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RESEARCH ARTICLE

Effects of Aqueous Methanolic Extract of Flax Seeds (*Linum usitatissimum*) on Serum Estradiol, Progesterone, Kidney and Liver Functions and Some Serum Biochemical Metabolites in Immature Female Rats

Nazir Ahmad*, Zia-ur-Rahman[1], Nafees Akhtar and Shujait Ali

Department of Theriogenology, [1]Department of Physiology & Pharmacology, University of Agriculture, Faisalabad, Pakistan
*Corresponding author: profnazir53@hotmail.com

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ABSTRACT

Effects of aqueous methanolic extract of Flax seeds (*Linum usitatissimum*) on serum estradiol, progesterone and some biochemical metabolites in immature female rats were investigated. Thirty six immature female rats were divided into 3 equal groups. Rats of group A served as controls, while those of groups B and C were given crude extract of Flax seeds orally @ 500 mg/kg and estradiol @ 40 µg/kg body weight, respectively, daily for 14 days. After 7 days, 6 rats from each group were euthanized, while the remaining 6 rats in each group were killed 14 days after treatment. Blood samples were taken for serum biochemical analysis, while ovaries were used for the determination of ovarian cholesterol contents and histological studies. Body weight of rats given Flax seeds extract was higher ($P<0.05$) compared to control rats and those given estradiol. Serum estradiol, progesterone, total proteins and total cholesterol contents were higher ($P<0.05$) in rats given Flax seeds extract and estradiol compared to controls. The ovarian cholesterol contents were reduced in rats of both treatment groups ($P<0.05$). Serum ALT and AST activities were higher in Flax seeds treated rats compared to control, while it was not so in estradiol treated group. There was no difference in serum urea concentrations among rats of three groups. In conclusion, aqueous methanol extract of Flax seeds increased serum estradiol, progesterone, total proteins, total cholesterol, ALT and AST activity, and decreased ovarian cholesterol levels, while it had no effect on kidney function in immature female rats.

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INTRODUCTION

Linum usitatissimum (Flax seeds), also known as Linseeds, are commonly used as a source of protein supplements in the rations of dairy animals. According to Petit *et al.* (2001), feeding of Flax seeds to dairy cows increased the first service conception rates by 17%. Flax seeds also show many health benefits (Khan *et al.*, 2010). These have been shown to have antioxidant property (Rhee and Brunt, 2011) and prevent diabetic complications (Makni *et al.*, 2011), while lignan concentrate extracted from flax seeds showed cardioprotective effects in rats (Zanwar *et al.*, 2011). Chemically, these seeds contain 41% oil, 20% proteins and 28% dietary fiber. They are rich in essential omega-3 fatty acid and polyunsaturated linolenic acid (Conners, 2000).

In a recent study, Dilshad (2009) observed significant increase in the body weight, uterine weight and the ovarian weight of mice given aqueous methanol extract of Flax seeds @ 200 or 300 mg/kg body weight for 25 days. This increase in the uterine and the ovarian weights was attributed to the possible phyto-estrogenic activity present in the Flax seeds. The present paper describes effects of aqueous methanolic extract of *L. usitatissimum* (Flax seeds) on serum estradiol, progesterone and some biochemical metabolites in immature female rats.

MATERIALS AND METHODS

Collection of plant materials: Seeds of *L. usitatissimum* (Flax seeds) were purchased from a local herbal shop. After drying in shade, these seeds were ground into fine

powder, which was used for the preparation of crude extract in 70% (v/v) solution of methanol in distilled water (aqueous methanol solution). For this purpose, about 100 g of the powdered material was soaked in 2 liters of 70% aqueous methanol solution for 5 days, with shaking twice daily. After 5 days, supernatant was separated through filtering using porous cloth. The remaining plant material was again soaked in another 2 liters of 70% aqueous methanol solution for 5 days and supernatant was again separated. The filtrates for the two collections were pooled and concentrated in a rotary evaporator at 40°C to get the crude extract.

Experimental rats: Thirty six immature female Sprague-Dawley rats (3-4 weeks of age), procured from the breeding colony being maintained at the National Institute of Health (NIH), Islamabad, Pakistan were used. These rats were acclimatized for 3-5 days before start of the experimental treatments. They were maintained under naturally prevailing climatic conditions and were provided with feed and water *ad libitum*.

Treatments & post treatment monitoring: Experimental rats were randomly divided into 3 groups A, B and C, with 12 rats in each group. Rats of group A were not given any treatment and served as negative control, and those of group B were given aqueous methanol extract of *L. usitatissimum* orally at the dose rate of 500 mg/kg body weight. Rats of group C were given Estradiol dipropionate (Injection Agofollin; BIOTIKA, Slovak Republic) S/C at the dose rate of 40 µg/kg body weight and served as positive control. The treatments were given for 14 days. After 7 days, six rats from each group were euthanized, while the remaining six rats from each group were euthanized after 14 days of treatment. Body weight and weights of ovaries and uterus were recorded. Blood samples were collected from rats of each group; serum was harvested and stored at -20°C for biochemical analysis. Right ovaries collected from these rats were used for the determination of ovarian cholesterol contents, while left ovaries were processed for histological examination.

Serum biochemistry: Serum concentrations of total proteins, total cholesterol, urea, ALT activity and AST activity were determined, using commercially available kits (Randox Laboratories Ltd, UK). Samples and standards were processed as per instructions of the manufacturer of the respective kit. The absorbance of samples and standards was measured using Chemistry Analyzer (BTS-330, Biosystems, Spain). The concentration of respective biochemical constituent was calculated through dividing absorbance of the sample by absorbance of the standard and multiplying by concentration of the standard. The detailed procedure has been described earlier (Ahmad *et al.*, 2012).

Hormone assay: Serum samples were also analyzed for estradiol and progesterone concentrations, through solid phase ELISA based on the principle of competitive binding, using commercial kits. These kits contained micro titer wells coated with a polyclonal antibody directed towards an antigenic site on the steroid hormone molecule, while hormone-horseradish peroxidase conjugate was provided for binding to the coated antibody. After processing the samples and standards as per instructions of the manufacturer of the kit, optical density of the samples and standards was determined at 450±10 nm wave length, 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 values of the standards.

For estradiol, commercially available estradiol ELISA kit (Reference No. EIA-2693, DRG Diagnostics, Germany) was used. The kit contained standards with estradiol concentrations of 0, 25, 100, 250, 500, 1000 and 2000 pg/ml. The sensitivity of the assay was 9.714 pg/ml and the cross reactivity was 0.05% for esteriol and 0.2% for estrone. The intra-assay and inter-assay coefficients of variation were <7.0 and <10.0%, respectively.

For progesterone, progesterone ELISA kit Reference No. EIA-1561 was used. The concentrations of standards were 0, 0.3, 1.25, 2.50, 5.00, 15.00 and 40.00 ng/ml. The minimum detection limit of the assay was 0.45 ng/ml and the cross reactivity for other steroids was <1.10%. The intra-assay and inter-assay coefficients of variation were <7.0 and <10.0%, respectively.

Determination of ovarian cholesterol contents: For the determination of cholesterol contents in the ovaries of rats, tissue samples were homogenized in 500 µL chloroform:methanol (2:1). After homogenization, 125 µL of 0.15M sodium chloride was added and samples were centrifuged at 2000g for 15 minutes. The lower phase was used for the determination of cholesterol contents using the analytical kit method, as described earlier.

Phytochemical screening of Flax seeds: The powdered material of Flax seeds was screened for the presence of various phytochemical constituents, viz. tannins, alkaloids, glycosides, saponins, reducing sugars, steroids and carbohydrates. The procedure already described by Evans (1996) was applied for tannins, alkaloids, steroids and saponins, while presence of glycosides, reducing sugars and carbohydrates was judged as per Parekh and Chanda (2007).

Histological studies: Tissue samples collected from ovaries of experimental rats were processed for histological studies, using the standard Hematoxylin and Eosin staining technique. Briefly, immediately after collection, tissue samples were preserved in 10% buffered formal saline solution. Specimens of 5 mm thickness were subjected to dehydration in ascending grades of ethanol, clearing in xylene and embedding in paraffin. About 5 µm thick sections were cut and stained with Hematoxylin and Eosin stains.

Statistical analysis: Mean values (±SE) were computed for various parameters. In order to ascertain the magnitude of differences in these parameters among various groups, the data were analyzed statistically through two way Analysis of Variance procedure, following completely randomized design. Duncan's Multiple Range Test was applied for multiple means comparisons, where necessary (Steel *et al.*, 1997).

RESULTS AND DISCUSSION

Mean values (±SE) of body and organs weights are shown in Table I. Mean body weight of rats given aqueous methanol extract of Flax seeds (128.58±4.54 g) was significantly higher (P<0.05) compared to rats of control group (119.33±6.23 g) and those given estradiol (111.67±5.33 g). However, rats given estradiol did not show significant difference in body weight from control rats. Similarly, the values for ovarian weight and uterine weight in rats given Flax seeds were higher compared to those for control rats, but the difference could not reach significance level. This indicated that Flax seeds increased the body weight significantly and the ovarian and uterine weight non-significantly over the controls.

Dilshad (2009) observed significant increase in the body weight, uterine weight and the ovarian weight of mice given aqueous methanol extract of Flax seeds @ 200 or 300 mg/kg body weight for 25 days; the increase was dose dependent, with dose rate of 300 mg/kg showed higher increase compared to 200 mg/kg. This increase in the uterine and the ovarian weights was attributed to the possible phyto-estrogenic activity of the Flax seeds. According to Thompson *et al.* (2004), Flax seeds contain secoisolariciresinol diglycoside, which is changed to phytoestrogens by the ruminal microflora in compound stomach animals and by the microflora of the hind gut in rats and mice. Circosta *et al.* (2001) have indicated that treatment of immature female mice with estradiol benzoate significantly increased body weight, uterine weight and uterine luminal epithelium compared to the controls.

As shown in Table 2, serum estradiol and progesterone concentrations were higher (P<0.05) in rats given extract of Flax seeds compared to those of control group. Rats given estradiol showed even higher (P<0.05) serum estradiol levels than rats of other two groups. Richter *et al*. (2010) also recorded increased production of estradiol by breast carcinoma cells (MCF7) stimulated with Flax seeds extract. Estrogen-like activity of Flax seeds was also evident in the histological sections of ovaries. Ovaries of rats treated with extract of *Linum usitatissimum* (Flax seeds) revealed the presence of multiple mature Graafian follicles, together with corpora lutea (Fig. 1 and Fig. 2a). Similarly, rats given estradiol injection also exhibited mature, as well as growing, follicles. However, no mature follicles were seen in rats of control group; these rats had only growing Graafian follicles, with a corpus luteum (Fig. 2b). Increased levels of serum progesterone in rats given extract of Flax seeds can be attributed to the corpora lutea seen on the ovaries in histological sections.

According to Dilshad (2009), ovarian cholesterol contents were significantly reduced in immature mice treated with aqueous methanol extracts of Flax seeds for 25 days; the effect increased as the dose of the extract was increased from 100 to 300 mg/kg body weight. In the present study, ovarian cholesterol contents were significantly reduced in rats treated with extract of Flax seeds @ 500 mg/kg for 14 days compared to control rats (Table 2). The same was true for rats treated with estradiol. Telefo *et al.* (1998) recorded a significant increase in serum estradiol levels with a simultaneous

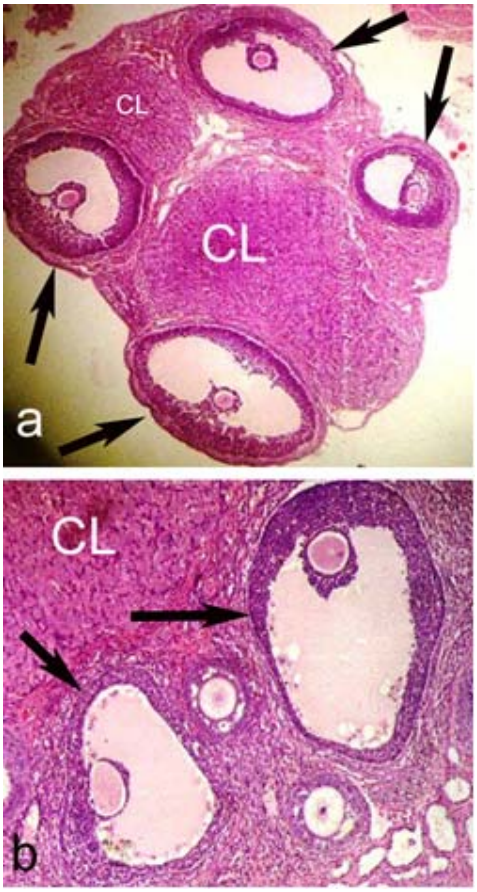

Fig. 1: Photomicrograph of the ovary of a rat treated with extract of Flax seeds showing mature Graafian follicles (arrows) and corpora luteum (CL). H & E; a: Lens 40 and b: Lens 100.

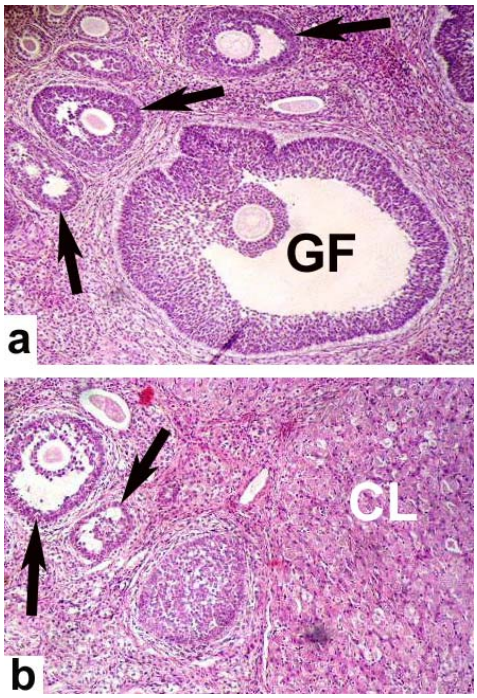

Fig. 2: Photomicrograph of the ovary of: a) a rat treated with extract of Flax seeds showing a mature Graafian follicle (GF) and many growing Graafian follicles (arrows); and b) a rat of control group showing a few growing Graafian follicles (arrows) and a corpus luteum (CL). H & E; Lens 100.

decrease in ovarian cholesterol in rats treated with aqueous extracts of estrogenic plants. It is well established that all steroid hormones are synthesized from cholesterol as precursor (Hafez and Hafez, 2006). Thus, the simultaneous increase in serum estradiol level and the decrease in ovarian cholesterol level indicate that the decrease in ovarian cholesterol has been due to its utilization in steroidogenesis for the biosynthesis of estradiol. Srivastava and Dasgupta (1980) observed a decrease in the ovarian cholesterol levels in immature female rats given treatment of Centchroman, a drug used to induce ovulation in ammenorrhoic women. Chinoy and Patel (2001) found that a block in the steroidogenic pathway caused a significant accumulation of cholesterol in the ovaries of mice treated with fluoride or aluminium.

Table 3 shows serum concentrations of various biochemical metabolites in control rats and those given Flax seeds extract and estradiol**.** Serum total protein contents were higher ($P<0.05$) in rats treated with Flax seeds (59.20±1.76 g/L) or estradiol (68.55±1.60 g/L) compared to the rats of control group (54.19±1.15 g/L). Estradiol treated rats had even higher serum total proteins compared to those given Flax seeds. This indicates that both *L. usitatissimum* and estradiol increased serum total protein concentrations. However, the effect of estradiol was more pronounced than that of the *L. usitatissimum*. Moreover, the effect of the *L. usitatissimum* was more apparent during the 2nd week of treatment, while that of estradiol was seen during the first week. An increase in the total protein contents in the uterus and ovaries of immature mice treated with extract of Flax seeds has been reported earlier (Dilshad, 2009). Whether increase in the protein contents in the serum is associated with the increase in the protein contents in the uterus and ovaries needs further investigations.

Serum total cholesterol concentrations were higher ($P<0.05$) in rats of both the treatment groups (Flax seeds and estradiol) compared to controls; the difference between the former two groups was also significant (Table 3). This indicates that treatment of rats with Flax seeds extract as well as estradiol significantly increased the serum total cholesterol concentrations; the effect of Flax seeds was higher compared to that of estradiol. It is well known that cholesterol is the precursor of all the steroid hormones including estradiol (Hafez and Hafez, 2006). It is possible that this increased serum total cholesterol during the early stages of the treatment (two weeks in this study) would be used for the synthesis of estrogens at some later stages.

Phytoestrogens from soybean and kudzu have been shown to decrease serum total cholesterol and non-high density lipoprotein cholesterol, with high density lipoprotein cholesterol (HDL-cholesterol) being unaffected (Guan *et al.*, 2006; Abuelgassim, 2010). In another experiment conducted at our laboratory (unpublished data), treatment of immature female rats with ethanolic extract of an estrogen-containing plant *Medicago sativa* for 22 days significantly reduced serum total cholesterol compared to control rats. It seems that duration of treatment is the possible cause of this variable effect. Short-term treatment of rats with estrogen-containing plants might increase serum total cholesterol, while long term treatment may have opposite effect.

Serum urea concentrations were lower in rats treated with Flax seeds or estradiol compared to controls (Table 3). However, the difference in serum urea concentrations among rats of three groups were statistically non significant, indicating that the treatments had no adverse effect on the kidney function. However, serum ALT and AST activities were higher in Flax seeds treated rats compared to control, while it was not so in estradiol injected group. This indicates that *L. usitatissimum* extract had adversely affected the liver function of rats, while estradiol injection had no such effect, as it did not affect serum ALT or AST activity compared to control rats.

Phytochemical screening of *L. usitatissimum* seeds revealed the presence of alkaloids, saponins and carbohydrates, while they gave negative reaction for tannins, glycosides, reducing sugars and steroids. Upon phytochemical screening of the ethanol extract of the plant *Balanites roxburghii* (Balanitaceae), Padmashali *et al.* (2006) observed positive results for alkaloids, glycosides, saponins, flavones and phenolic compounds. Petroleum ether, chloroform, ethanol and distilled water extracts of this plant showed antifertility activity in female albino rats at doses of 300 and 600 mg/kg body weight orally. Among these, the ethanol extract was found to be the most effective in causing abortifacient activity.

In conclusion, aqueous methanol extract of Flax seeds increased serum estradiol, progesterone, total proteins, total cholesterol, ALT and AST activity, and decreased ovarian cholesterol levels, while it had no effect on kidney function in immature female rats. The seeds contained alkaloids, saponins and carbohydrates.

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Table 1: Effect of Flax seeds extract and estradiol on body and organs weights in immature female rats

Parameters	Days of treatment	Group A (Control)	Group B (Flax seeds)	Group C (Estradiol)	Overall mean
Body weight (g)	7	105.83±9.39	117.83±5.36	96.50±1.96	106.72±4.0b
	14	132.83±3.15	139.33±3.98	126.83±5.41	133.00±2.63a
	Mean	119.33±6.23b	128.58±4.54a	111.67±5.33b	119.86±3.25
Ovarian weight (mg)	7	18.83± 6.23	32.33±5.13	30.33±2.81	30.50±2.21b
	14	39.50±2.10	48.17±3.88	46.67±3.27	44.78±1.95a
	Mean	34.17±2.62	40.25±3.89	38.50±3.21	37.64±1.89
Uterine weight (mg)	7	23.33±3.99	23.83±1.60	25.67±3.15	24.28±1.67
	14	22.33±0.42	27.67±2.62	34.50±1.86	28.17±1.52
	Mean	22.83±1.89b	25.75±1.44ab	30.08±2.19a	26.22±1.16

Values (mean±SE) with different letters within a row or a column for each parameter differ significantly ($P<0.05$).

Table 2: Effect of Flax seeds extract and estradiol on serum hormones and ovarian cholesterol contents in immature female rats

Parameters	Days of treatment	Group A (Control)	Group B (Flax seeds)	Group C (Estradiol)	Overall mean
Serum estradiol (pg/ml)	7	21.08±3.28	30.68±6.85	101.18±11.56	50.98±9.68a
	14	14.77±3.67	21.35±2.0	50.50±4.11	28.87±4.19b
	Mean	17.93±2.53c	26.02±3.68b	75.84±9.62a	39.93±5.53
Serum progesterone	7	12.85±1.18	30.72±1.84	31.40±2.11	24.99±2.29
(ng/ml)	14	16.13±2.44	24.12±3.81	24.02±3.14	21.42±2.08
	Mean	14.49±1.39b	27.42±2.25a	27.71±2.39a	23.21±1.55
Ovarian cholesterol	7	27.52±5.22	16.99±0.48	18.76±0.54	21.09±1.99
(mg/dL)	14	36.70±3.66	17.14±0.77	20.66±1.81	24.83±2.44
	Mean	32.11±3.34a	17.07±0.43b	19.71±0.95b	22.96±1.59

Values (mean±SE) with different letters within a row or a column for each parameter differ significantly (P<0.05).

Table 3: Serum concentrations of various biochemical constituents in control rats and those given extract of Flax seeds and estradiol

Parameters	Days of treatment	Group A (Control)	Group B (Flax seeds)	Group C (Estradiol)	Overall mean
Total proteins (g/L)	7	53.45±2.03	54.50±1.59	68.79±3.22	58.92±2.13b
	14	54.92±1.22	63.90±1.48	68.31±0.98	62.38±1.51a
	Mean	54.19±1.15c	59.20±1.76b	68.55±1.60a	60.65±1.32
Total cholesterol	7	46.29±2.60	92.04±4.51	68.22±2.54	68.85±4.88b
(mg/dL)	14	64.94±3.12	114.15±3.36	75.82±3.81	84.97±5.45a
	Mean	55.61±3.41c	103.09±4.28a	72.02±2.47b	76.91±3.85
Urea (mg/dL)	7	34.84±1.80	27.06±1.17	31.55±1.89	31.15±1.18b
	14	38.22±1.87	36.98±1.69	38.48±2.23	37.89±1.07a
	Mean	36.53±1.34	32.02±1.79	35.01±1.74	34.52±0.97
ALT activity (U/L)	7	24.45±1.02	23.77±1.51	17.07±0.36	21.76±0.99b
	14	20.19±1.51	30.20±2.42	24.56±1.27	24.99±1.39a
	Mean	22.32±1.08b	26.98±1.67a	20.82±1.29b	23.37±0.89
AST activity (U/L)	7	14.05±0.88	18.42±1.67	19.16±0.46	17.21±0.82a
	14	12.09±0.47	13.96±1.22	10.29±0.59	12.12±0.58b
	Mean	13.07±0.56b	16.19±1.19a	14.73±1.38ab	14.66±0.66

Values (mean±SE) with different letters within a row or a column for each parameter differ significantly (P<0.05).

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