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## The Effects of Malaysian Herb, *Labisia pumila* var. *alata* on Oestrous Cyclicity and Reproductive Parameters of Nulliparous Rats

(Kesan Herba Malaysia, *Labisia pumila* var. *alata* ke atas Kitaran Oestrus dan Parameter Reproduksi Tikus Nuliparus)

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### ABSTRACT

Numerous nutraceutical products containing the powdered or extracted parts of *Labisia pumila* (Myrsinaceae) have been widely available for years in Malaysia, aimed at women of reproductive age. However, there is scarce of information concerning the effects of this plant on the reproductive function of nulliparous females prior to the present study. The toxicity potential of *Labisia pumila* var. *alata* (LPA) on oestrous cycle and reproductive parameters was evaluated in groups of 40 virgin rats. They were administered with LPA at the doses of 0 (control), 20, 200 or 1000 mg/kg/day for duration of three weeks. The results obtained indicated that the administration of LPA at all dose levels did not cause mortality nor show noticeably any treatment-related signs of toxicity on the physical appearance, behaviour and body weight of all the rats studied. The pattern and length of oestrous cyclicity as well as the changes in reproductive hormones were statistically comparable among groups. No indications of abnormalities in the histology of uterus and vagina were observed. However, the presence of ovarian follicular cysts has raised apprehension that requires further investigation. The current findings suggested that oral treatment of LPA were associated with toxicity concerns.

**Keywords:** *Labisia pumila* var. *alata*; nulliparous; oestrous cycle; ovarian cyst; rat

### ABSTRAK

Pelbagai produk nutraseutik yang mengandungi serbuk atau ekstrak *Labisia pumila* (Myrsinaceae) terdapat secara meluas di Malaysia sejak sekian lama, disasarkan kepada wanita dalam usia reproduktif. Walau bagaimanapun, terdapat sedikit maklumat mengenai kesan tumbuhan ini ke atas fungsi reproduktif betina nuliparus sebelum kajian ini dijalankan. Potensi ketoksikan *Labisia pumila* var. *alata* (LPA) telah dinilai ke atas kitaran oestrus dan parameter reproduktif bagi sekumpulan 40 tikus dara. Tikus tersebut diberikan LPA pada dos 0 (kawalan), 20, 200 or 1000 mg/kg/hari selama tiga minggu. Keputusan yang diperolehi menunjukkan bahawa pemberian dos LPA pada semua peringkat tidak menyebabkan kematian, juga tidak menunjukkan sebarang tanda ketoksikan yang ketara pada keadaan fizikal, perlakuan dan berat badan semua tikus yang dikaji. Corak dan tempoh kitaran oestrus serta perubahan hormon reproduktif adalah setanding secara statistik dalam kalangan semua kumpulan rawatan. Tiada indikasi ketaknormalan yang diperhatikan dalam histologi uterus dan vagina. Walau bagaimanapun kehadiran sista folikel ovari telah menimbulkan kebimbangan yang memerlukan kajian lanjut. Penemuan dalam kajian ini menunjukkan bahawa rawatan oral LPA dikaitkan dengan kebimbangan kesan toksik.

**Kata kunci:** Kitaran oestrus; *Labisia pumila* var. *alata*; nuliparus; sista ovari; tikus

### INTRODUCTION

*Labisia pumila* or locally known as kacic fatimah is one of the most popular herbs that is receiving high demand by the local populace in Malaysia. The popularity of this herb is manifested by the emergence of many commercial products in the Malaysian market including capsules, tablets, health drinks and tonics containing this herb. *L. pumila* has been utilized by the Malays and aboriginal women of reproductive age for numerous purposes particularly related to the reproductive health. Traditionally, water decoctions of the root or whole plant have been given to pregnant woman prior to childbirth for the main purpose of inducing and facilitating labor (Burkill 1966; Stone 1988). It is also used as a postpartum medication (Stone

1988) for the recovery of the post-gravid uterus, to delay fertility, to regularize blood circulation and to help regain body strength (Muhamad & Mustafa 1994). Its other folkloric uses include treatment of dysentery, flatulence, dysmenorrhea, gonorrhoea, rheumatism, hemorrhoid and help to firm the abdominal muscles (Jamia et al. 2003). Many believe that this herb is a phytoestrogen and thus is able to enhance libido (Asiah et al. 2007), alleviate fatigue, promote emotional well being and assist women in achieving fuller breasts and tighten vaginal muscles (Ayida et al. 2006).

The highly popular nature of *L. pumila* amongst women warrants a detailed scientific study particularly on its toxicological potential in the females. Couple with the

fact that there is still dearth of information on the safety profile of this herb particularly on the female reproductive function, the present study was designed to assess the possible effects of this herb on the reproductive parameters of female rats. This work was also to reflect on conditions where *L. pumila* is consumed by non-pregnant women for the sustenance of their reproductive health.

## MATERIALS AND METHODS

### PLANT MATERIAL

Plant material used in this study was *L. pumila* var. *alata* (LPA). It was chosen among the three popular varieties, since this variety is the most commonly used by the Malay community in the country. The aqueous extract of LPA was prepared by the Chemical Engineering Pilot Plant (CEPP) from Universiti Teknologi Malaysia (UTM), Skudai, Johor, Malaysia. For dosing, the ready powdered extract was weighed and reconstituted with distilled water at different dosages; 0 (control), 20, 200 and 1000 mg/kg/day.

### ANIMALS AND EXPERIMENTAL DESIGN

Female, virgin, healthy Sprague Dawley rats weighing between 150 and 160 g and 8 and 9 weeks old were procured from the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia (USM) Health Campus, Kelantan, Malaysia. After the acclimatisation period of 14 days, the rats were randomly selected to be housed into four experimental groups ( $n=10$ ). They were maintained at  $22\pm 2^\circ\text{C}$  (relative humidity of 50-60%) with 12 h light/dark cycle (lights on from 0700 to 1900 h). The animals had free access to commercially obtained pelleted rat chow and distilled water *ad libitum*. The conduct of this study was approved by the Universiti Sains Malaysia Animals Research and Ethics Committee (No. 013).

The present study began by looking at the oestrous cyclicity of female rats for two weeks to provide a baseline data for regularity of cycling. The procedure of obtaining vaginal smear in this study was adopted with modifications from Marcondes et al. (2002) and Voipio and Nevalainen (1998). The oestrous cycle was divided into a sequence of phases; proestrus (12-14 h), estrus (25-27 h), metestrus (or diestrus I) (6-8 h) and diestrus (or diestrus II) (55-57 h) that were distinguished by the cytological proportion among cornified, leukocytes and nucleated epithelial cells. Females demonstrating 4 to 6 days oestrous cycle were judged to have a normal cycle. It was determined by counting the number of days from the first estrus in a cycle to the next estrus phase in a subsequent regular cycle. Those showing irregular cycles were excluded from the evaluation.

The females were also observed daily for mortality, moribundity, general appearance and behaviour throughout this study. Additionally, daily body weight was recorded for all animals. Following that, females were exposed

to different doses of LPA or vehicle (distilled water) by gavaging on the first day of diestrus at approximately the same time in the morning for duration of three weeks. Vaginal samples were continuously inspected daily during this period. Number of animals completing at least four regular oestrous cycles was documented. The average oestrous cycle length of animals was also calculated.

On the first day of diestrus upon completion of three weeks test period, all females were anesthetized and immediately laparotomized. Blood samples were collected for serum hormonal assays. Autopsy was performed by which all visceral organs were inspected macroscopically with verification of abnormalities were conducted by the independent toxicologist. Only the reproductive organs i.e. ovary, uterus and vagina were removed, weighed and fixed in 10% neutral buffered formalin solution prior to histopathological examination. All animals were sacrificed under overdose of anaesthesia.

### HISTOPATHOLOGY OF REPRODUCTIVE ORGANS

The aforementioned reproductive organ tissues were serially sectioned at 5  $\mu\text{m}$  thickness, embedded and stained with hematoxylin and eosin. The sections from each organ were examined both qualitatively and quantitatively under a light microscope attached to an image analysis system (Olympus, Japan). Histopathological changes noted in treated animals were compared with the control and all deviations from normal were recorded. These interpretations were validated by the independent histopathologist from the same institution.

### SERUM HORMONE LEVELS

Upon withdrawing of blood, the samples were allowed to clot before centrifugation at 3000 rpm for 15 min to obtain serum. Enzyme-linked immunosorbent assay (ELISA) hormonal kits were utilized in this study to measure 17- $\beta$  oestradiol, progesterone and free testosterone (IBL-Hamburg GmbH, Germany). These hormonal kits were based on the competition principle and the microplate separation. The microplate ELISA reader (U Quant Biotek Instrument, USA) at wavelength of 450 nm was used to measure the optical density (O.D). The inter and intra-assay variations of each hormone were less than 15%.

### STATISTICAL ANALYSIS

All numerical data were analyzed using Statistical Package for Social Sciences (SPSS Inc. Chicago, Illinois, USA version 20). GLM repeated measures were applied in analyzing data on body weight of females. Other data, for instance, weight gain, percentage change in body weight, absolute and relative reproductive organ weights, measurements of all histopathological quantitative parameters and all serum hormones analysis were analyzed by one-way ANOVA. Additionally, the regularity of oestrous cycle was tested by Chi square whereas Kruskal Wallis test was applied to assess the length of oestrous cycle.

## RESULTS

## GENERAL HEALTH AND BODY WEIGHT

There were no treatment-related signs of toxicity and deleterious effects of LPA on all female rats during the pre- and three-week treatment periods. Their body weights were increased and consistent among all groups over the study period.

## OESTROUS CYCLICITY

All females displayed normal regular oestrous cycle during the two weeks pre-treatment period. Furthermore, LPA did not substantially alter the oestrous cycle of rats during the treatment period. Twenty-six of 30 treated females (86.67%) and 90% (9/10) of the control animals showed regular cycles during the three weeks test period (Table 1).

The length of the oestrous cycle during treatment period was not considerably different between treated animals and the control group. However, one female of the control group exhibited extended estrus (3 days). Furthermore, one female of Group 2 (LPA 20 mg/kg/day) showed prolonged proestrus (2 days) whereas three females of Group 4 (LPA 1000 mg/kg/day) displayed either an extended estrus (3 days), proestrus (2 days) or even shortened oestrous cycle (a total of 3 days) during the treatment period with LPA (Table 1). These abnormal cycles, however, were only detected once throughout the entire experiment.

## GROSS EXAMINATION AND ORGAN WEIGHTS

On gross inspection at autopsy, there were no unusual judgements on the general condition of rats in both treated and control groups. Besides demonstrating a good external anatomy, their visceral organs were also in normal appearance. The reproductive organ weights of ovaries, uterus and vagina of all animals studied did not exhibit any statistical differences.

## HISTOPATHOLOGY

The histopathological findings showed that there was no concrete evidence of LPA-related deformities in the reproductive organs examined. However, histopathology of the ovary indicated that one female of the control group, which was the same animal that experienced irregular oestrous cycle was observed to have follicular cysts. Furthermore, another female from Group 2 (LPA 20 mg/kg/day) and four animals of the highest dose group (LPA 1000 mg/kg/day) (Figure 1) that represented 16.67% (5/30) of the total LPA-treated females also showed follicular cysts in their ovaries. Out of this number, two females of the LPA 1000 mg/kg/day dose group (Animal-3 and Animal-5) were the same animals that previously displayed irregular cycles. None of cystic follicles was observed in animals of Group 3 (LPA 200 mg/kg/day). The follicular cysts showed stromal hyperplasia and collagenised cortex. Other animals without follicular cysts showed good features of the ovarian cortical and medullary regions (Figure 2). Statistical

TABLE 1. Summary of observations on female rats after receiving daily administration of LPA for three weeks

Parameters	Group 1 DW/Control	Group 2 20 mg/kg/ day	Group 3 200 mg/kg/ day	Group 4 1000 mg/kg/ day	P-value
Oestrous cyclicity:					
- Pre-treatment period					
No. of females with normal cycles.	10/10	10/10	10/10	10/10	-
Length of oestrous cycles (days) <sup>b</sup> .	4.50 (0.63)	4.50 (1.00)	4.50 (0.13)	4.50 (0.63)	n/s
- During LPA treatment period					
No. of females with normal cycles.	9/10	9/10	10/10	7/10	-
Length of oestrous cycles (days) <sup>b</sup> .	4.50 (0.50)	4.50 (0.63)	4.75 (0.50)	5.00 (0.63)	n/s
No. of females with extended estrus	1/10	0/10	0/10	1/10	-
No. of females with extended proestrus	0/10	1/10	0/10	1/10	-
No. of females with shortened cycle	0/10	0/10	0/10	1/10	-
Histopathology (quantitative analysis):					
No. of females with follicular cysts	1/10	1/10	0/10	4/10	-
Hormone level:					
17- $\beta$ oestradiol (pg/mL) <sup>a</sup>	9.40 $\pm$ 3.38	9.25 $\pm$ 1.51	9.42 $\pm$ 1.00	12.80 $\pm$ 2.15	n/s
Progesterone (ng/mL) <sup>a</sup>	16.99 $\pm$ 3.64	33.61 $\pm$ 6.01	44.25 $\pm$ 9.79	27.32 $\pm$ 7.10	n/s
Free testosterone (pg/mL) <sup>a</sup>	0.13 $\pm$ 0.06	0.18 $\pm$ 0.06	0.21 $\pm$ 0.06	0.48 $\pm$ 0.23	n/s

n = 10 for each group.

<sup>a</sup>Mean  $\pm$  SEM (for parametric data)

<sup>b</sup>Median (IQR) (for non-parametric data)

n/s Statistically not significant between all groups,  $p > 0.05$



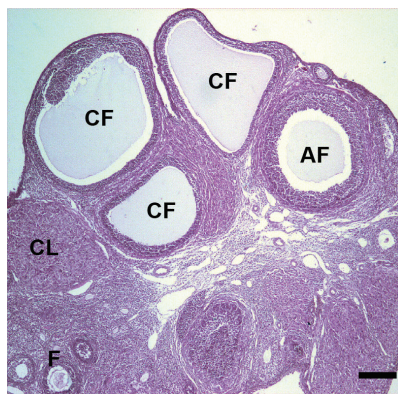


FIGURE 1. Histopathology of ovary showing cystic follicles (CF). The photomicrograph was taken from a female treated with LPA 1000 mg/kg/day (Animal-5). Note the follicular cysts showing larger follicles with thin granulosa cell layer. Note also the atretic secondary follicle (AF) with follicular debris. Others are normal follicle (F) and normal corpora lutea (CL). Bar: 100  $\mu$ m. Magnification: 40 $\times$

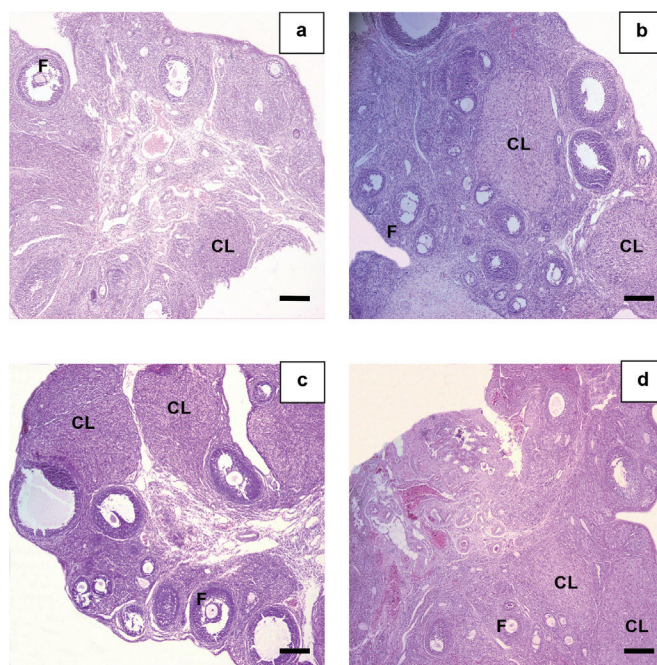


FIGURE 2. Histology of normal ovaries showing well developed follicles (F) and corpora lutea (CL). The photomicrographs were arranged in accordance to treatment groups (a) control/DW, (b) LPA 20 mg/kg/day, (c) LPA 200 mg/kg/day and (d) LPA 1000 mg/kg/day. Bar: 100  $\mu$ m. Magnification: 40 $\times$

analysis on the measurement of the number and diameter of corpora lutea were not significantly different in all groups of animals. Separately, the histological evaluations of uteri and vagina for all LPA-treated animals did show any considerable variations as these organs looked comparable with no indication of abnormalities when compared among all groups of animals.

#### ANALYSIS OF HORMONES

Statistical evaluation showed that the changes in the serum levels of oestradiol, progesterone and free testosterone were not significantly different in all studied rats (Table 1).

#### DISCUSSION

A previous sub-acute toxicity evaluation has shown that *L. pumila* at high doses ranged from 250 to 1000 mg/kg was associated with some toxic manifestations i.e. irregular values of liver function parameters and abnormal histoarchitecture of liver, kidney and lungs of rats (Singh et al 2009). Prior to this, petroleum ether extract of this plant was found to cause sinusoidal degeneration of liver, as a consequence from inflammation in the renal tubules (Effendy et al. 2006). In contrast, earlier studies discovered that aqueous extract of LPA did not possess teratogenic potential (Wan Ezumi et al. 2005) nor deleterious effects

on both dams and fetuses of rats macroscopically (Wan Ezumi et al. 2007). Moreover, the genotoxicity evaluation of LP using micronucleus assays did not demonstrate any mutagenic potency at any dose level tested (Zaizuhana et al. 2006).

The present study showed that exposure of rats daily to LPA of up to 1000 mg/kg did not cause any considerable deleterious effect as all females survived throughout the entire experiment. Their general physical health, behavior and body weights were not significantly disrupted by LPA at all dose levels. These findings are in agreement with the results of the previous studies where LPA did not affect general condition of female rats (Singh et al. 2009; Wan Ezumi et al. 2007, 2005).

As the characterization of oestrous cycle is one of the valuable measures to help clarifying numerous toxicological issues in females (Goldman et al. 2007), this study discovered that LPA did not statistically disrupt the regularity of 4-6 days oestrous cycle pattern of rats. Even though the cases of irregular cycles were noted in most of the treatment groups including the control but none in the 200 mg/kg dose group, these effects were however, not significant statistically. As noted in the U.S. Environmental Protection Agency (EPA) (1996), occasionally control animals can also exhibit irregular cycles.

Apart from the regularity of oestrous cycle pattern, the length of each cycle phase was equally important to be considered for the determination of the oestrous cyclicity status. The variability in the duration of cycles noted in the five irregular cycle rats (1 control and 4 LPA-treated animals) of this experiment, however, did not significantly alter the mean cycle length of all animals. The alteration in the length of oestrous cycle phase might be resulted by several factors for instance, as the effects of polycystic ovarian syndrome (PCOS) (Manneras et al. 2007). As in this study, there were no noticeable histopathological changes of ovary in the five above mentioned rats except for the presence of ovarian follicular cysts in three of them (1 control, 2 of 1000 mg/kg dose group) whereas other cases of follicular cysts were detected in rats without any noticeable symptoms. Bogovich (1991) reported that adult laboratory rats can spontaneously develop ovarian cysts compared to immature rats.

The detection of four cases of follicular cysts in the LPA 1000 mg/kg/day dose group might be highly correlated with the elevated levels of serum free testosterone and oestrogen of the same group. However, the reason of similar occurrence of cysts noted in the control and LPA 20 mg/kg/day dose groups could not be further elucidated since both the oestradiol and free testosterone levels of these groups were relatively lower than those of the higher dose groups (LPA 200 and 1000 mg/kg/day). In contrast, none of animals in the Group 3 (LPA 200 mg/kg/day) showed presence of cystic follicles.

Many researchers speculated that formation of cysts might be associated with endocrine disturbances. Nevertheless, etiology and pathogenesis of follicular cyst in human and animal species remain presently unclear

and may be considered to be multi-factorial (Manneras et al. 2007; Shirwalkar et al. 2007). As being studied in rats, Shirwalkar et al. (2007) had warned that exogenous oestrogen could cause deleterious effects on the ovarian physiology and endocrinology which eventually induce cystogenesis, loss of follicle pool and early senescence. Other studies also stated that rats with polycystic ovaries demonstrated a reduction trend in estrogen and progesterone levels but increased in androgens (Manneras et al. 2007) when compared to the control intact females. As being reported to show estrogenic effects (Manneras et al. 2010), LPA in the present study had also increased the oestradiol levels of rats receiving higher doses but however, this was not statistically significant. Additionally, the free testosterone level also showed an increment as the dose increased. Even these did not reach significance, but the trend shown might be correlated with the determination of bioactive peptide (4.3 kDa) as an aphrodisiac marker in *L. pumila* plant. Taken *E. longifolia* for instance, the presence of the similar bioactive peptide in the plant could increase the testosterone level in rat leydig cell (Asiah et al. 2007). Hence, it is possible to suggest that the administration of LPA might also cause elevation in the testosterone level of female rats as being observed in this study. The rise in free testosterone is associated with increase in libido and sexual interest in females. Nevertheless, George et al. (2014) could not find significant differences in the scores of female sexual function index from the women supplemented with *L. pumila* tablet for 12 weeks.

## CONCLUSION

Under conditions of this study, LPA did not statistically pose any significant toxic effects on the reproductive system of female rats. The incidence of several ovarian cystic follicles found in the highest dose group, however, needs to be investigated as a progression of this study. This finding suggests that LPA could be utilized by females with caution.

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