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Effects of Eurycoma longifolia Standardized Extract on Estradiol Valerate-Induced Teratogenicity in Female Rats

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Abstract

Estradiol valerate, a synthetic steroidal estradiol is used to treat female reproductive disorders as well as contraception. Consumption of estradiol valerate may produce abortional and teratogenic effects. Possible effects of the standardized extract of Eurycoma longifolia, TAF 273, on foetal development in female rats exposed to estradiol valerate were therefore investigated. Pregnant female rats on day 12 of gestation were administered with a single subcutaneous injection of estradiol valerate (0.15 mg/kg). TAF 273 was administered orally at doses of 25, 50 and 100 mg/kg body weight from day 12 to day 19 of gestation followed by termination of pregnancy on day 21. A decreased the number of live foetuses with high number of post-implantation loss

and early resorption were observed. Administration of 100 mg/kg TAF 273 resulted in high number of live foetuses but with decreased body weight and crown-rump length possibly due to competition for space for embryonic development and blood supply. Kyphosis, scoliosis and kinky tail following treatment with estradiol valerate were apparent. Eurycoma longifolia may have potential in ameliorating teratogenic effects of estradiol valerate by reducing external abnormalities in the rat foetuses and may have prospects in the treatment of female-linked disorders and complications.

***Key Words*:**Eurycoma longifolia; TAF 273; Teratogenicity; Estradiol Valerate; Foetus.

1 Introduction

Teratogenic effects during pregnancy can be induced by a variety of factors primarily affecting the balance of maternal hormones. Among problems associated with the lack of natural oestrogen in the body include irregularity of the menstrual cycle, decreased reproductive capacity and also effects on bone formation in women. However, excessive levels of oestrogen can be a risk leading to breast and uterine cancers and birth defects and foetal loss following pregnancy. Estradiol valerate, a synthetic and steroidal estradiol, has been used to treat female reproductive disorders associated with low levels of oestrogen. It is sometimes administered during in vitro fertilization (IVF) to support fertility procedures by thickening and stabilizing the uterine lining continuing up to week 12 of pregnancy to maintain appropriate hormonal environment for embryo transfer.(1) Exposure to estradiol valerate during early pregnancy has since been known to produce abortional effects especially in cases of unknown pregnancy. Teratogenic effects have also been detected at toxic levels of the agent.(2) A traditional plant, Eurycoma longifolia, growing in abundance in Malaysia and countries of South East Asia, is known for its positive reproductive effects and a standardized extract, TAF 273, has been shown to alleviate hormone-related disorders in males. Enhancements in sperm quality and spermatogenesis are some of the most notable effects observed. (3, 4) However, little is known on its potential towards the female reproductive system. The present work aimed to inves-

tigate the possible effects of TAF 273, a standardized extract of Eurycoma longifolia, on the prevention or amelioration of estradiol valerate-induced teratogenicity effects in foetus of rats.

2 Literature Review

2.1 Teratogenicity

Teratogenicity is defined as a property or capability to induce malformations. Teratogenic agents can cause malformations or abnormalities in the embryo during foetal development (5) by crossing the placenta. The teratogenic effects from exposure to drugs or potential teratogenic agents can take place during different periods of pregnancy. Gestation in rats can be divided into implantation (gestation day 1-7), organogenesis (gestation day 8-14) and foetal development (gestation day 14-20) periods. Exposure to teratogens at specific periods may result in multiple manifestations of defects. Consumption of substances from plant origin has been associated with teratogenic effects making it unsafe for administration during pregnancy.(6, 7) The malformations produced include growth retardation, skeletal and visceral malformations. A few factors can lead to congenital malformations in the developed foetus including the exposure of the mother to environmental agents and chemical pollutants resulting in genetic alterations.(8) Congenital malformations can cause foetus mortality and morbidity. Some teratogenic agents cause toxicity towards the development of the foetus without inducing maternal toxicity. This may interfere with the later development of an individual especially in cases of exposure to endocrine disruptors.(9)

2.1.1 Estrogen

Estrogen or 17-estradiol is a major female sex hormone that regulates the female reproductive system. It is produced mainly in the ovary via conversion from testosterone by aromatase.(10) It is crucial in maintaining the reproductive cycle, acting together with progesterone in maintaining pregnancy. Therefore, any disturbance in the balance of estrogen levels in females may directly lead to female reproductive disorders.(11) It is known that exposure

to excess exogenous estrogen during a hormone-sensitive period in prenatal environment can result in abnormalities and perturbation in female reproductive system and function.(12, 13) Many previous studies have demonstrated that excess in foetal estrogen can give rise to chronic diseases in adulthood such as breast cancer.(14-16) Estradiol valerate (EV) forms free 17-estradiol (E2) in the body, hence it is commonly used as contraceptive drugs, agents assisting in vitro fertilization (IVF), hormone replacement therapy (HRT) as well as to treat female animal models with reproductive disorders. Induction with EV in female rats at different stages of development (neonatal, prepubertal, pubertal and adult) has resulted in the formation of cystic follicles, a distinct characteristic for polycystic ovarian syndrome (PCOS). Little is known on the action of EV as a teratogenic agent therefore creating the opportunity for exploration on its adverse effects in foetus development.

2.2 Eurycoma longifolia

Eurycoma longifolia Jack (Genus: Eurycoma; Family, Simaroubaceae), popularly known as 'Tongkat Ali is cultivated throughout the tropics (17) and is considered popular for its beneficial effects as means for fertility enhancement .

2.2.1 Common Uses

A variety of ailments have been treated using extracts from various parts of the plant. The multiple applications of the roots have shown to relieve conditions of aches and fever in malaria, reduce glandular swelling and ease symptoms of dysentery. Various formulations of the roots of the plant have been consumed as health supplements (18-20), but the most significant use of Eurycoma longifolia is as an aphrodisiac to enhance sexual desires in men with problems of impotence. The leaves have been traditionally applied to treat gum diseases and ulcers, as well as some sexually transmitted diseases like gonorrhea and syphilis. (17)

2.2.2 Activity of Bioactive Constituent

The many uses of Eurycoma longifolia in traditional and modern treatments are contributed mainly by secondary metabolites pos-

sessed by the plant. Phytochemical studies on Eurycoma longifolia, particularly from the roots, have led to the identification, isolation and characterization of a wide array of chemical compounds including quassinoids such as eurycomanone, squalene derivatives, canthine-6-one, l-carboline alkaloid, biphenylneolignans and pasakbumi-B.(17) These compounds isolated from Eurycoma longifolia, are useful as anti-tumor promoting, anti-cancer, anti-bacterial, anti-oxidant, anti-parasitic, anti-malarial, anti-inflammatory, anti-ulcer, anti-pyretic and anti-arthritic agents as well as possessing cytotoxic and aphrodisiac effects.(17, 21) Some past studies involving biological activities of the plant extracts supported the value of Eucoma longifolia application. Cytotoxicity activities of Eurycoma longifolia extract against in vitro culture of chloroquine-resistant Plasmodium falciparum showed impressive potencies comparable to cytotoxicity on KB cells.(22) Another study by Ang and Sim (23) has reported that root extracts processed using chloroform, methanol, water and butanol increased mounting frequency of the treated animals in a dose-dependent manner. Recent studies on fertility of male rats indicated that eurycomanone possess the ability to enhance the production of testosterone thus leading to improved spermatogenesis and enhanced fertility.(3, 4) Previous study by Abdulghani et al. (24) showed that the extract has potential in ameliorating polycystic ovarian syndrome (PCOS) in female rats exposed to testosterone propionate which later corresponds to a study by Abdul Rahman et al., that reported the potential of the extract in the regulation of the female rats oestrous cycle.(25) Treatment with Eurycoma longifolia standardized extract during the period of organogenesis did not cause any teratogenic effects in female rats suggesting the safety of usage of doses below 100 mg/kg in pregnancy.(26)

3 Methodology/Materials

3.1 Chemicals

Estradiol valerate (USP, CAT no. 1254009) was purchased from Nanolife Quest (Shah Alam, Malaysia). The test chemical was dissolved in sesame oil and was freshly prepared daily before treatment.

3.2 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. Extraction and fractionation of the prototypes were specifically done by chromatographic separation following the methods of Low et al.(27)

3.3 Animals

Twenty-five nulliparous female and eight sexually matured male Sprague-Dawley rats, aged between 10 to 12 weeks were used. The animals were placed in polypropylene cages under standard laboratory conditions at a room temperature of 22 4 0C, humidity of 30-70% with 12 hours light/dark cycle. All animals were fed with standard diet and tap water ad libitum throughout the experiment. The study was performed according to Animal Study Guidelines and was ethically approved by the Integrated Centre for Research Animal Care and Use (ICRACU), International Islamic University Malaysia [(IIUM/ IACUC Approval/ 2015/ (5) (31)].

3.4 Selection of Dose

The no-observed adverse effect level (NOAEL) of the extract was shown as 100 mg/kg body weight/day by Low et al.(26) The Eurycoma longifolia standardized extract, TAF 273, were prepared at concentration of 25 mg/kg, 50 mg/kg and 100 mg/kg body weight to investigate effects of increasing dosage. Estradiol valerate at a dose of 0.15 mg/kg was chosen in this present work following recommendation from a previous study by Behnam-Rasouli and Nikravesh.(2)

3.5 Treatment

The possible teratogenic effects on the development of foetus in female rats exposed to estradiol valerate (EV) was explored. This study was undertaken to further evaluate the possible effects of different doses of Eurycoma longifolia standardized extract (TAF 273) on the developmental toxicity induced by excessive estrogen levels in pregnant rats. Female rats were housed with male rats at

the start of the pro-oestrus stage at a ratio of 3:1. The presence of vaginal plug or sperm in the vaginal smear the following morning confirmed the success of mating and was considered as day 0 of the gestation period (GD0). Pregnant rats were weighed individually and randomly divided into five treatment groups of 5 rats each as follows: (i) 10 ml/kg of distilled water (p.o.), (ii) 0.15 mg/kg EV (s.c.) on GD12, (iii) 0.15 mg/kg EV (s.c) on GD12 + 25 mg/kg TAF 273 (p.o.) from GD12 to GD19, (iv) 0.15 mg/kg EV (s.c) on GD12 + 50 mg/kg TAF 273 (p.o.) from GD12 to GD19 and (v) 0.15 mg/kg EV (s.c) on GD12 + 100 mg/kg TAF 273 (p.o.) from GD12 to GD19. The TAF 273 extracts were prepared daily in distilled water and administered using oral feeding gavage. Pregnancy was terminated on GD21 by injection of 15 mg/kg of xylazine (Sigma Aldrich) and 30 mg/kg ketamine (Sigma Aldrich) intraperitoneally (i.p) subsequent to which a caesarean section was performed.

3.6 Maternal Evaluation

Maternal body weight was recorded before termination of the pregnancy on G21. The uterus (gravid uteri) and ovaries were removed, dried in absorbent paper and weighed. The site for implantation were counted through the transparent uterine wall. The uterine horns were cut along the anti-mesometrial (greater) curvature for macroscopic examination of position of late resorptions. Live or dead fetuses were subsequently numbered from the ovarian end of each horn. The live foetus was determined by observation of breathing or response to sound or touch. The number of implantations, resorptions and foetuses were necessary to calculate the post-implantation loss using the following formula:

Post-implantation loss (%) = [(no. of dead foetus + no. of resorption) / total no.implantations]$\times$ 100.(28)

3.7 Foetus Evaluation

Live foetuses were removed from the uterus and separated from its amniotic sac and placenta and dried on a bloating paper. Foetal parameters such as number of live foetus, weight of foetus, crown-rump-length and tail length were measured. All foetuses were then

examined for sex and external anomalies including kyphosis, scoliosis, kinky tail and growth retardation.

3.8 Statistical Analysis

The SPSS 18.0 software was used for statistical analyses of data obtained and results were presented as mean ± SD. One-way analysis of variance (ANOVA) and Tukeys test were used as measures to evaluate the significant differences between the groups. The statistical difference was indicated with $p \leq 0.05$.

4 Results and Findings

4.1 Maternal Body Weight

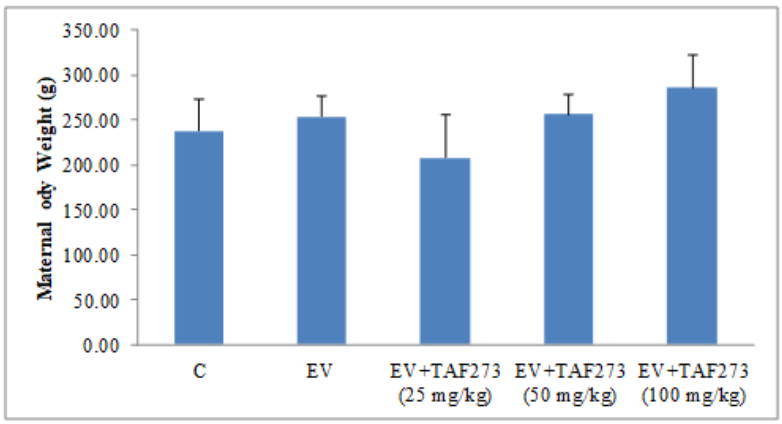

Figure 1: Maternal body weight on GD21 following exposure to EV and treatment with TAF 273 (25, 50 and 100 mg/kg) from GD12-GD19 (n=5). Values are expressed as mean ± SD.

Pregnant rats in all groups survived until the scheduled euthanasia. The effects of treatment with estradiol valerate and Eurycoma longifolia standardized extract (TAF 273) on mean weights of pregnant rats are shown in Figure 1. Although not significant, the mean values for the control and experimental groups on GD21 showed a slight increase in body weight for dams treated with EV alone (253.75 ± 23.24 g) compared to the control group (237.71 ± 35.96

g). It was observed that the body weight of dams increased in a dose-dependent manner and100 mg/kg recorded the highest mean body weight of 285.49 ± 36.58 g. TAF 273 at 50 mg/kg and 25 mg/kg resulted in mean weights of 256.07± 21.91 g and 207.93 ± 46.81 g respectively. There was a significant ($p<0.05$) difference in body weight of dams between groups treated with 25 mg/kg and 100 mg/kg of TAF 273.

Table 1. Reproductive parameters exhibited at gestation day 21 following exposure of pregnant rats to estradiol valerate (EV) and Eurycoma longifolia standardized extract (TAF 273).

			Prior single injection of 0.15 mg/kg of EV		
Parameters	**Control**	**EV (0.15 mg/kg)**	**TAF 273 (25 mg/kg)**	**TAF 273 (50 mg/kg)**	**TAF 273 (100 mg/kg)**
No. of dams	5	5	5	5	5
Implantation					
Total (N)	54	46	45	48	53
Implantation/dam	10.8 ± 1.52	9.2 ± 2.39	9.0 ± 3.71	9.6 ± 2.39	10.6± 1.79
Live foetuses					
Total (N)	53	43	43	42	52
Live foetuses/dam	10.6 ± 2.88	8.6 ± 2.07	8.6 ± 2.30	8.4 ± 2.70	10.4± 2.70
Dead foetuses					
Total (N)	-	-	-	-	-
Resorptions					
Total (N)	1	3	2	6	1
Resorptions/dam	0.2 ± 0.45	0.6 ± 0.55	0.4 ± 0.90	1.20 ± 2.17	0.2± 0.45

Values are expressed as mean ± SD, n=5

Fig. 2 Boost converter

4.2 Number of Live Foetuses

Exposure to EV and treatments with TAF 273 at all doses had no significant effect on the number of live foetuses recovered compared to those in the control group. In EV alone-treated group, a slight reduction in the number of live foetuses was observed (8.6 ± 2.07) when compared to the control group (10.6 2.88). In TAF 273 treatment groups, the number of live foetuses in dams treated with 25 mg/kg and 50 mg/kg were comparable to those in EV-treated group, with 8.6 ± 2.30 and 8.4 ± 2.70 foetuses respectively. In contrast, treatment with 100 mg/kg TAF 273 showed an increased in the number of live foetuses (10.4 ± 2.70 foetus) which are comparable to those in control group. This is in accordance with the study done by Low et al.(26), where they found that the number

of live foetuses was significantly increased ($p<0.01$) following treatment with TAF 273 during the period of organogenesis. In addition to this, the reduction in the number of live foetus observed in EV-treated group in the present study shows agreement to the study done by Benham-Rasouli and Nikravesh(2). The results are also in accord with that of a study using a lower dose of EV (5 g) administered intradermally on gestation day 7, which showed a significant reduction ($p<0.01$) in litter size.(29)

4.3 Post-implantation Loss and Resorptions

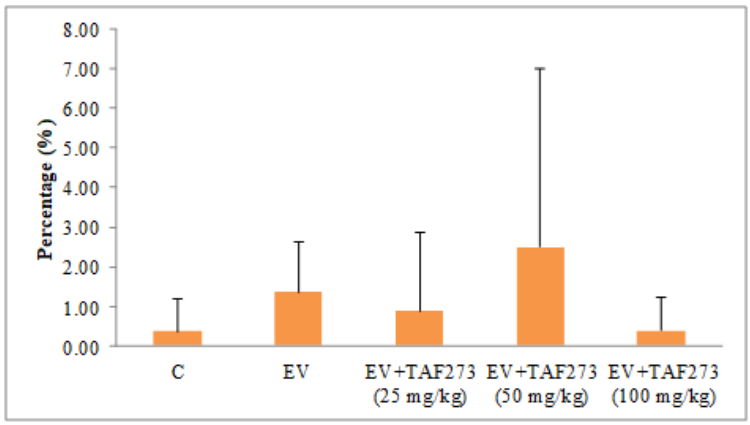

Figure 2. Percentages of post-implantation loss following exposure to EV and treatment with TAF 273 (25, 50 and 100 mg/kg) from GD12-GD19 (n=5). Values are expressed as mean ± SD.

It was previously reported that a single injection of 0.15 mg/kg EV during the first half of pregnancy (gestation day 0, 2, 4, 6, 8 and 10) increased the abortion rate of pregnant rats. Exposure of estradiol valerate on the first half of pregnancy (on gestation day 6) has been done (data not shown) prior to the present study in order to prove the high incidence of abortion. Similarly, no pregnancies were observed in all treated dams and inflammation and haemorrhage of the uteri were observed suggesting implantation to have occurred before abortion. Some of the dams experienced vaginal bleeding after injection of EV on GD6. It was then decided that the injection of EV commenced on GD12 to investigate the effects

of the Eurycoma longifolia standardized extract on foetal development upon exposure to EV. Figure 2 shows the initial number of successful implantation to enable the calculation and the presentation of the number of foetal post-implantation loss. Exposure to EV was done during the second half of pregnancy on gestation day 12 (GD12) according to a previous study done by Behnam-Rasouli and Nikravesh.(2). Therefore, results on the number of corpora lutea, pre-implantation loss and number of early resorption were not included in this present study as the exposure begins after implantation.

Resorptions were found in all groups with no statistical difference observed (Table 1). The control group and treatment group with high dose of TAF 273 (100 mg/kg) showed the lowest mean values of resorption among all groups with similar mean values of 0.20 $\pm$0.45 per dam. Treatment with mid-dose of TAF 273 (50 mg/kg) recorded the highest mean value of 1.20$\pm$ 2.17 followed by EV alone-treated group with a mean resorption of 0.60 $\pm$ 0.55 per dam. Mean resorption following administration of 25 mg/kg TAF 273 was 0.40 $\pm$ 0.90 per dam. Similar to the trend in resorption, treatment with 50 mg/kg TAF 273 also recorded the highest mean percentage of post-implantation loss followed by EV alone-treated group with 2.50 $\pm$ 4.52 % and 1.36 $\pm$ 1.24 % compared to the control group (0.37 $\pm$ 0.83) respectively. The mean percentage of post-implantation loss in the group treated with 25mg/kg and 100 mg/kg of TAF 273 were 0.88$\pm$ 1.99 % and 0.38 $\pm$ 0.85 % respectively. Authors note that the variability in some groups are high for some treatments as appropriate in any animal study.

4.4 Foetal Weight

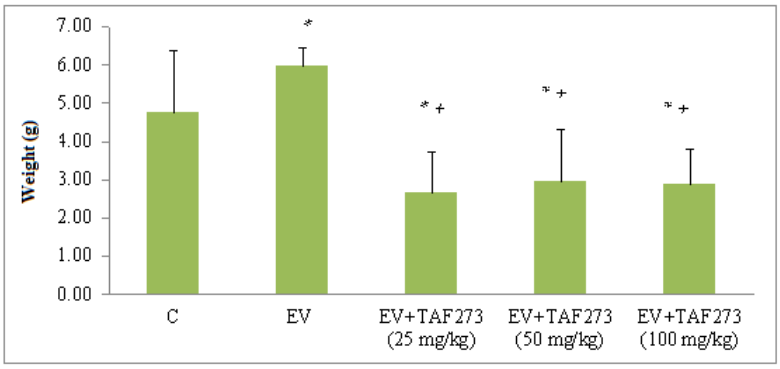

Figure 3. Body weight of foetus following exposure to EV and treatment with TAF 273 (25, 50 and 100 mg/kg) from GD12-GD19. Data are expressed as mean SD. * $p < 0.001$ compared to control group (C), + $p < 0.001$ compared to EV alone-treated group (EV).

As shown in Figure 3, the mean body weight of the foetus was significantly reduced ($p<0.05$ and $p<0.001$) in all TAF 273 treatment groups compared to that of control and EV alone-treated group. The EV alone-treated group recorded the highest foetus body weight with a mean value of 5.95 0.49 g compared to the control group (4.75 ± 1.64 g). The results showed that the foetus weight was influenced by the number of litter size. In the EV alone-treated group, the number of litter size was reduced with increased in the litter mass. This showed that the foetus in the EV alone-treated group received enough supply of nutrition since there were more spaces in the uterus due to a lower number of foetuses therefore resulting in an increase in the foetus weight.(30) The significant decreased observed in the foetus body weight in all TAF 273 treated groups may be due to the effect on the availability of space in the uterus for embryonic development as well as insufficient blood supply for nutrition.(31-33) The reduction may also serve as an indication that the foetus experienced intrauterine growth restriction.

4.5 Foetal Crown-rump Length

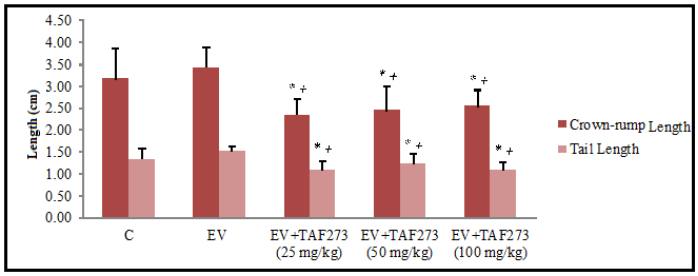

Figure 4. Crown-rump length and tail length following exposure to EV and treatment with TAF 273 (25, 50 and 100 mg/kg) from GD12-GD19. Data are expressed as mean SD. * $p < 0.05$ compared to control group (C), + $p < 0.05$ compared to EV alone-treated group (EV).

The EV alone-treated group recorded the highest means value of crown-rump length (3.44 $\pm$ 0.45 cm) compared to the control group (3.18 $\pm$ 0.70 cm) (Figure 4). The crown-rump length was significantly ($p<0.05$) lower in groups that received treatment of 25 mg/kg, 50 mg/kg and 100 mg/kg TAF 273, with mean values of 2.36 $\pm$ 0.35 cm, 2.45 $\pm$ 0.56 cm and 2.55$\pm$ 0.38 cm respectively. The reduction in crown-rump length observed in the treatment groups whose mother were treated with various doses of TAF 273 was probably due to the reduction of body weight of the foetuses. As mentioned earlier, this might also serve as an indication for intrauterine growth restriction. A representation of crown-rump length can be seen in Figure 5a.

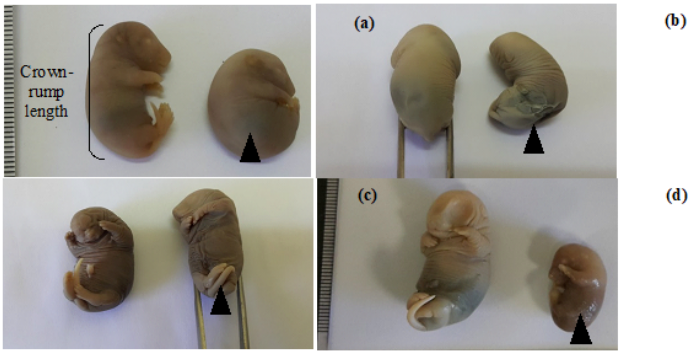

Figure 5: Photographs showing (a) foetus with kyphosis (arrowhead), (b) foetus with scoliosis (arrowhead), (c) foetus with kinky tail (arrowhead) and (d) foetus with growth inhibition (arrowhead).

4.6 Foetal Tail Length

The foetal tail length was significantly ($p<0.05$) lower in the TAF 273 treatment groups whose mothers received 25 mg/kg, 50 mg/kg and 100 mg/kg extract of Eurycoma longifolia standardized extract, TAF 273, compared with the control and EV-treated group (Figure 5). The tail length recorded in the treatment groups were 1.10 $\pm$ 0.20 cm, 1.23 $\pm$ 0.23 cm and 1.10 $\pm$0.18 cm for 25 mg/kg, 50 mg/kg and 100 mg/kg TAF 273 respectively. In the EV alone-treated group, the tail length was recorded at a higher value (1.52$\pm$0.12 cm) than that of the control group (1.35$\pm$ 0.23 cm). Several studies with different plant extracts have also reported the same findings where reduction in foetal weight and crown-rump length have associations with a reduced tail length.(34, 35) This connection was not fully understood but the reduction in the length of the tail may probably be due to the inhibition of growth of different parts of the body.

4.7 Sex of Foetus

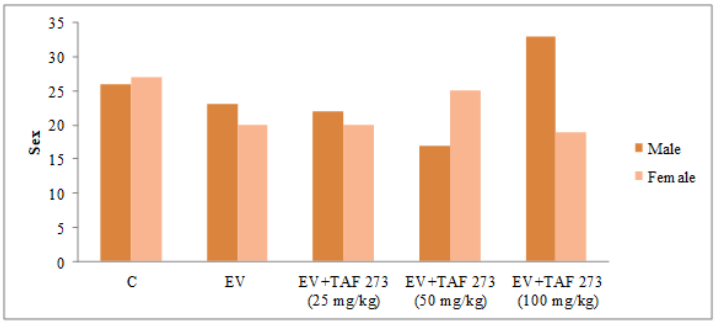

Figure 6. Sex of foetus following exposure to EV and treatment with TAF 273 (25, 50 and 100 mg/kg) from GD12-GD19.

The sex of the foetus was determined by observation of anogenital distance (AGD). The AGD in females is usually smaller than the males. The number of male foetuses was increased in the group treated with 100 mg/kg of TAF 273 with 33 males recorded out 52 foetuses (Figure 6). The control group recorded a balanced ratio of male and female foetuses (26 and 27) similar to pregnant rats treated with EV alone (23 and 20) and TAF 273 at 25 mg/kg treatment group (23 and 20). A record of 25 females out of 42 foetuses was noted for the group treated with 50 mg/kg TAF 273. Determination of sex in polytocous species with multiple offspring was suggested to be influenced by maternal diet which has high glucose content, fatty acids and postprandial triacylglycerol (TAG), following which, an increased chance of male offspring are produced rather than females.(36) The high proportion of male foetuses in the group treated with 100 mg/kg TAF 273 might probably be due to the maternal diet before and during conception thus not related to the treatment.

4.8 External Malformations of the Foetus

Table 2. Foetal abnormalities exhibited at gestation day 21 following exposure of pregnant rats to estradiol valerate (EV) and Eurycoma longifolia standardized extract (TAF 273).

Parameters	Control	EV (0.15 mg/kg)	Prior single injection of 0.15 mg/kg of EV		
			TAF 273 (25 mg/kg)	TAF 273 (50 mg/kg)	TAF 273 (100 mg/kg)
No. of foetus observed	53	43	43	42	52
Kyphosis	6 (11.32%)	15 (34.88%)	10 (23.26%)	10 (23.81%)	6 (11.54%)
Scoliosis	15 (28.30%)	20 (46.51%)	5 (11.63%)	8 (19.05%)	4 (7.69%)
Kinky tail	2 (3.77%)	9 (20.93%)	4 (9.30%)	11 (26.19%)	19 (36.54%)
Growth inhibition	16 (30.19%)	-	28 (65.11%)	30 (71.43%)	33 (63.46%)

Values are expressed as mean ± SD, n=5

4.8.1 Kyphosis and Scoliosis

Kyphosis or body humpback is a condition in which the spine in the upper back has an excessive curvature (Figure 5a). Administration of EV to pregnant rats on GD12 induced an increase in the percentage of external malformations of the foetus in the form of kyphosis exhibited by 15 out of 43 foetuses (34.88%) (Table 2). In the groups treated with 25 mg/kg and 50 mg/kg TAF 273, the incidence of kyphosis was the same with 10 foetuses recorded for each. Control and treatment group of 100 mg/kg TAF 273 each recorded 6 foetuses with kyphosis. Scoliosis, on the other hand, is an occurrence of abnormal lateral curvature of the spine (Figure 5b). Scoliosis seemed more evident in the group treated with EV alone with the incidence of 46.51 % (20 out 43 foetuses) followed by the control group at 28.30 % (15 out of 53 foetuses). Scoliosis incidences in the TAF 273 treatment groups were 11.63 % (5 out 43 foetuses), 19.05 % (8 out of 42 foetuses) and 7.69 % (4 out of 52 foetuses) for doses of 25 mg/kg, 50 mg/kg and 100 mg/kg respectively. The skeletal malformations in the form of kyphosis and scoliosis observed in this present work might be due to congenital factors or restricted food supply to foetus. A study in different species has found that deficiency of vitamin B6 induced scoliosis in 75% of birds.(37) It might also be caused by the positioning of the foetus and the limited space in the uterus especially in the EV alone treatment group, where the foetuses were observed to have a higher body weight and length.(38)

4.8.2 Kinky Tail

Incidence of kinky tail (Figure 5c) in TAF 273 treatment groups increased in a dose-dependent manner with 4 (9.30 %), 11 (26.19

%) and 19 foetuses (36.54 %) for 25 mg/kg, 50 mg/kg and 100 mg/kg doses respectively (Table 2). In the group administered with EV alone, 9 out 43 (20.93%) foetuses displayed kinky tail which was observed to be higher than the control group (3.77 %). The incidence of kinky tail is correlated to the length of the tail. A reduction in tail length may lead to a higher percentage of kinky tail. Interference of the drug during ossification process might be the reason of kinky tail formation.(39)

4.8.3 Foetal Growth Inhibition

Foetal growth inhibition (Figure 5d) was observed in control (30.19%) and in all TAF 273 treatment groups. In the latter, foetal growth inhibition was more pronounced in the group treated with 50 mg/kg TAF 273 (71.43%) (Table 2). The growth inhibition for treatment groups with 25 mg/kg and 100 mg/kg were 65.11 % and 63.46 % respectively. Intrauterine growth restriction (IUGR) or growth retardation is a condition where the foetus fails to reach its full potential of genetic growth potential.(40, 41) Reduction in body weight and length of the foetus can be an indicator of IUGR.(42) In the present study, treatment with TAF 273 at doses of 25 mg/kg, 50 mg/kg and 100 mg/kg caused foetal growth retardation indicated by a statistically significant ($p<0.05$) reduction of both foetus weight and crown-rump length. As pregnancies were synchronized in all rats, the observed IUGR of the foetus in this study may probably be associated to impaired glucose supply to the foetus rather than the reduction in gestational length or preterm delivery.(34) This is associated with reduction of placental efficiency and development in relation to maternal-foetuses exchange (33, 35) as a smaller placenta with a lower blood flow may cause a significant hypoxia resulting in growth retardation of the foetus.(38, 43) The placental weight and length were not recorded in this current study but the parameters may provide an insight to the growth inhibition observed, showing functional connection between the mother and foetus. Nevertheless, the growth inhibition observed in this experiment could perhaps resulted from effects of Eurycoma longifolia standardized extract (TAF 273) and/or spontaneous or naturally occurring conditions in the female pregnant rats. A similar pattern of growth inhibition was observed in the control group but with a

smaller number of foetuses exhibiting the characteristics. This was supported by studies where the malformations induced were also sporadically observed in the foetus from the control group.(44-46)

5 Conclusion

In conclusion, the developmental toxicity induced by estradiol valerate was evidenced by a marked reduction in the litter size as well as an increase in the number of foetuses with external morphology defects. The present study also suggests that Eurycoma longifolia at a dose of 100 mg/kg may have the potential in ameliorating teratogenic effects of estradiol valerate by reducing external abnormalities in the rat fetuses. The increased incidence of growth inhibition may have to be investigated further to identify plausible causes. Application of plant extracts in between gestation day 12 and 19 has enabled the study of anti-teratogenic effects without the loss of foetus through spontaneous abortion and this experimental model may prove to be beneficial in the extension of the investigation into perturbation of pathways important to foetal growth. The standardized extract of the Eurycoma longifolia seemed to have potential in the protection against teratogenic effects resulting from hormonal imbalance and may have prospects in the treatment of female-linked disorders and fertility procedures.

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