous female Sprague-Dawley rats of 10 weeks of age. The animals were placed in polypropylene cages under standard laboratory conditions at room temperature of (22 ± 4) °C, humidity of 30%–70% with 12 h light/dark cycle. All animals were fed with standard diet and tap water *ad libitum* throughout the experiment. The study was performed according to the Guidelines for Animal Study and ethically approved by the Integrated Centre for Research Animal Care and Use, International Islamic University Malaysia [IIUM/IACUC Approval/2015/(5)(31)].

2.3. Oestrous cycle

Vaginal smears of the female rat were examined daily for a 2-week period which covers at least 3 cycles to allow for the selection of females of normal duration of oestrous cycle of 4–5 days. The animals with regular cycle were selected and randomized into control and treatment groups ($n = 6$ per group). Rats in the treatment groups received the extract orally at different dose levels of 2.5, 5.0, 10.0, 25.0, 50.0 and 100.0 mg/kg body weight for 5-day starting from the oestrus stage while the control group received distilled water in a similar manner. The oestrous cycle pattern of both control and treatment groups and body weights were monitored daily and recorded throughout the 5 days treatment and after withdrawal of treatment. The stages of oestrous cycle were determined by daily examination of vaginal smears according to Organisation for Economic Co-operation and Development guidelines [14]. Vaginal smears were obtained on daily basis between 9.00 a.m. and 10.30 a.m. and observed under the light microscope at 4× and 10× magnification.

2.4. Blood sampling and hormonal assay

Blood samples were collected during the oestrus stage by retinol orbital puncture using haematocrit capillary tube. The blood was left to clot for at least one hour. Separation of serum was done by centrifugation at 3 000 r/min for 10 min and this was later stored at −80 °C before being used for the determination of oestradiol, testosterone and progesterone levels using ELISA kit (Cusabio, China) according to the manufacturer's instructions.

2.5. Histology

Animals from each group were sacrificed at oestrus stage following withdrawal of treatment following the ethical procedure. The right ovaries were excised from each rat, cleaned of fatty layers and fixed in 10% buffered formalin solution for at least 24 h before the preparation of the histology slide. Sections were cut using a rotary microtome at 5 μm thickness and later placed on a glass slide and stained with haematoxylin and eosin (H&E). Photomicrographs of the ovary sections were taken at 10× and 100× magnification.

2.6. Statistical analysis

Data obtained are presented as the mean ± SD. For statistical evaluation, general linear model (repeated measure), Tukey's honest significant difference and One-way ANOVA test were applied. A P-value <0.05 was considered significant in this study.

3. Results

3.1. Oestrous cycle

Table 1 showed the results of the effects of TAF 273 on the oestrous cycle of female rats during the 5-day treatment and after withdrawal of the treatment. No significant differences ($P > 0.05$) were recorded in the duration of oestrous cycle for the control and treatment groups. In the group of rats treated with 100 mg/kg extract, the length of oestrus stage was increased when compared with control group while the dioestrus stage was increased in the group treated with 50 mg/kg extract. The length of pro-oestrus and metoestrus stages in all groups remained unchanged throughout the study and after withdrawal of the treatments.

Table 1
Effects of TAF 273 extract administered for 5 days on the duration of oestrous cycle in different groups of rats (day).

Group	Dose (mg/kg body weight)	Oestrous cycle	Pro-oestrus	Oestrus	Metoestrus	Dioestrus
0	Control	4.83 ± 0.75	1.00 ± 0.00	1.50 ± 0.55	1.00 ± 0.00	1.33 ± 0.52
1	2.5	4.17 ± 0.98	1.00 ± 0.00	1.33 ± 0.47	1.00 ± 0.00	1.17 ± 0.37
2	5.0	4.33 ± 0.82	1.00 ± 0.00	1.50 ± 0.12	1.00 ± 0.00	1.00 ± 0.00
3	10.0	4.33 ± 0.52	1.00 ± 0.00	1.17 ± 0.37	1.00 ± 0.00	1.17 ± 0.37
4	25.0	4.33 ± 0.52	1.00 ± 0.00	1.33 ± 0.52	1.00 ± 0.00	1.00 ± 0.00
5	50.0	4.83 ± 0.98	1.00 ± 0.00	1.17 ± 0.37	1.00 ± 0.00	1.67 ± 0.75
6	100.0	4.33 ± 0.52	1.00 ± 0.00	1.33 ± 0.47	1.00 ± 0.00	1.00 ± 0.00

Values are expressed as mean ± SD, $n = 6$.

3.2. Body weight and organs weights

Body weights as well as that of the uterus and ovaries are shown in Table 2. There was a significant ($P < 0.05$) increase in body weights of the group treated with 2.5 mg/kg and 100 mg/kg extract when compared to the control group. Except for the group treated with 25 mg/kg, all other groups recorded an increase of body weight which were however not significant ($P > 0.05$) as compared to the control group. Results also demonstrated that the wet weight of the uterus following treatment with 2.5 mg/kg extract showed significant ($P < 0.05$) increase when compared with the control group. The increase in uterus weights was also observed in other treatment groups but data did not indicate significant change from that of the control group. The weights of ovaries did not show any significant changes for all groups although some groups recorded higher weights of the ovaries when compared to the control group.

Table 2

The effects of TAF 273 extract on body weight, uterus and ovaries weight of female rats (g).

Group	Dose (mg/kg body weight)	Day 0	Day 5		
		Body weight	Body weight	Uterus weight	Ovaries weight
0	Control	189.42 ± 10.77	199.43 ± 10.54	0.44 ± 0.04	0.09 ± 0.04
1	2.5	234.48 ± 22.42	253.03 ± 8.31*	0.78 ± 0.38*	0.15 ± 0.10
2	5.0	198.25 ± 11.79	207.28 ± 6.69	0.42 ± 0.06	0.11 ± 0.02
3	10.0	190.08 ± 4.69	198.37 ± 1.70	0.50 ± 0.04	0.08 ± 0.02
4	25.0	210.45 ± 14.73	208.94 ± 6.16	0.51 ± 0.15	0.10 ± 0.05
5	50.0	194.13 ± 7.16	207.07 ± 5.12	0.48 ± 0.11	0.10 ± 0.05
6	100.0	234.45 ± 29.49	236.79 ± 6.90*	0.48 ± 0.04	0.13 ± 0.02

Values are expressed as mean ± SD, $n = 6$; *: $P < 0.05$ significantly different compared to control.

3.3. Reproductive hormones

The effects of TAF 273 on serum testosterone, oestradiol and progesterone are shown in Table 3. A notable increase was found in testosterone, oestradiol and progesterone levels in the treatment groups although they were not statistically significant when compared to the control group.

Table 3

The effects of TAF 273 extract on reproductive hormones in female rats (ng/mL).

Group	Dose (mg/kg body weight)	Testosterone	Oestradiol	Progesterone
0	Control	4.02 ± 2.16	0.62 ± 0.47	0.33 ± 0.52
1	2.5	4.74 ± 1.28	0.93 ± 0.27	0.34 ± 0.35
2	5.0	5.30 ± 2.44	0.67 ± 0.39	0.34 ± 0.83
3	10.0	4.17 ± 1.94	0.95 ± 0.21	0.78 ± 1.02
4	25.0	4.73 ± 1.80	0.88 ± 0.23	0.52 ± 0.21
5	50.0	3.70 ± 0.83	0.98 ± 0.25	0.24 ± 0.58
6	100.0	4.18 ± 0.74	1.12 ± 0.11	0.42 ± 0.48

Values are expressed as mean ± SD, $n = 6$.

3.4. Histological observations

There were no physiological and histological changes in the ovaries of the control and treated animals. The analysis of the cross section of the ovaries (Figure 1) shows normal morphology of the ovaries and the presence of ovarian follicles at various stages of development. Table 4 summarized the number of primary, secondary and Graafian follicles in the control and treatment groups. There was a significant decrease ($P < 0.05$) in the number of secondary follicles of group treated

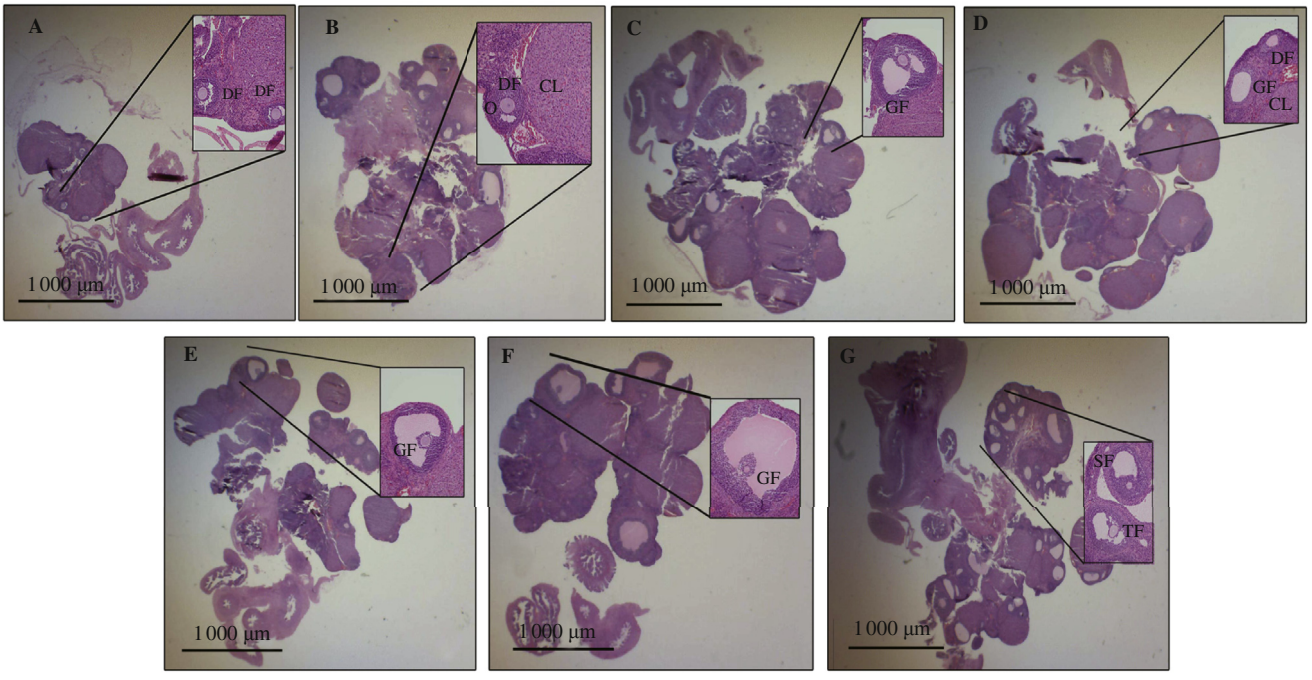

Figure 1. Cross sections of the ovaries of A: Group 0 (control), B: Group 1 (2.5 mg/kg), C: Group 2 (5.0 mg/kg), D: Group 3 (10.0 mg/kg), E: Group 4 (25.0 mg/kg), F: Group 5 (50.0 mg/kg) and G: Group 6 (100.0 mg/kg) showing normal appearance of the ovary and the development of different phases of follicles. DF: Developing follicle; CL: Corpus luteum; O: Oocyte; GF: Graafian follicle; SF: Secondary follicle; TF: Tertiary follicle (H&E staining). (In box: 100× magnification) (10× magnification).

with 10 mg/kg TAF 273 extract as compared to the control group. However, no significant differences in other treatment groups were found. Oedema, cystic follicles or arrested oocytes were not found suggesting that the extract did not exert any toxic effects to the reproductive organs of the experimental animals.

Table 4

The effects of TAF 273 extract on primary, secondary and Graafian follicles counts of rat ovaries.

Group	Dose (mg/kg body weight)	Primary follicles	Secondary follicles	Graafian follicles
0	Control	2.17 ± 0.98	1.67 ± 1.03	0.50 ± 0.55
1	2.5	3.67 ± 1.21	2.67 ± 0.52	1.14 ± 1.17
2	5.0	2.67 ± 1.03	1.50 ± 0.55	1.33 ± 0.52
3	10.0	1.50 ± 0.55	0.33 ± 0.82*	0.17 ± 0.41
4	25.0	2.17 ± 0.41	1.17 ± 0.75	0.67 ± 0.52
5	50.0	2.00 ± 0.89	0.67 ± 0.52	1.17 ± 0.41
6	100.0	1.83 ± 0.75	1.50 ± 0.55	1.00 ± 0.89

Values are expressed as mean ± SD, $n = 6$; *: $P < 0.05$ significantly different compared to control.

4. Discussion

The effects of TAF 273 extract on female reproductive organs and functions were evaluated in the present study. Since there is little information regarding the effects of the TAF 273 on the female reproductive cycle and ovarian functions, the current doses were selected based on a similar study using TAF 273 that exhibited the reproductive toxicity and teratology effects of the plant in rats of both sexes. The study has found that 100 mg/kg induced slight toxicity but concluded it to be the no-observed adverse effect level [15].

Oestrous cycle is a rhythmic reproductive cycle in sexually matured female mammals where many physiological, biochemicals, morphological and histological changes take place in the ovaries [16]. Normal oestrous cycle sequence consists of pro-oestrus, oestrus, metoestrus (dioestrus I) and dioestrus (dioestrus II) where the appearance of different types of cell characterizes each stage and represents the follicular and luteal phases of the reproductive cycle. A pro-oestrus smear is dominated by nucleated epithelial cells while an oestrus smear primarily consists of cornified cells without nucleus. Following that, a metoestrus smear will consists of the same proportion of leukocytes, cornified and nucleated epithelial cells and a dioestrus smear consists primarily of leukocytes. Rats and mice are commonly used as models to study the effects of compounds due to the spontaneous ovulation and shorter length of the oestrous cycle in the females. A cycle is considered irregular and/or abnormal if the sequence is not followed [17]. It is however still considered as normal if the rats have an extended 1 or 2 days of oestrus and dioestrus but a longer period of dioestrus (4 days or longer) and/or oestrus (3 days or longer) will indicate that the oestrous cycle of the rats are being disturbed [18].

Oestrous cycle has been used as principal tool in the determination of reproductive cycle and also in reproductive toxicology studies [19,20]. The lining cells of the vagina are very sensitive to the fluctuations of the circulating hormones levels which make it a valuable marker for indication of disruption of the hormonal balance in the ovary [14]. The current study revealed that TAF 273 extracts did not cause any adverse effects on the normal cycle of the rats as all animals exhibited the normal 4–5 days oestrous cycle with no significant changes to the cycle observed.

The inconsistency of the hormones levels observed in this experiment showed that there is a wide variation of serum levels in each individual which may be influenced by body weights and other factors. However, the administration of TAF 273 extracts did not cause any significant changes ($P > 0.05$) to the serum levels of testosterone, oestradiol and progesterone in the experimental groups as compared to the control group. In a previous study, TAF 273 extracts have been proven to increase testosterone levels which improved spermatogenesis of male rats [13]. Testosterone serum levels evaluated in this study did not show any significant elevation when compared to the control group. This is in contrast to the effects of high levels of testosterone in women which are known to cause hyperandrogenism, one of the main causes for polycystic ovarian syndrome. Oestradiol is a primary female hormone which is responsible for the growth and development of the reproductive organs while progesterone regulates monthly menstrual cycle and prepares the body for conception and pregnancy [21]. Levels of oestradiol and progesterone in all groups remained normal thus indicating that the extract may have a potential ability to maintain the normal balance of the reproductive hormones.

The ovary consists mainly of three endocrine tissues; the stroma, the follicles and the corpus luteum [16]. The net weight of the ovary is contributed by the weight of these tissues. The number of ovarian follicles can also indicate ovarian toxicity following treatment with plant extracts [22]. Absence of gonadotropins or steroidal hormones may cause a reduction in ovarian weight leading to the disruption of follicular activity [23]. This study showed that ovarian weight and follicular development in the ovaries of the treatment groups were not inhibited. The number for primary, secondary and Graafian follicles were not significantly altered as compared to the control group. This is supported by the normal concentrations of testosterone, oestradiol and progesterone in the serum.

Plant extracts have been found to cause imbalances and alterations towards the female reproductive hormones by affecting the hypothalamus–pituitary–gonadal axis or the reproductive organs thereby inhibiting ovarian steroidogenesis [24]. This can cause irregularity of the ovarian functions which may be in the form of erratic oestrous cycle, interruptions to reproductive functions and inhibition of ovulation [25]. Current study on short-term treatment (5-day) of standardized *E. longifolia* extract, TAF 273 on female rats showed that the extract did not alter nor disrupt the regularity of the oestrous cycle. The regular cycle was maintained even after the withdrawal of the treatment. To date, there is no standard protocol on the minimum time required for the detection of toxic implications to the female reproductive functions or hypothalamus–pituitary–gonadal axis. Toxicity study in rats at the duration of 2.5–3 ovarian cycles is sufficient for identification of ovarian toxicity as suggested by Sanbuissho *et al.* [26]. However, study by Mirsky *et al.* [25] showed that the adverse effects on various organs of rats following the repeated treatment with the aromatase inhibitor, exemestane can be identified as early as 4 days based on microscopic evaluation of the oestrous cycle alone. This supported our findings on the effects of TAF 273 extracts on the oestrous cycle of female rats during the 5 days treatment.

In conclusion, the present study shows that all doses of the TAF 273 extracts tested did not interfere with the normal oestrous cycle of the female rats. Further investigation on the effects of the standardized extract will allow the identification of possible ovarian toxicity pathways and probable potentials of the extract in the management of women infertility.

Conflict of interest statement

We declare that we have no conflict of interest.

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