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Effect of Flax Seeds (*Linum usitatissimum*) on Uterine and Ovarian Protein Contents, Ovarian Cholesterol, Serum Estradiol and Onset of Puberty in Immature Female Mice

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ABSTRACT

The effects of aqueous methanolic extract of Flax seeds (FS) on body and organ weights, uterine and ovarian protein contents, ovarian cholesterol concentration, serum estradiol concentration, age at vaginal opening and estrus in immature female mice were investigated. One hundred and twenty immature female Balb/c mice (27 days old) were divided in to four equal groups A, B, C and D. Mice of group A served as untreated control, while mice of groups B, C and D were given orally the extract of FS at the dose rates of 100, 200 and 300 mg/kg body weight, respectively for 25 days. Onset of vaginal opening and estrus were monitored for mice of each group. Six mice of each group were euthanized at day 5, 10, 15, 20 and 25 of treatments. Overall mean values for body, ovarian and uterine weights were higher in mice given higher doses of extract (200 or 300 mg/kg) compared to control group or those given low dose (100 mg/kg; $P<0.05$). The same was true for the ovarian and uterine protein contents, while an opposite trend was seen for ovarian cholesterol contents. In mice treated with FS, highest serum estradiol levels were reached 5-10 days earlier compared to mice of control group. Similarly, vaginal opening and estrus were recorded at an early age in mice given higher doses of extract. It was concluded that FS extract increased serum estradiol, uterine and ovarian protein contents, decreased ovarian cholesterol and enhanced onset of puberty in immature female mice. © 2012 Friends Science Publishers

Key Words: Flax seeds; Mice; Organs weight; Ovarian and uterine proteins; Ovarian cholesterol; Serum estradiol; Puberty

INTRODUCTION

Livestock play an important role in agricultural economy of Pakistan. Late attainment of puberty is, however, one of the important factors adversely affecting the reproductive efficiency of livestock. Although modern facilities have replaced traditional practices in some parts of developed world, ethnoveterinary practices (EVP) still have significant contributions to animal health in areas where farmers either do not have access to the modern medicine or they have faith in the traditional medicine. In this regard, livestock farmers use various indigenous plants and other materials such as FS, dried dates, raw eggs etc. to hasten onset of puberty and improve fertility of dairy animals (Njidda & Isidahomen, 2011; Bilal *et al.*, 2012).

Ethnopharmacological studies have shown that a number of medicinal plants produce secondary metabolites which may affect reproduction in man and animals (Sakamoto *et al.*, 1988). Flax seeds/linseeds (*Linum usitatissimum* L.), locally called *Alsi,* are traditionally used as a protein supplement in livestock feed. Flax seeds exhibit antioxidant property (Rhee & Brunt, 2011), and are useful in preventing hyperlipidaemia and diabetic complications in rats (Makni *et al.*, 2011). Flax seeds also suppress atherosclerosis (Prasad, 2009), and cardioprotective effects of lignan concentrate extracted from flax seeds have been seen in rats (Zanwar *et al.*, 2011). Moreover, FS have been shown to reduce growth of human breast tumor cells (MCF-7) in anthymic mice (Chen *et al*., 2009).

Feeding Flax seeds (*Linum usitatissimum)* to dairy cows increases first service conception rate by 17% (Petit *et al*., 2001). A recent survey has shown that FS are used by the local farmers for the treatment of retention of foetal membranes, silent estrus and delayed puberty in cattle and buffaloes (Dilshad, 2009). The present study evaluates the effects of aqueous methanolic extract of FS on body weight, uterine and ovarian weights, uterine and ovarian protein contents, ovarian cholesterol concentration, serum estradiol concentration, and onset of puberty in immature female mice.

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MATERIALS AND METHODS

Collection and extraction of flax seeds: Flax seeds were purchased from the local market and ground. The powder was used for the preparation of aqueous methanolic extract, using 70% aqueous methanol solution, as described elsewhere (Ahmad *et al.*, 2012).

Experimental mice: One hundred and twenty (120) immature female Balb/c mice (20 days old) were obtained from the breeding colony being maintained at the National Institute of Health (NIH), Islamabad, Pakistan. At NIH, all pups were sorted by sex at birth (day 1) and redistributed so that all litters were standardized to 10 female pups per dam. Mice were weaned at day 19 of age. All animals were housed under controlled lighting (12 h light: 12 h dark) and temperature (22±2°C) conditions. Mice were given fresh water and NIH-31 rodent diet (Table I) *ad libitum*. All animal procedures complied with NIH animal care guidelines.

After 7 days of acclimatization (27 days of age), mice were treated either with desired concentration of the extract (treatment groups) or distilled water (control group). The aqueous methanol extract was dissolved in distilled water to obtain desired concentration to be given to each group of experimental mice.

Experimental design: The experimental mice were divided into four groups A, B, C and D, with 30 mice in each group. Mice of group A served as control and were given distilled water. Mice of groups B, C and D were orally given the extract of FS at the dose rates of 100, 200 and 300 mg/kg body weight, respectively, for 25 days.

Post treatment monitoring: From the day of the start of treatment (day 27 of age), live weight for each mouse was recorded at 5 days intervals. Vaginal appearance of all mice was observed daily for coloration and degree of distention at the time of medication. After vaginal introitus, vaginal lavage was taken daily from each mouse to determine the age at first estrus (puberty) judged from the cornified smear (Cooper *et al.,* 1993).

Blood collection: Six mice from each group were euthanized at day 5, 10, 15, 20 and 25 of treatments by cervical dislocation. For this purpose, mice were anesthetized in glass desiccators containing a swab of methoxyflurane (Metofane; Pitman-Moore, Inc.). Before killing, the blood was collected in a tube; the serum was separated and stored at 4°C for estradiol analysis.

Collection of uterus and ovaries: After incision of the skin and the abdominal muscles, uterus along with ovaries was removed. Both uteri and ovaries were blotted free of blood and weighed separately. Two ovaries from control and each treated group were randomly selected and fixed in Bouin solution for histological studies.

Estradiol quantification: Serum estradiol was quantified by using commercially available ELISA kit (Estradiol Enzyme Immuno Assay test kit, Catalog No: BC- 1111, BioCheck, Inc., USA), which had 100% cross reactivity for estradiol (E_2). The BioCheck E_2 EIA is based on the principle of competitive binding between E_2 in the sample and E_2-HRP conjugate for a constant amount of rabbit anti-Estradiol. After processing the samples and standards as per instructions of the manufacturer of kit, optical density of the samples and standards was determined at 450±10 nm wavelength, using a Microsrtrip Reader (Stat-Fax-303, Awareness Technology, Inc.). The concentration of the hormone in the sample was determined from a standard curve generated between the concentrations and optical density of the standards.

Biochemical studies: The uterine and ovarian tissues were processed for the determination of proteins and cholesterol contents. For this purpose, ovaries and uterine samples were separately homogenized in 10 volumes of TS buffer (0.25 M sucrose, 1 mM EDTA and 10 mM Tris-HCl, pH 7.4). The homogenate was then centrifuged at 6000 × *g* at 4°C for 15 minutes and supernatant was used for the determination of proteins in both uteri and ovarian tissues and cholesterol contents in ovarian tissue (Telefo *et al.,* 1998).

Protein contents in the ovarian and uterine tissues were estimated by using protein assay kit (Catalog No., 500-0207; Quick Start Bovine Serum Albumin Standard set, 2×2 mL each, with concentrations of 2.0, 1.5, 1.00, 0.75, 0.5, 0.25 & 0.125 mg/mL). The microassay protocol for the protein determination was performed by using 300 µL microplate assays. Total protein level was expressed as mg/100 g fresh tissue weight.

Ovarian cholesterol concentrations were estimated by using Cholesterol assay kit (Breur & Breur Diagnostic Laboratories, USA) and expressed as mg/100 g fresh tissue weight. The principle of this assay kit is the determination of cholesterol after enzymatic hydrolysis and oxidation (Artiss & Zak, 1997) at 546 nm.

Histological studies: The preserved ovaries from mice of each group were dehydrated in graded alcohols, cleared in benzene, embedded in paraffin, serially sectioned at 5 µm and then stained with hematoxylin and eosin. Ovarian sections were examined under light microscope for determining various developmental stages of follicles (Chen *et al.,* 1981).

Statistical analysis: The data were analyzed by using General Linear Model (GLM) and means were compared through Least Significant Difference test. For this purpose, Statistical Package for Social Sciences (SPSS, Version 10, Chicago, IL, USA) was applied.

RESULTS AND DISCUSSION

Body weight: Oral administration of different doses of aqueous methanol extract of FS extract significantly affected the body weight (Table II). At the 5th day of treatment, mice of groups C and D showed higher body weight compared to those of groups A and B. This trend was maintained till the end of the study. On overall basis, the mice of group D were heaviest, followed by those of

Table I: Composition of mice feed (%)

Ingredients	Quantity
Corn starch	62.5
Corn oil	10.0
Glucose	5.0
Casein	12.5
Mineral + vitamin mix	10.0
Total	100

Table II: Effects of different doses of aqueous methanolic extract of Flax seeds on body and organ weights of immature female mice (Mean ± SE)

Parameter	Treatment groups	Duration of treatment (Days)					Overall mean
		5	10	15	20	25	
Body weight (g)	A (Control)	18.69 ± 0.04	19.93 ± 0.02	22.44 ± 0.05	24.72 ± 0.02	24.89 ± 0.21	19.66 ± .08c
	B (100 mg/kg)	17.77 ± 0.43	19.76 ± 0.24	21.93 ± 0.15	24.42 ± 0.20	24.68 ± 0.50	19.36 ± 0.23d
	C (200 mg/kg)	20.28 ± 0.19	22.48 ± 0.15	25.85 ± 0.19	26.8 ± 0.21	27.50 ± 0.20	21.43 ± 0.16b
	D (300 mg/kg)	20.43 ± 0.19	23.79 ± 0.12	26.03 ± 0.11	26.82 ± 0.11	28.57 ± 0.12	21.81 ± 0.12a
	Mean	19.29 ± 0.21E	21.49 ± 0.13D	24.06 ± 0.13C	25.69 ± 0.18B	26.41 ± 0.26A	
Ovarian weight (mg)	A (Control)	7.43 ± 0.07	11.27 ± 0.09	12.70 ± 0.12	14.43 ± 0.12	17.30 ± 0.07	12.63 ± 0.09d
	B (100 mg/kg)	7.57 ± 0.09	11.64 ± 0.12	13.39 ± 0.11	17.75 ± 0.11	17.82 ± 0.11	13.63 ± 0.11c
	C (200 mg/kg)	12.42 ± 0.12	18.65 ± 0.12	18.87 ± 0.09	19.22 ± 0.13	18.88 ± 0.16	17.61 ± 0.12b
	D (300 mg/kg)	12.53 ± 0.10	19.29 ± 0.11	19.28 ± 0.04	19.44 ± 0.09	19.56 ± 0.11	18.02 ± 0.09a
	Mean	9.99 ± 0.095E	15.21 ± 0.11D	16.06 ± 0.09C	17.71 ± 0.11B	18.39 ± 0.11A	
Uterine weight (mg)	A (Control)	24.3 ± 0.09	77.55 ± 0.148	69.82 ± 0.14	64.94 ± 0.102	62.77 ± 0.199	59.88 ± 0.135d
	B (100 mg/kg)	25.73 ± 0.11	79.39 ± 0.145	72.57 ± 0.217	76.77 ± 0.14	71.85 ± 0.54	65.26 ± 0.23c
	C (200 mg/kg)	74.8 ± 0.12	77.41 ± 0.07	82.23 ± 0.103	84.22 ± 0.13	93.68 ± 0.126	82.47 ± 0.11b
	D (300 mg/kg)	80.49 ± 0.16	78.71 ± 0.181	92.97 ± 0.198	96.7 ± 0.179	106.07 ± 0.579	90.99 ± 0.26a
	Mean	51.33 ± 0.12E	78.27 ± 0.136D	79.4 ± 0.165C	80.66 ± 0.138B	83.59 ± 0.361A	

Table III: Effects of different doses of aqueous methanolic extract of Flax seeds on tissue biochemical metabolites and serum estradiol in immature female mice (Mean ± SE)

Parameter	Treatment groups	Duration of treatment (Days)					Overall mean
		5	10	15	20	25	
Uterine protein contents (mg/100g)	A (Control)	11.02 ± 0.02	14.33 ± 0.02	18.20 ± 0.01	18.61 ± 0.03	18.37 ± 0.04	16.11 ± 0.03d
	B (100 mg/kg)	11.55 ± 0.02	15.07 ± 0.03	18.96 ± 0.03	19.00 ± 0.037	19.12 ± 0.02	16.74 ± 0.03c
	C (200 mg/kg)	14.43 ± 0.04	18.26 ± 0.02	19.18 ± 0.02	19.77 ± 0.02	20.22 ± 0.02	18.37 ± 0.02b
	D (300 mg/kg)	18.20 ± 0.03	18.33 ± 0.05	19.58 ± 0.04	19.86 ± 0.02	20.48 ± 0.02	19.29 ± 0.03a
	Mean	13.80 ± 0.03E	16.50 ± 0.03D	18.98 ± 0.03C	19.31 ± 0.03B	19.55 ± 0.02A	
Ovarian protein contents (mg/100g)	A (Control)	8.32 ± 0.01	10.23 ± 0.01	11.92 ± 0.01	12.03 ± 0.05	12.08 ± 0.02	10.92 ± 0.02d
	B (100 mg/kg)	8.48 ± 0.01	11.83 ± 0.02	11.94 ± 0.01	12.19 ± 0.03	12.74 ± 0.01	11.44 ± 0.02c
	C (200 mg/kg)	10.44 ± 0.02	12.00 ± 0.03	12.64 ± 0.01	12.98 ± 0.02	13.05 ± 0.02	12.22 ± 0.02b
	D (300 mg/kg)	12.02 ± 0.02	12.37 ± 0.02	13.01 ± 0.03	13.31 ± 0.01	13.46 ± 0.02	12.83 ± 0.02a
	Mean	9.82 ± 0.01E	11.61 ± 0.02D	12.38 ± 0.02C	12.63 ± 0.02B	12.83 ± 0.02A	
Ovarian cholesterol contents (mg/100g)	A (Control)	3.54 ± 0.01	2.67 ± 0.01	2.13 ± 0.01	1.92 ± 0.01	1.79 ± 0.01	2.41 ± 0.01a
	B (100 mg/kg)	3.38 ± 0.01	2.27 ± 0.01	2.03 ± 0.03	1.88 ± 0.01	1.74 ± 0.01	2.26 ± 0.01b
	C (200 mg/kg)	2.34 ± 0.01	1.88 ± 0.01	1.82 ± 0.01	1.77 ± 0.01	1.73 ± 0.01	1.91 ± 0.01c
	D (300 mg/kg)	1.91 ± 0.01	1.79 ± 0.01	1.74 ± 0.01	1.71 ± 0.01	1.73 ± 0.01	1.78 ± 0.01d
	Mean	2.79 ± 0.01A	2.15 ± 0.01B	1.93 ± 0.01C	1.82 ± 0.01D	1.75 ± 0.01E	
Serum estradiol contents (pg/ml)	A (Control)	17.16 ± 0.08	13.33 ± 0.07	26.38 ± 0.07	35.60 ± 0.076	37.84 ± 0.09	26.06 ± 0.08b
	B (100 mg/kg)	14.50 ± 0.08	20.70 ± 0.09	26.39 ± 0.09	37.62 ± 0.11	27.51 ± 0.07	25.34 ± 0.09c
	C (200 mg/kg)	12.35 ± 0.09	28.46 ± 0.09	38.45 ± 0.10	24.25 ± 0.02	26.23 ± 0.05	25.95 ± 0.07b
	D (300 mg/kg)	12.74 ± 0.14	26.87 ± 0.67	22.77 ± 1.33	37.18 ± 0.38	34.06 ± 0.36	26.73 ± 0.58a
	Mean	14.19 ± 0.10E	22.34 ± 0.23D	28.50 ± 0.40C	33.66 ± 0.15A	31.41 ± 0.14B	

Values with different lower case letters in a column for each parameter differ significantly (P<0.05)
Values with different upper case letters within a row differ significantly (P<0.05)

group C and A. However, the mice of B group attained lesser weight than the control and the mice of other two groups (P<0.05). This indicates that significant increase in the body weight was obtained with the administration of higher doses (200 & 300 mg/kg) of the FS extract. Similar observations have been recorded by Ahmad *et al*. (2012) in rats treated with aqueous methanolic extract of FS.

Ovarian weight: Maximum increase in ovarian weight was recorded between day 5 and 10 of treatment in mice of D and C groups, while maximum ovarian weight gain in group B was between day 15 and 20 of treatment. However, mice of group A attained maximum ovarian weight during the day 20 and 25 (Table II). Thus, higher doses of extract accelerated ovarian growth at an early age compared to low

dose or the control. Overall, maximum mean ovarian weight (18.02 ± 0.09 mg) was recorded for the D group, followed by the C (17.61 ± 0.12 mg) and B (13.63 ± 0.11 mg). The lowest ovarian weight (12.63 ± 0.09 mg) was recorded in mice of control group (P<0.05). These findings are in contrast to those of Ahmad *et al*. (2012), who were unable to record any significant effect of FS treatment (500 mg/kg body weight for 14 days) on ovarian weight in immature rats. Besides species differences, variations in the dose or duration of treatment can be blamed for these discrepancies between the two studies. According to Tou *et al*. (1999), long term administration of FS at the rate of 10% in diet increased ovarian weight compared to control, increased serum estradiol and enhanced onset of puberty in rats. However, short term treatment had no such effects.

Uterine weight: A noticeable difference in the uterine weight of all groups was recorded right at day 5 of treatment, when the uterine weight in mice of groups C and D was about 3 times higher than that for groups A and B (Table II). Mice of groups A and B had the highest uterine weight at day 10 of experiment. However, in groups C and D, uterine weight kept on increasing till the last day of treatment. Overall mean uterine weight of all groups differed significantly (P<0.05), values being highest in group D and lowest in control group. Ostrovsky (1962) and Ostrovsky and Kitts (1963) also recorded distinct increase in uterine weight in female rats orally given red clover and subterranean clover.

When immature female rats were treated with estradiol benzoate, a significant increase was observed in the body and uterine weight (Circosta *et al.*, 2001). Flax seeds are rich in seciosolariciresinol diglycoside, which is converted to phytoestrogens by rumen micro organisms in ruminant species and by hind gut microorganisms in non ruminants (Thompson *et al*., 2004). Thus, the increase in body weight, ovarian and uterine weight of mice treated with higher doses of FS extract can be attributed to the phyto-estrogenic activity of these seeds. It is well known that estrogens have protein anabolic effects and thus increase nitrogen retention and stimulate tissue growth (Hafez & Hafez, 2006). Stimulation of rat's uterus with estrogenic compounds resulted in the initiation of cascade of biochemical events which result in macromolecular synthesis (DNA, RNA, proteins) causing growth of uterus (Katzenellenbogen *et al.*, 1979) and other organs.

Uterine and ovarian protein contents: Mice of group D (fed highest concentration of FS extract) had higher mean uterine protein contents at each day of treatment compared to those of other treatment groups (fed lower concentrations of FS extract) as well as the control group (Table III). The overall mean uterine protein contents were highest (19.24±0.03 mg/100 g) in mice of group D, while the lowest vale was for mice of control group (16.11 ± 0.03 mg/100 g), with all groups differed significantly from one another (P<0.05). Ovarian protein contents showed the similar trend as for uterine protein contents (Table III).

As far as could be ascertained, this is the first report on the effect of FS on uterine or ovarian protein contents. Ahmad *et al*. (2012), however, observed a significant increase (P<0.05) in the serum total protein contents in immature rats given FS extract compared to controls. Whether this increase in the total protein contents in the blood is associated with increased total protein levels in the uterus or ovaries is not clear.

Ovarian cholesterol content: With increasing dose rate, cholesterol content in the mice ovaries decreased as the duration of treatment was increased. Overall minimum mean value of ovarian cholesterol content (mg/100 g ovary) was recorded in mice of group D (1.78 ± 0.01) and maximum was in control group A (2.41 ± 0.01), the difference being significant (P<0.05; Table III).

Thus, the ovarian cholesterol was low in the mice groups given FS extract. The possible reason may be that steroidogenesis is dependent on the availability of cholesterol substrate. Even if other factors such as gonadotropin stimulation and expression of steroid biosynthetic enzymes are in place, insufficient cholesterol substrate can limit steroidogenesis. Ovaries rely on a combination of substrate sources to support steroidogenesis, including cholesterol uptake from plasma lipoproteins, mobilization of intracellular cholesterol ester stores, and *de novo* cholesterol synthesis (Wade *et al.,* 2002). The utilization of cholesterol in steroidogenesis decreased the amount of available cholesterol content in early pubertal mice. Chinoy and Patel (2001) also recorded that a block in the steroidogenic pathway in mice treated with fluoride or aluminium individually or in combination resulted in significant accumulation of cholesterol in the ovaries.

Previous studies (Guan *et al.*, 2006; Abuelgassim, 2010) have also indicated that phytoestrogens of kudzu and soybean decreased total cholesterol and LDL-cholesterol, while HDL-cholesterol was not affected. Moreover, Flax seeds prevent hyperlipidaemia in diabetic rats (Makni *et al.*, 2011).

Serum estradiol concentrations: Highest mean estradiol concentration (37.84±0.09 pg/mL) in group A was observed at day 25 of treatment, while it occurred at day 20 in mice of group B (37.62 ± 0.11 pg/mL) and D (37.18 ± 0.37 pg/mL) and at day 15 in mice of group C (38.45 ± 0.10 pg/mL). It shows that in mice treated with FS, highest serum estradiol levels were reached 5-10 days earlier compared to mice of control group. On overall basis, serum estradiol was the highest in mice of group D and lowest in those of group B, differences among all groups were significant (Table III). The increased estradiol levels in mice treated with FS can be attributed to their phyto-estrogenic activity.

The current results support those of Tou *et al*. (1999) and Ahmad *et al.* (2012), who observed increased estradiol levels in rats treated with FS compared to controls. Similar activity of FS has also been reported by Richter *et al*. (2010) in stimulation of breast carcinoma cells (MCF7) with FS extract through estradiol production.

Fig. 1: Histogram of the ovary of a mouse of group D at day 32 of age showing corpora lutea and developing follicles

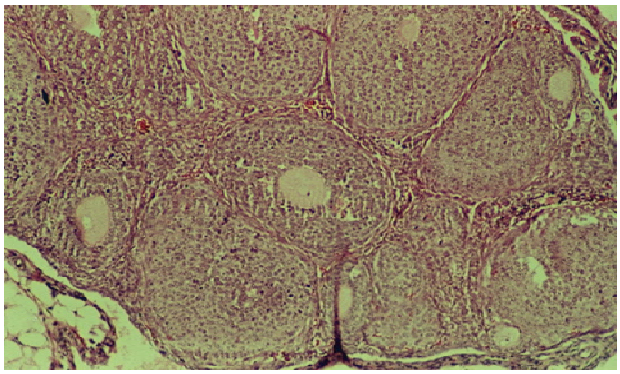


Fig. 2: Histogram of the ovary of a mouse of group C at day 42 of age showing mature and developing follicles

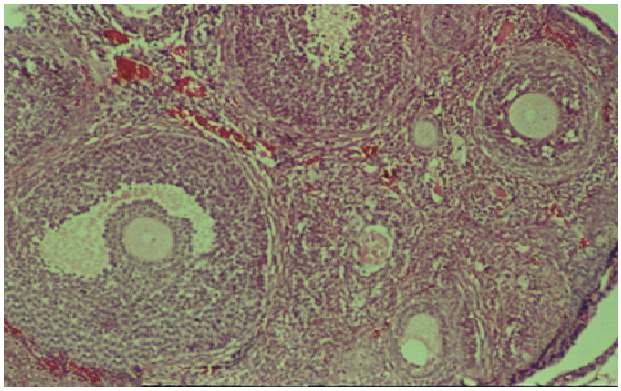
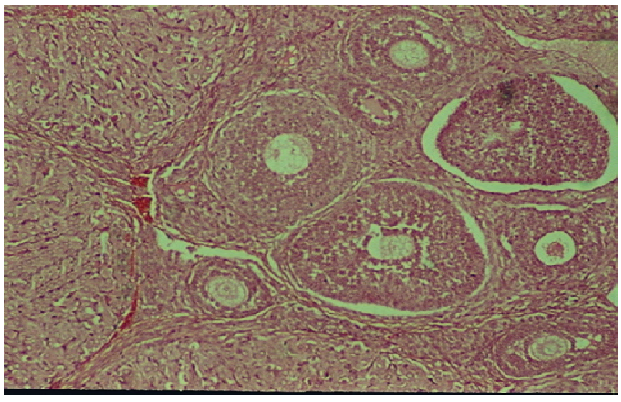


Fig. 3: Histogram of the ovary of a mouse of group A at day 42 of age showing developing follicles



Vaginal opening and estrus: Effect of feeding 3 concentrations of FS extract to mice was variable for the onset of vaginal opening (VO) and estrus. Vaginal opening was seen at significantly lower age ($P<0.05$) in mice of groups D (30.5 days) and C (31.2 days) compared to mice of groups B (33.4 days) and A (34.9 days). The difference in the age of vaginal opening between groups C and D and A and B was non-significant. This indicates that vaginal opening occurred at lower age in mice given 200 or 300 mg/kg FS extract compared to mice of control group or those given low dose (100 mg/kg) of the extract. A similar trend was seen for the onset of first estrus (puberty), recorded at the lowest age of 34.1 days in mice of group D and the highest age of 48.8 days in the control group.

Onset of puberty in a female is the complex interaction between age, body weight, ovarian and uterine growth and secretion of gonadotropins including FSH and LH. During the pre-pubertal period, increased concentrations of FSH and LH stimulate growth and development of follicles on the ovaries. Previously, it has been reported that larger doses of estrogen suppress the secretion of pituitary gonadotropins and thus inhibit the follicular growth, whereas small doses of estrogens were found to enhance the follicular development (Smith & Bradbury, 1961). The increased ovarian weight and follicular development recorded in mice given FS extracts may be explained as the phytoestrogenic properties of the plant extracts which have been reported to possess weak estrogenic effects. Thus, mice treated with high doses of FS extract showed higher ovarian and uterine growth, showed higher serum estradiol levels earlier and attained puberty earlier compared to mice of control group. Histological examination of the ovaries also revealed the presence of corpora lutea and growing follicles (Fig. 1) in the ovarian sections of mice of group D as early as day 5 and day 10 after treatment (32-37 days of age). In mice of group C, mature Graafian follicles (Fig. 2) were seen at Day 15 of treatment (42 days of age). Only developing follicles were seen in mice of groups B and A at day 42 of age (Fig. 3), while mature follicles in mice of these two groups were seen at day 20 of treatment (47 days of age). Murthy *et al.* (1997) found that immature mice showed early vaginal opening, premature cornification of vagina and increased uterine weight when treated with benzene extract of *Hibiscus rosa sinensis* flowers @ 125 and 250 mg/kg body weight intraperitoneally.

In conclusion, FS extract increases serum estradiol, uterine and ovarian protein contents, decreased ovarian cholesterol and enhanced onset of puberty in immature female mice.

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