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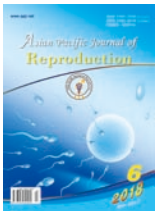

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Effects of *Salacia lehmbachii* ethanol root bark extract on estrous cycle and sex hormones of female albino rats

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ABSTRACT

Objective: To evaluate the effect of *Salacia lehmbachii* (*S. lehmbachii*) ethanol root bark extract on estrous cycle and sex hormones in female rats. **Methods:** Forty-eight virgin rats with regular 4-day cycle were grouped into four and each group was further subdivided into 'a' and 'b' (n=6). Each group was orally treated for 28 days with 2 mL of distilled water (control), ethanol root bark extract of *S. lehmbachii* in doses of 250, 500 and 750 mg/kg/body weight (groups 2, 3, 4, respectively). Estrous cycle was determined daily using the vaginal smear method. Rats in 'a' subgroups were weighed and sacrificed on the 29th day, and blood was collected for serum generation which was used for hormonal assay and sex organs were removed and weighed. Rats in 'b' subgroups were discontinued from treatment for 2 weeks and the parameters above were reassessed. **Results:** The mean length of estrous cycle and duration of diestrous of treated rats were prolonged dose dependently compared to control. The increase was significant (P<0.05) at 500 and 750 mg/kg. The other estrous phases were shortened in the same pattern. Relative weights of sex organs were reduced significantly (P<0.05) at the highest dose. Sex hormones levels were significantly (P<0.05) reduced compared to control. The above changes reverted towards the control values two weeks post treatments. **Conclusions:** Ethanol root bark extract of *S. lehmbachii* (high doses) has antifertility effect in female rats as it prolongs the estrous and diestrous cycle, and reduces serum sex hormones levels. The observed alterations were reversible.

1. Introduction

From the earliest days of mankind, medicinal plants have been used for diverse health issues including controlling pathological conditions and physiological activities. In the course of preclinical evaluation of these plants for safety, some of them have been reported to interfere with female reproductive functions especially the estrous cycle[1,2]. Commonly, such interference is either expressed as a change in normal components of vaginal smear and disruption in the frequency of particular stages of the estrous cycle or both[3]. Xenobiotics may affect estrous cycle by acting at different levels of hypothalamic-pituitary axis resulting in alterations in the levels of gonadotrophins, namely leutinizing hormones (LH) and follicle stimulating hormones (FSH) or at ovarian level to inhibit ovulation[4].

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Ovulation is fundamental to successful establishment of pregnancy, but may also impact the developmental potential of resultant embryos. The ovulatory cascade of events is complex and triggered by a surge of LH which stimulates the activity of multiple intracellular signaling pathways in granulosa cells culminating in altered transcriptional complexes mediating expression of ovulatory genes[5,6]. The observed effects of LH are mainly mediated by adenylate cyclase and increased cyclic adenosine monophosphate (cAMP). The cAMP in turn via cAMP-dependent protein kinase affects three distinct steps which are crucial in the ovulatory process. They include stimulation of steroidogenesis, induction of cyclooxygenase (COX)/ lipooxygenase leading to increased synthesis of prostaglandins/leukotrienes and stimulation of plasminogen activator which catalyzes the conversion of plasminogen to plasmin. The involvement of cyclooxygenase enzymes signifies that ovulation is an inflammatory process. Thus, it may be blocked by chemicals with anti-inflammatory properties especially when they are administered at high doses before the LH surge[7]. Such chemicals are present in some medicinal plants that are used locally for the treatment of diverse ailments. An example of such plants is *Salacia lehmbachii* (*S. lehmbachii*).

S. lehmbachii is a small flowering tree of 3-5 meters in height. It is one of the 52 species in the genus Salacia belonging to family of Celastraceae[8]. The plant is commonly used by the local dwellers in some parts of Southeastern Nigeria and Cameroun particularly the Bakassi forest reserves, for the treatment of febrile illnesses like malaria. It is known in vernacular as 'eba-enang-enang' (peoples of Akwa Ibom and Cross River States) and 'ora-mmanu' (the Igbos), all of Nigeria. The median lethal dose of the root bark extract of the plant in albino rats is above 5 000 mg/kg while its chemical constituents include alkaloids, glycosides, flavonoids, tannins, saponins and polyphenols[9]. The plant has a wide range of pharmacological actions including analgesia and antiinflammation[9], inhibition of male sex hormones[10] and antifertility in male rats[11]. The hepatotoxic, hematotoxic, embryotoxic and teratogenic potentials of the plant have also been reported[12–14]. Though, the root bark extract of *S. lehmbachii* is reported to have antifertility effect in male rats, till date there has not been study to evaluate similar effect in female rats. The present study was undertaken to evaluate the effect of the ethanol extract of the plant's root bark on the estrous cycle and sex hormones in female albino rats.

2. Materials and methods

2.1. Collection and identification of plant material

The roots of *S. lehmbachii* were purchased from Watt market, a local market in Calabar, capital of Cross River State, Nigeria. The plant was authenticated by the Department of Botany of the University of Calabar where a voucher specimen with herbarium number 688 was deposited.

2.2. Preparation of the extract

The roots were washed with clean water to remove dirt and dried in their lengths in an electric oven, and thermostatically controlled at 40 ℃ for 12 h. The root bark powder of the plant was obtained following an earlier described method[10]. The dry ethanol extract was derived from a two-staged Soxhlet extraction of the root bark powder using ethanol (99.8%, BDH Chemical Limited, England) as solvent and the resultant extract solution dried into dry powder following the earlier described method[10]. The solid extract which formed the yield was weighed and preserved in a clean and dry container until required for the experiments. The extract was constituted in distilled water to give the doses required for the study.

2.3. Experimental animals

Twelve weeks old virgin rats, 48 in number and weighing between 170-190 g were purchased from the animal house of the Department of Pharmacology, University of Calabar and housed in plastic cages with wire gauzed top. Each cage contained six rats which were properly identified with dilute picric acid. The animals were acclimatized for seven days to normal laboratory conditions [relative humidity: (50±5)%; temperature: (28±2) ℃ and 12 h of light-dark cycle] before the start of the experiment and maintained at the same conditions throughout the duration of the study. They were fed with standard rat chow (Agro-Feeds, Calabar) and water (Water board, Calabar) *ad libitum*. The guidelines on Care and Use of Laboratory Animals were followed (NIH Publication, No. 85-23, revised 1985). The study protocol (No: 010PA21016, dated 24/10/2016) was approved by the Research and Ethical Committee of the Faculty of Basic Medical Sciences, University of Calabar, Nigeria.

2.4. Determination of the estrous cycle

Estrous cycle of rats was determined between 7 a.m. and 9 a.m. each day using the vaginal smear method[15]. Vaginal lavage of rats carried out with 10 μL of normal saline (NaCl 0.9%) using a plastic pipette resulted in vaginal fluid, a drop of which was placed on a glass slide and examined under a light microscope unstained, without a cover slide and condenser lens and using 40 × objective lens. The proportion of characteristic cell types (leucocytes, cornified and epithelial cells) was used to determine the phases of estrous cycle and a different slide was used per animal[16]. After confirming regular 4-day cyclicity for sixteen days (4 cycles), the animals were selected for this study.

2.5. Animal treatment

The effects of the extract on estrous cycle of female rats were assessed following the earlier described protocol[17] but with slight modifications. Rats with regular 4-day cycle were unbiasedly shared into 4 groups of twelve rats per group. Each group was further

subdivided into 'a' and 'b' (n=6). Group 1 (Control) received 2 mL of distilled water; groups 2, 3 and 4 had 250, 500 and 750 mg/kg body weight of the ethanol root bark extract of *S. lehmbachii*. All treatments were through a gavage starting from proestrous phase and lasted 28 days (7 estrous cycles). The length of estrous cycles and duration of each phase of the cycle were recorded as described previously[18]. Twelve hours after the last treatment, rats in all the 'a' subgroups were weighed before being sacrificed under chloroform anaesthesia and blood was collected via cardiac puncture into plain bottles for generation of serum which was used for hormonal assay. The uterus and ovaries were dissected out, cleaned of blood in between filter papers, and weighed (absolute weight). For the rats in the 'b' subgroups, all administrations were withdrawn for two weeks and their estrous cycles were studied for another 28 days (post-extract period), at the end of which, the sex hormones, weight of animals and that of uterus and ovary were reassessed. The aim of this later exercise was to evaluate the reversibility of observed changes in the first part of the study.

2.6. Relative organ weights

The relative weights of the harvested organs of each rat in 2.5 g above were calculated as earlier documented using the formular below[19,20]:

Relative organ weight (mg/100 g body weight) = Absolute organ weight (g) × 100 / Body weight of rat on sacrifice day (g)

2.7. Hormonal assay

Sera generated from the collected blood samples by allowing them stand for 3 h to ensure complete clotting, centrifuging the clotted samples at 3 000 rpm for 10 min and aspirating off the serum with sterile needles and syringes were used for hormonal analysis. The enzyme-linked immunoassay technique was used for the assay. The procedures in the hormone assay kits (Life Science Inc, USA) were followed according for LH and FSH, estradiol and progesterone[21].

2.8. Statistical analysis

Results were expressed as mean ± stand error (mean ± SE) of six observations. Means were analyzed using a one-way ANOVA followed by Student's test to compare the difference between the control and treated values and P<0.05 was considered significant.

3. Results

Estrous cycle length was dose dependently prolonged in treated rats compared to control (Table 1). Treated rats changed estrous cycle length from 4 days (Control) to (4.31±0.35) days, (5.41±0.94) days and (6.63±1.01) days at doses of 250, 500 and 750 mg/kg respectively. The extract-induced elongation of estrous cycle was significant (P<0.05) only at high doses (500 mg/kg and 750 mg/kg).

Table 1

Effect of ethanol root bark extract of *S. lehmbachii* on rat estrous cycle.

Extract	Length of rats estrous cycle (d)	
	After 24 days of treatment	2 weeks after withdrawal of treatment
Control (2 mL DW)	4.00 ± 0.00	4.00 ± 0.00
250 mg/kg ESL	4.31 ± 0.35	4.00 ± 0.00
500 mg/kg ESL	5.41 ± 0.94[a]	4.71 ± 0.44
750 mg/kg ESL	6.63 ± 1.01[a]	5.23 ± 1.21[a]

Values are Mean ± SEM; n=6; DW= Distilled water; a = significant difference from 250 mg/kg of ESL at P<0.05; ESL = Ethanol root bark extract of *Salacia lehmbachii*.

The duration of dietrous phase was prolonged while that of other phases (proestrous, estrous, metestrous) were shortened also dose dependently (Table 2). *S. lehmbachii* ethanol root bark extract increased the duration of diestrous from 25.00 (% no. of days) in control rats to 41.06 and 47.72 at doses of 500 and 750 mg/kg body weight respectively, showing significance (P<0.05) only at high doses.

Table 2

Effect of ethanol root bark extract of *S. lehmbachii* on phases of rat estrous cycle (% number of days).

Extract	Proestrous	Estrous	Metestrous	Diestrous
Control (2 mL DW)	25.00	25.00	25.00	25.00
250 mg/kg ESL	24.71	25.00	24.41	25.88
500 mg/kg ESL	18.85[a]	21.08[a]	19.01[a]	41.06[a]
750 mg/kg ESL	17.94[ab]	16.92[ab]	17.42[ab]	47.72[ab]

Values are Mean ± SEM; n=6; DW= Distilled water; a = significant difference from 250 mg/kg ESL, P<0.05; b = significant difference from 500 mg/kg ESL, P<0.05; ESL = Ethanol root bark extract of *Salacia lehmbachii*.

The relative weights of rats uteri and ovaries were significantly (P<0.05) different from control only in rats treated with 750 mg/kg body weight (Table 3).

Table 3

Effect of ethanol root bark extract of *S. lehmbachii* on relative weights of rat uterus and ovary (mg/100g body weight).

Extract	After 24 days of treatment		2 weeks after withdral of treatment	
	Uterus	Ovary	Uterus	Ovary
Control (2 mL DW)	0.151±0.030	0.059±0.001	0.149±0.021	0.059±0.002
250 mg/kg ESL	0.153±0.041	0.058±0.001	0.153±0.041	0.058±0.001
500 mg/kg ESL	0.155±0.023	0.060±0.003	0.152±0.021	0.061±0.013
750 mg/kg ESL	0.179±0.031[a]	0.145±0.002[a]	0.151±0.031	0.065±0.022

Values are Mean ± SEM; n=6; DW = Distilled water; a = significant difference from 250 mg/kg ESL, P<0.05; ESL = Ethanol root bark extract of *Salacia lehmbachii*.

Serum concentrations of FSH, LH, progesterone and estradiol were dose dependently reduced by the extract and at high doses (500 and 750mg/kg body weight), and the percentage reduction was

significantly (P<0.05) different of the control value (Table 4).

Reassessment of each of the above parameters after two weeks of withdrawal of treatments gave values showed a reversal toward the control values (Tables 1, 3, 5).

4. Discussion

Estrous cycle in rats occurs rapidly between 4 to 5 days and the events within the cycle are regulated by gonadotropin releasing hormone from the hypothalamus, gonadotropins from the anterior pituitary gland and sex hormones from the gonads[22]. The cyclic vaginal change which characterizes estrous cycle is an index of good functioning of the neuroendocrine- reproductive system and ovarian activity, while a distortion of normal estrous cycle indicates disruption of ovarian progesterone and estrogen balance[1,2]. In our study, the length of the estrous cycle was dose dependently and significantly (P<0.05) increased in the extract-treated groups compared to control. The mean cycle lengths of 4.31 and 5.41 days from rats treated with 250 and 500 mg/kg body weight of the extract aggree with the results of other investigators who recorded a mean cycle length of 5.4, 4.5 and 4.4 days[23–25]. The cycle length of 6.63 days from rats treated with high dose (750 mg/kg) of the extract clearly show a prolonged cycle length and this value was higher but statistically insignificant (P>0.05) to that from 500 mg/kg–treated groups.

The observed increase in estrous cycle length in this study implies impaired fertility and could be ascribed to the prolonged diestrous and shortening of the other phases also noted in this study. Other workers had also recorded the increased estrous cycle length and prolonged die strus with shortening of the other estrous phases following the administration of other plant extracts and xenobiotics. This includes studies on *Garcinia kola* seeds[17], *Trichosanthes cucumerina*[26], *Ixora coccinea*[27], *Mimosa pudica*[28], artemether[29] and amodiaquine[30]. A perculiarity of the plants and drugs mentioned above as well as S. lehmbacii being investigated is anti-inflammatory activity[31–35,11].

The prolongation of estrous cycle and diestrous phase observed in this study is an indication of impairment of ovulation, and also is regarded as an inflammatory process[36]. Some researchers had earlier used anti-inflammatory drugs to block ovulation[37]. Anti-inflammatory drugs inhibit the COX enzymes thereby preventing prostaglandins synthesis. There are two important isozymes of COX enzyme (COX-1 and COX-2) and studies carried out on COX-2 deficient mice showed defective ovulation, implying that COX-2 is involved in ovulation[38]. Since the extract has anti-inflammatory action as earlier mentioned, it may have blocked ovulation in this study by inhibiting COX-2 enzyme thereby inhibiting prostaglandin synthesis.

The hypothalamic-pituitary-gonadal axis as already mentioned plays an important role in reproduction. Gonadotrophins (LH, FSH) in females cause follicles to mature, develop into pre-ovulatory follicles and secrete estrogen[7]. In this study, the levels of gonadotrophins were significantly reduced indicating distortion of pituitary-ovarian axis. This finding aggrees with the earlier report that the same extract disrupts the pituitary-testicular axis in male rats resulting in reduced levels of gonadotrophins[16]. FSH stimulates the growth and maturation of ovarian follicles by acting directly on the receptors located on the granulosa cells. The observed reduction in its levels may impair the process of folliculogenesis and delay maturation of the follicle. LH stimulates secretion of sex steroids from the gonads and its surge in females stimulates ovulation. Reduction of its serum level could disrupt ovulation either by decreasing the number of mature follicles or altering the pattern of estrous cycle[39]. Therefore, the observed reduction in the serum LH levels may be due to inhibitory effect of the extract on LH release which then disrupts ovulation process in treated rats. Also, there is a possibility of the extract causing dysregulation in the hormonal secretion via its effect on the anterior pituitary or hypothalamus. Our findings aggree with the results of other workers who also reported decreased release of LH and FSH in rats treated with various extracts[2,10]. The proestrus surge of LH which is responsible for ovulation requires high estrogen levels, and the subsequently formed corpus luteum secretes progesterone which

Table 4

Effect of ethanol root bark extract of *S. lehmbachii* on serum levels of sex hormones in female rats.

Extract	Progesterone (ng/mL)	Estradiol (pg/mL)	LH (mIU/mL)	FSH (mIU/mL)
Control (2 mL DW)	52.60 ± 2.11	691.14 ± 0.69	2.88 ± 0.79	3.11 ± 0.44
250 mg/kg ESL	5 0.95 ± 1.21	678.50 ± 1.12	2.78 ± 1.01	3.04 ± 0.86
500 mg/kg ESL	33.10 ± 1.28[a]	520.41 ± 1.18[a]	1.33 ± 0.92[a]	2.46 ± 0.71[a]
750 mg/kg ESL	28.60 ± 0.55[ab]	430.61 ± 1.10[ab]	0.93 ± 0.65[ab]	2.03 ± 0.60[ab]

Values are Mean ± SEM; n=6; DW = Distilled water; ESL = Ethanol root bark extract of *Salacia lehmbachii*; a = significant difference from 250 mg/kg ESL, P<0.05; b = significant difference from 500 mg/kg ESL, P<0.05; LH = Leutinizing hormone; FSH = Follicle stimulating hormone.

Table 5

Effect of ethanol root bark extract of *S. lehmbachii* on serum levels of sex hormones in female rats after two weeks of withdrawal of treatments.

Study group	Progesterone (ng/mL)	Estradiol (pg/mL)	LH (mIU/mL)	FSH (mIU/mL)
Control (2 mL DW)	52.60 ± 2.11	691.14 ± 0.69	2.88 ± 0.79	3.11 ± 0.44
250 mg/kg ESL	52.25 ± 1.00	678.50 ± 1.12	2.78 ± 1.01	3.04 ± 0.56
500 mg/kg ESL	48.96 ± 0.04	648.66 ± 1.18	2.70 ± 0.92	2.91 ± 0.71
750 mg/kg ESL	45.88 ± 0.55[a]	610.74 ± 1.10[a]	2.41 ± 0.30[a]	2.71 ± 0.52[a]

Values are Mean ± SEM; n=6; DW = Distilled water; a = significant difference from 250 mg/kg ESL, P<0.05; ESL = Ethanol root bark extract of *Salacia lehmbachii*; LH = Leutinizing hormone; FSH = Follicle stimulating hormone.

regulates menstruation and prepares the female for conception amongst other things[40]. Estrogen and progesterone have a feedback inhibitory effect on gonadotropin releasing hormone secretion in the hypothalamus, and it is this inhibition that prevents the mid cycle LH surge and ovulation. In this study, the serum levels of estrogen and progesterone were significantly reduced in the extract-treated rats. The reduction in estrogen levels may be due to inhibitory effect of the extract on gonadotropins as FSH stimulates maturation of the Graafian follicle while LH causes the follicles to synthesize testosterone which is then converted to estrogen by aromatase[41]. It may also be attributed to a decreased aromatase activity or substrate supplementation during its synthesis as earlier reported[42]. Estrogen level reduction may be a direct toxic effect on follicular cells as the extract was reported to cause such toxicity in the thecca cells of the rats testes[11]. Similar results were obtained following administration of *Cnidoscolous aconitifolius* leaf extract[43]. The decreased progesterone level is probally caused by poor synthesis because of deficient corpus luteum resulting from lack of ovulation, or a direct toxic effect on the corpus luteum. Administration of water based extract of combined *Lepidagathis longifolia* and *Phyllagathus rotundifolia* also reduced both progesterone and estrogen levels in rats[44]. The insignificant difference in the relative weights of uteri and ovaries in rats treated with low doses of the extract implies that the changes produced by the extract did not affect the organs' relative weights. However, the significant reduction of the organs weight at high dose of the extract (750 mg/kg) may be caused by the observed reduction in estradiol levels since this hormone is directly responsible for the growth and development of reproductive organs[45].

Certain biocative compounds found in plant extracts can affect estrous cycle, conception and reproduction. Typical examples are alkaloids and flavonoids which have been shown to reduce plasma concentrations of LH, FSH, estradiol and progesterone[43–45]. Therefore, the presence of these phytochemicals in the extract as previously reported[9] may account for the observed alterations in female reproductive hormones levels.

The toxicity of a medication may be reversible or irreversible depending on the type of interactions between the drug and the organism. When interaction brings about reversible toxic effects, the only determining factor is the toxicant's concentration at the site of action whereas irreversible toxicity occurs when biomacromolecules bind covalently with the toxicant[46]. The reversible effect of the extract on estrous cycle observed in this study is an indication that the cell injury that led to the changes was not permanent but reversible. It is likely that the absorbed extracts were adequately cleared such that plasma level fell below the minimum effective concentration. Other researchers also reported reversed antifertility action from the seeds, roots and pulp extracts of *Momordica charantia* after withdrawal of therapy in rats[1,2].

From our findings, the root bark of *S. lehmbachii* prolongs estrous cycle length, increases diestrous duration and reduces sex hormones levels in female albino rats, and thus could impair fertility. We advice cautious usage of the plant on women of reproductive age especially those with reduced fertility.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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