

Journal of Complementary and Integrative Medicine

Volume 7, Issue 1

2010

Article 21

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Recommended Citation:

Zanwar, Anand A.; Aswar, Urmila M.; Hegde, Mahabaleshwar V.; and Bodhankar, Subhash L. (2010) "Estrogenic and Embryo-Fetotoxic Effects of Ethanol Extract of *Linum usitatissimum* in Rats," *Journal of Complementary and Integrative Medicine*: Vol. 7: Iss. 1, Article 21.

DOI: 10.2202/1553-3840.1381

Available at: <http://www.bepress.com/jcim/vol7/iss1/21>

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Estrogenic and Embryo-Fetotoxic Effects of Ethanol Extract of *Linum usitatissimum* in Rats

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Abstract

The objective of the study was to investigate estrogenic and progestogenic activity of ethanol extract of *Linum usitatissimum* (LU-EE) in immature rats and its resultant effect on pregnancy in adult rats. Estrogenic activity was carried out by vaginal cornification, vaginal opening and by rodent uterotrophic assay. Effect of LU-EE on pregnancy in rats was studied on female Wistar rats by oral administration of LU-EE to pregnant female rats from day 1-7 of pregnancy. Further progestogenic activity was studied by histamine-induced decidualoma formation. Blood hormones were estimated for further confirmation. LU-EE (500, 1000 mg/kg) caused a significantly ($P < 0.001$) increase in uterine weight in immature rats. LU-EE (500, 1000 mg/kg) produced cornification of epithelium suggesting estrogenic activity. Oral administration of LU-EE to pregnant female rats from day 1-7 of pregnancy at the doses of 500 and 1000 mg/kg body weight resulted in complete termination of pregnancy. Estimation of estrogen in blood showed significant increase in estrogen level in LU-EE (1000 mg/kg) treated rats ($P < 0.001$). It can be concluded that LU-EE possesses estrogenic activity and the estrogenic activity of LU-EE appears to be responsible for fetocidal effect.

KEYWORDS: abortive, flaxseed, *Linum usitatissimum*, phytoestrogens, pregnancy

Author Notes: Acknowledgements The authors would like to acknowledge Dr. S. S. Kadam, Vice-Chancellor and Dr. K. R. Mahadik, Principal, Poona College of Pharmacy, Bharati Vidyapeeth University, Pune, India, for providing necessary facilities to carry out the study. We are also thankful to Dr. P. B. Ghorpade, Principal, Scientist and Linseed breeder, All India Co-ordinated Research Project on linseed, for authentication of sample.

INTRODUCTION

Consumption of flaxseed has potential health benefits due to reported anticancer effects (Chen et al 2002), antiviral, bactericidal (Collins et al 2003), antioxidant (Kitts et al 1999) and anti-inflammatory activities (Kinniry et al 2006). Moreover flaxseeds have been reported to be useful in the treatment of diabetes (Prasad 2000), hypercholesterolemic menopause (Lemay 2002) and atherosclerosis (Prasad 1997). Flaxseeds are used for food in various forms. Whole flax means the seeds of flax used in the powder form. The oil of flax is extracted by cold press method. The remaining mass of oil is called 'cake' in India and 'meal' in other countries. In India a preparation called 'Chuteny' is essential component of meal. When they are consumed the metabolism of lignan occurs in the liver and precursor secoisolaricinol diglucoside (SDG) is released. This compound has been reported to have anticancer effect (Thompson et al 1966a; Thompson et al 1966b) in mammary tumor growth. The bacteria present in the colon convert SDG to enterolactone and enterodiols, which are called mammalian lignans.

Mammalian lignans i.e. enterolactone and enterodiols are thought to exert protective effects in breast cancer by interfering with endogenous sex hormone metabolism during pregnancy. Flaxseed has been shown to cause a dose-related lengthening of the rat estrous cycle (Orcheson et al 1998; Tou et al 1998; Tou et al 1999). Dietary flaxseed (10%) caused lowered birth weight of rat pups and female offsprings with shorter anogenital distance, greater uterine and ovarian relative weights and lighter body weight at puberty, lengthened estrous cycle (Tou et al 1998). Mammalian lignans are structurally similar to 17- β -estradiol and exhibit estrogen-like or antiestrogen-like properties depending on dose, duration of administration and stage of development (Tou et al 1998; Tou et al 1999; Ward et al 2001). Disruption of the estrogen balance during pregnancy may have deleterious effects on the establishment and maintenance of pregnancy (Collins et al 2003). Maternal exposure to a diet containing flaxseed during pregnancy and lactation altered reproductive indices in both male and female offsprings (Tou et al 1998; Tou et al 1999). Among females, exposure to a 10% flaxseed diet resulted in a shortened anogenital distance. All of these changes were due to estrogenization. Flaxseed can potentially alter reproduction, depending on the dose and timing of exposure (Tou et al 1999). It has been stated that flaxseed is not suitable for consumption by pregnant women (Dey and Yuan 2003). In recent published reports suggest that, fetus can be very sensitive to flax oil (Rao et al 2007). Earlier report of defatted flaxseed or SDG has shown that phytoestrogenic activity may interfere with pregnancy outcome (Tou 1998).

Flaxseed or oil supplementation is claimed to be useful in treatment of hypercholesterolemia or other non-communicable diseases. But it has not been extensively studied for its effects in physiological conditions like pregnancy. The

objective of the study was to investigate estrogenic and progestogenic activity of ethanolic extract of *Linum usitatissimum* (called as LU-EE) in immature rats and effect of estrogenic activity on pregnancy in adult rats.

MATERIALS AND METHODS

Collection and authentication of plant

Fresh seeds of *Linum usitatissimum* were purchased from local flax supplier of Pune, Maharashtra State, India. The seed of *Linum usitatissimum* was identified and authenticated at College of Agriculture, Nagpur, India and voucher specimen was deposited at the institute.

Drugs and chemicals

Estradiol benzoate (Sigma Chemicals, St. Louis, USA), hydroxyprogesterone caproate (Sun-Pharmaceuticals, India), histamine dihydrochloride (Ozone International, Mumbai, India), anesthetic ether (TKM Pharma, Hyderabad, India), olive oil (SOS Cuetara SA, Madrid, Spain), absolute alcohol (Changshu Yangyuan Chemicals, China) were purchased from respective vendors. Petroleum ether (60-80 °C), hydrochloric acid, sodium hydroxide and sodium chloride of analytical grade were purchased from S.D. Fine-Chem. Ltd, Mumbai, India.

Preparation of ethanolic extract of *Linum usitatissimum*

The seeds of *Linum usitatissimum* were crushed and milled. These seeds were defatted by petroleum ether in soxhlet apparatus. The marc was then hydrolyzed with 1 M aqueous sodium hydroxide for 1 h at room temperature by constant rotation, followed by extraction with 50% ethanol. Then solution was acidified to pH 3 using 1 M hydrochloric acid. The filtrate was dried on tray dryer at 50 °C. The powdered of ethanolic extract was dissolved in distilled water to prepare the drug solution of different concentration and used for pharmacological studies.

Preliminary phytochemical screening

The preliminary phytochemical analysis for LU-EE was carried for the alkaloid (Mayer's, Hager's, Dragendorff's, Wagner's test), flavonoids (Shinoda test), steroids (Salkowski, Liberman-Burchard, Libermann's test), phenolic compounds, glycosides and volatile oils.

Experimental animals

Either sex of Wistar rats (150-200 g), immature female rats (50-55 g) or Swiss albino mice (18-23 g) were purchased from National Toxicology Centre, Pune, India. They were maintained at a temperature of $25\pm 1^\circ\text{C}$ and relative humidity of 45 to 55% under 12h light: 12h dark cycle. The animals had free access to food pellets (Chakan Oil Mills, Pune, India) and water was available *ad libitum*.

Research protocol approval

The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) constituted in accordance with the rules and guidelines of the Committee for the Purpose of Control and Supervision on Experimental Animals (CPCSEA), India.

Acute oral toxicity study

Swiss albino mice of either sex were subjected to acute toxicity studies as per guideline (AOT No. 425) suggested by Organization for Economic Co-operation and Development 2001. The mice were observed by housing them individually in the polypropylene metabolic cages continuously for 2 h for behavioral, neurological and autonomic profiles and for any lethality during next 48 h.

Estrogenic activity

Effect of LU-EE on vaginal cornification

The method described by the Vogel and Vogel 2002 was used. Immature female Wistar rats weighing 55 to 60 g were ovariectomized. They were maintained for about one week on standard laboratory diet and water *ad libitum*. The animals were divided into four groups containing 6 animals per group. Group I- vehicle (distilled water, 10 ml/kg), Group II -LU-EE (500 mg/kg), Group III- LU-EE (1000 mg/kg) and in Group IV- estradiol (0.5 $\mu\text{g}/\text{animal}$, s.c) in olive oil. LU-EE was administered orally. The LU-EE was administered twice daily on two following days at 10:00 a.m. and 5:00 p.m. and continued 5:00 p.m. of the third day and at 10:00 a.m. on the fourth day. On fourth day the smears were prepared on a glass slide and stained for 10 min with 5% aqueous methylene blue solution. The smears were examined microscopically and scored according to the following guidelines:

- 0- Diestrus phase- mainly leukocytes with few epithelial cells
- 1- Metestrus phase-many leukocytes among few cornified cells
- 2- Proestrus phase- Presence of nucleated or nucleated plus cornified cells
- 3- Estrus phase- Presence of cornified cells only.

Only animals showing score 2 or 3 were considered to be positive for estrogenic activity.

Effect of LU-EE on vaginal opening in immature rats

Immature female Wistar rats (55-60 g) were divided into four groups (n=6) viz; Group- vehicle (distilled water, 10 ml/kg), Group II - LU-EE (500 mg/kg), Group III- LU-EE (1000 mg/kg) and Group IV- estradiol (0.5 µg/animal, s.c.) in olive oil. LU-EE was given orally. The dose was administered once daily for 5 days. On day 6th vaginal opening was observed (Laurence and Bacharach 1964).

Evaluation of LU-EE by rodent uterotrophic assay in immature rats

Immature female rats (50-55g) were divided into four groups (n=6) viz; Group I- vehicle (distilled water), Group II- LU-EE (500 mg/kg), Group III- LU-EE (1000 mg/kg) and Group IV- estradiol (0.5 µg/animal, s.c.) in olive oil. LU-EE was given orally. The LU-EE was administered once daily for 28 days. On day 29, the animals were sacrificed and the uterus weights were determined (Diel et al 2002).

Effect of LU-EE on pregnancy in rats

Estrous cycle in female rats

Female Wistar rats (150 -200 g) were used for the study. The animals were housed in group of 4 animals per cage. Every morning between 8:00 to 9:00 a.m. vaginal fluids were collected by inserting the tip of dropper filled with 1-2 ml of normal saline [sodium chloride (NaCl) 0.9%] into the rat vagina. A drop of vaginal fluid smeared on the slide. Unstained vaginal smear was observed under light microscope, with 10 and 40 x objective lenses. Three types of cells could be recognized: round and nucleated as epithelial cells; irregular ones without nucleus as cornified cells; and the little round as leukocytes. The proportion among them was used for the determination of the estrous cycle phases (Marcondes et al 2002).

Effect of LU-EE on pregnancy in rats

Adult female Wistar rats (150-200 g) were selected for the pregnancy study. Vaginal smear of female rats were examined daily. The rats in proestrous phase of estrous cycle were housed overnight for mating with adult Wistar male rats (150-200 g) of known fertility. Vaginal smears of these female rats were examined the following morning for evidence of copulation; the presence of thick clump of spermatozoa in vaginal smear indicated pregnancy. The day on which spermatozoa were observed in the vaginal smear was designated as day 1 of pregnancy. The pregnant rats were separated out. The pregnant rats were divided into four groups (n=6) viz; Group I- vehicle (distilled water, 10 ml/kg), Group II- LU-EE (250 mg/kg), Group III- LU-EE (500 mg/kg) and Group IV- LU-EE (1000 mg/kg). LU-EE was given orally from day 1 to day 7 of pregnancy.

The animals were laparotomized under light ether anesthesia on day 10 of pregnancy. Implantation sites on both horns of uterus were recorded. The abdominal wounds were sutured layer by layer and animals were allowed to go term. After delivery, numbers of pups born were noted. Those rats, which didn't deliver, were laparotomised on day 25 and uteri were examined for resorption of implantation sites (Badami et al 2003; Bodhankar et al 1974).

Hormonal estimation in pregnant rat

Separate sets of pregnant rats were selected for hormonal estimation. The LU-EE was administered to the rats at a dose of 1000 mg/kg. The number of pregnant animals in each group was six. The control group of animal received the vehicle (distilled water) only. LU-EE was administered orally from day 1 to 7 of pregnancy.

The animals were laparotomized under light ether anesthesia on day 10 of pregnancy. Implantation sites on both horn of uterus were recorded. The abdominal wounds were sutured layer by layer. On 14th day of pregnancy blood was withdrawn by retroorbital puncture method. Animals were allowed to go to term.

E2 estradiol was estimated by CLIA: IMMULITE, fully automated immunoassay analyzer DPC, USA and progesterone was estimated by MEIA/FPIA: AxSYM fully automated immunoassay analyserabbott, USA in the blood sample drawn on 14th day of pregnancy. Estradiol values are expressed in pg/ml and progesterone values are expressed in ng/ml.

Progestogenic activity

Effect of LU-EE on histamine induced decidual formation

Adult female Wistar rats weighing 200 to 250 g were ovariectomized. They were allowed to recover for two days. Animals were divided into 3 groups containing 6 animals each. Group I-vehicle (distilled water, 10ml/kg), Group II-LU-EE (1000 mg/kg p.o.) and Group III-hydroxyprogesterone caproate (0.04 mg/animal, s.c.). The treatment period was for 14 days. All these groups treated with 0.5 µg estradiol per animal once daily s.c. for 4 days, followed by 9 days of vehicle, LU-EE (1000 mg/kg) and hydroxyprogesterone (0.04 mg/animal s.c.) administration. On 5th day of progesterone, vehicle and LU-EE treatment uterine horns were exposed and 1.0 mg histamine dihydrochloride was injected into lumen of one horn while in other horn (control), vehicle (distilled water) was injected. The animals were sacrificed on day 14th after the last treatment. Both uterine horns were removed and weighed. The degree of decidual formation was evaluated by the percent increase in the weight of the histamine-injected uterine horn as compared with the control horn (Laurence and Bacharach 1964; Vogel and Vogel 2002).

Statistical analysis

Data was expressed as Mean ± S.E.M. and statistical analysis was carried out by One-way ANOVA using Graph Pad InStat version 3.00 for Windows Vista™ BASIC, Graph Pad Software, San Diego California USA, www.graphpad.com. *P* value < 0.05 was considered as significant.

RESULTS

Acute toxicity studies

In acute oral toxicity studies, no changes in the behavior and autonomic profiles and no mortality were observed in all treated and control groups of the mice up to the dose of 5000 mg/kg.

Estrogenic activity

Effect of LU-EE on vaginal cornification

The smears were examined microscopically and scored according to the guidelines. Control group showed estrus score-0, estradiol (0.5 µg/animal) treated

rats showed estrus score-3. Oral administration of LU-EE (500 mg/kg) showed estrus score-2, while LU-EE (1000 mg/kg) showed estrus score-3. (Table 1)

Effect of LU-EE on vaginal opening in immature rats

All the control rats showed closed vaginas, whereas LU-EE (500, 1000 mg/kg) showed open vaginas after 5 days of treatment. (Table 1)

Estimation of LU-EE by rodent uterotrophic assay in immature rats

Oral administration of the LU-EE (500, 1000 mg/kg) caused a significant increase in uterine weight in immature rats ($P < 0.001$ compared to estradiol group). The uteri of these rats were inflated and full of fluid resembling the proestrous/estrous uterus. (Table 1)

Table No. 1: Effect of LU-EE on rodent uterotrophic assay (uterine weight), vaginal opening and vaginal cornification (estrus score).

Groups	Treatment (dose, mg/kg body weight)	Uterus weight (mg) (Mean \pm S.E.M.)	Vaginal opening	Vaginal cornification (Estrus score)
Group I	Control	0.31 \pm 0.02	Vagina not Open	0
Group II	Estradiol (0.5 μ g/animal)	0.66 \pm 0.01**	Vagina Open	3
Group III	LU-EE (500 mg/kg p.o.)	0.46 \pm 0.03**	Vagina Open	2
Group IV	LU-EE (1000 mg/kg p.o.)	0.47 \pm 0.00**	Vagina Open	3

Values are mean \pm S.E.M., n=6 in each group; Statistical analysis by One-way ANOVA followed by *post hoc* Tukey’s test using Graphpad Instat software ** <0.01 compared to standard group.

Estrus score

- 0- Diestrus phase- mainly leukocytes with few epithelia cells
- 1- Metestrus- Presence of mixture of leukocytes and epithelial cells
- 2- Proestrus phase- Presence of nucleated or nucleated plus cornified cells
- 3- Estrus phase- Presence of cornified cells only.

Effect of LU-EE on pregnancy in rats

The total numbers of implants in LU-EE (250, 500, 1000 mg/kg) treated 6 rats per group were 43, 41 and 40 respectively. On the other hand total number of implants was 51 in control group. The numbers of pups delivered were 47 in control group and 40 in LU-EE (250 mg/kg) treated group. The rats treated with LU-EE (500 and 1000 mg/kg) did not deliver any litters. Laparotomy of rats on day 25 showed the resorption of the implantation sites. (Table 2)

Table No. 2: Effect of LU-EE on the number of implantation sites and pups delivered in rats.

Groups	Dose (mg/kg p.o.)	No. of rats showing presence of spermatozoa	No. of rats showing implantation on day 10	No. Implants in individual rats	No. of pups delivered	Percentage of reduction in pregnancies
Group I	Vehicle (10 ml/kg)	6	6	9,11,5,10,8,8 (51)	9,10,5,8,8,7 (47)	0
Group II	250	6	6	8,8,6,9,7,5 (43)	8,7,5,9,6,5 (40)	0
Group III	500	6	6	9,6,4,10,7,5 (41)	0,0,0,0,0,0 (0)	100
Group IV	1000	6	6	5,9,7,4,9,6 (40)	0,0,0,0,0,0 (0)	100

(Total number of implantation sites and number of pups born are shown in parentheses in six rats)

Progestogenic activity

Effect of LU-EE on histamine induced decidual formation

Administration of histamine 1 mg/kg in hydroxyprogesterone caproate (0.04 mg/animal s.c.) treated group for 9 days showed increase weight of uterine horn due to decidual formation in LU-EE (1000 mg/kg) treated group compared to control group. (Table 3)

Table No. 3: Effect of LU-EE on decidual formation in rats

Groups	Treatment (dose, mg/kg body weight)	Histamine induced decidual formation (Uterus weight in mg)
Group I	Control	0.21 ± 0.00
Group II	LU-EE (1000 mg/kg)	0.19 ± 0.00
Group III	Hydroxyprogesterone caproate (0.04 mg/animal)	0.24 ± 0.01*

Values are mean ± S.E.M., n=6 in each group; Statistical analysis followed by One-way ANOVA followed by *post hoc* Tukey's test using Graphpad Instat software; *P* value *<0.05 compared to control group.

Hormonal estimation in pregnancy

Estimation of estrogen in blood of pregnant rat treated with LU-EE (1000 mg/kg) on 14th day of pregnancy showed high estrogen level 33 pg/ml compared to 18 pg/ml of control group. The progesterone estimation was below measurement level in both the groups.

DISCUSSION

Flaxseed is a rich dietary source of phytoestrogens (Obermeyer et al 1995). The adverse effects of various phytoestrogens (not including those found in flaxseed) on reproduction in livestock were reviewed by Kaldas and Hughes 1989. Environmental endocrine active compounds, including phytoestrogens, can have pronounced effects on reproduction and fertility. Evidence is mounting that there are multifactorial causes for adverse reproductive outcomes. Genotypic susceptibilities and/or deficiencies of nutrients such as folic acid coupled with exposure to teratogens could account for some birth defects. This would explain the difficulties in establishing the causality of these events. There are many synthetic drugs available in the market as abortive or contraceptive pills. So exploration of drugs having no adverse effect on reproductive function is need of current time (Flynn et al 2003).

The present study was carried out to evaluate the estrogenic activity of LU-EE. The evidence of estrogenic activity is based on vaginal cornification, vaginal opening and rodent uterotrophic assay in immature rats. Both estradiol benzoate (0.5 µg per animal) and LU-EE (500 and 1000 mg/kg) showed proestrus and estrus phases in the vaginal smear of animal and vaginal opening indicating estrogenic activity. Repeated administration of estrogen induces a dose dependent increase of uterine weight in overioctimized female rats (Vasudeva and Sharma 2007). In the present investigation LU-EE (500 and 1000 mg/kg) showed significant increase in uterine weight indicating estrogenic activity. It is known that administration of estrogen has uterotrophic effects in ovariectomized female rats and mice. Such effects are associated with growth and proliferation of endometrial microvilli on the apical surface as well as increase in cell number (Jordan 1985). Administration of estrogens to pregnant animals has been found to cause detrimental effects on pregnancy (Tou et al 1998). Hence present study was extended to evaluate effect of LU-EE on pregnancy.

In the present investigation LU-EE (250 mg/kg p.o. 7 days) did not show any deleterious effect on number of implantation sites and number of pups born compared to control. Interestingly when dose of LU-EE was increased (500 and 1000 mg/kg p.o.) the number of implants formed was less than control group moreover such rats failed to deliver pups indicating fetocidal activity. The necropsy of uterus indicated resorption of foetuses. Traditionally flaxseeds are not given to pregnant women. The fetocidal activity of LU-EE (500 and 1000 mg/kg) observed in the present study supports the traditional ethnobotanical practice of abstinence of flaxseed in pregnancy. Further evidence of estrogenic activity was derived from the observation of significant high level of estrogen concentration in blood on day 14 of pregnancy in the LU-EE (1000 mg/kg) treated rats.

On other hand failure to detect measurable progesterone in the blood sample indicated that LU-EE did not possess progesteric activity. The deciduoma test is based on the fact that the endometrium of estrogen-primed progesterone treated rodent is sensitive to local stimuli such as scratching (Astwood 1939), chemical irritation and electrical stimulation and produces a deciduoma, as equivalent to maternal placental tumor (Vogel and Vogel 2002). In the present study, hydroxyprogesterone caproate injection showed increase in uterine weight as compared to control group and the weight of histamine-injected uterine horn decreased compared to control group, in LU-EE treated group confirming lack of progesterone like activity.

The high maternal estrogen levels during pregnancy are prevented from exerting hormone toxicity on the fetus by both sex hormone-binding globulin (SHBG) and alpha-fetoprotein (AFP) in humans and by AFP in rats. By binding to estrogen, these proteins prevent the free estrogen from interacting with receptors to produce hormonal effects. Those estrogens not extensively bound to AFP tend to be potent estrogenic toxins, capable of disrupting normal reproduction in the rat (Tou et al 1998). The results of present study thus indicated that LU-EE may be acting as estrogen which may be responsible for the termination of pregnancy.

It is a well known fact that for implantation and sustenance of pregnancy, equilibrium of secretion of estrogen and progesterone is necessary. Any imbalance in the levels of these hormones can cause anti-implantation or can result in resorption of pups. Compounds disturbing hormonal functions may evoke infertility (Hiremath et al 1999; Vasudeva and Sharma 2006,2007a,b; Padmashali et al 2006). In this study, the change observed in the uterus of animals treated with various extracts support an unfavorable uterine milieu. Therefore, the fetocidal activity may be due to estrogenic activity of the LU-EE causing expulsion or resorption of the implants by the uterus. LU-EE at a dose of 500 mg/kg body weight was found to possess 100% of fetocidal activity. The fetocidal activity may be due to estrogenic activity of the LU-EE.

Preliminary phytochemical studies indicated the presence of flavonoids and saponin glycosides in LU-EE. The adverse effects of saponins on animal reproduction have long been known and have been ascribed to their abortifacient, antizygotic and anti-implantation properties and flavonoids have been reported to possess antifertility activity (Padmashali et al 2006; Francis et al 2002; Badami et al 2003; Hiremath et al 1999). The observed effect of the LU-EE might be due to the presence of such compounds. Therefore, the fetocidal activity of LU-EE might be due to the presence of such compounds and potential estrogenicity of LU-EE. Plant products exhibiting estrogenic activity and producing antifertility effects are known in literature (Badami et al 2003; Vasudeva and Sharma 2006; 2007a,b).

CONCLUSIONS

Finally it can be concluded that LU-EE has estrogenic activity but no progestogenic activity. Presence of saponin glycosides and flavonoids and estrogenic activity of LU-EE produced resorption in pregnant rats. Caution is suggested for consuming flaxseed during pregnancy. Further purification and isolation of pure moiety responsible for fetocidal activity in LU-EE is required.

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