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RESEARCH ARTICLE

Evaluation of Antifertility Potential of *Entada rheedii* Spreng Seed Kernel Extract in Female Wistar Rats

Jayshree Dawane, Linpadmaja Thakur and Sonali Suryawanshi*

Department of Pharmacology, BVDTU Medical College, Pune – 411043, Maharashtra, India; suryawanshi.sonali@bharatividyapeeth.edu

Abstract

Background: Population growth has created a global dilemma, putting burden on economic, social, and ecological resources. *Entada rheedii* Spreng (ERS) seeds have many medicinal properties. In folkloric medicine, seeds are used as contraceptives, but no studies available. **Aim:** To evaluate antifertility potential of ERS seed kernel extract in female Wistar rats. **Method:** 90 Female Wistar rats with regular oestrus cycles and proven fertility were included. Methanol extract of ERS seed kernel was used in dose of 200 mg/kg and 400 mg/kg by oral route. Part I-Reproductive outcome rats were kept for mating after 8 days' initial drug treatment which continued for 21 days, number of litters was measured. Mothers were observed for regain of fertility and pubs for teratogenicity. Part II-Animals kept for mating followed by 7 days' drug treatment, Laparotomy done on the 10th day, number of implants checked and after delivery litter size counted. Part-III Abortifacient effect rats kept for mating. Laparotomy done on day 10, drug treatment was given for 21 days and again laparotomy was done and resorption of implants was measured. Blood withdrawal done from the rats of reproductive outcome for hormonal estimation. Data analyzed with Graph Pad Prism 6. **Results:** The control rats showed a significant increase in litter numbers compared to treated animal in both the doses of ERS low ($P < 0.001$) and high ($P < 0.001$). Pubs appeared normal and reversal of effect in the form of gain of fertility after 21 days of treatment was evident. The treatment with ERS extract resulted in a statistical increase in the resorption index ($P < 0.001$) suggesting a failure of embryonic development. Anti-implantation and abortifacient action were seen with both doses of ERS. **Conclusion:** Results show that ERS seed kernel has potential and reversible antifertility effect in female Wistar rats.

Keywords: Abortifacient Effect, Antifertility, Anti-implantation, *Entada rheedii* Spreng Seed Kernel, Reproductive Outcome

1. Introduction

The explosion of population in developing nations has heightened the need for efficient birth control methods. Many methods are available for contraception. The current population is 1.42 billion[1] with an increase in population, adversely affecting the quality of life[2]. The population growth rate is towards the higher side and if it remains the same, the load on the basic systems like public transport, electricity services, education and other essential things will also increase tremendously[3]. India is a developing country, and the socio-economic crisis is extreme, therefore population control is an important problem that needs attention. Education about birth control and effective, cheap and easily available contraceptives with less adverse effects will be very useful[4]. Research on female fertility regulation focuses mainly on the development of oral active antifertility drugs[5]. Primarily, most research efforts are directed toward the discovery of synthetic oral contraceptives with mild to moderate adverse effects. In 2019, the global scenario indicates that around 1.1 billion women were using one or the other methods of contraception for family planning and 50% of them were using hormonal contraceptives[6]. In India, the use of contraceptives was reported to be 47.7 per cent in 2017 and only 9 % use oral contraceptives[7]. Hesitancy to use oral contraceptives is because of the adverse

*Author for correspondence

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effects. Adverse effects encountered during the use of oral contraceptives like headache, weight gain, breast tenderness, acne, and mood swings, including hormonal imbalance hirsutism, hypertension, thromboembolism, and increased incidence of cervical, breast and other cancers which limits the use of them for long term. Ancient literature mentions many plants having the contraceptive effect, but very little attention has been paid to the plant materials[8]. Therefore, efforts need to be directed to develop novel and potent antifertility medications with minimal adverse effects.

Entada rheedii seeds are used as a folkloric medicinal plant for its narcotic, emetic, febrifuge, alexiteric and antiperiodic. It was also found to be effective in the treatment of jaundice, diarrhoea, musculoskeletal problems and mumps[9]. As per the literature, *E. rheedii* seed kernel can be used as a remedy for cerebral haemorrhage and oral contraceptives[10,11]. The seed kernel consists of a high quantity of Triterpenoids and saponins have been observed to enhance antifertility action[12]. Triterpenoid saponins are glycosylated triterpenes present in many dicotyledonous plant species[13], triterpenes are of interest due to their utility in different medical conditions as antiviral, anticancer, anti-inflammatory or wound-healing properties[14]. Antifertility effects of lupeol were recently evaluated and the contraceptive property has been identified[15,16]. The following research study was planned to examine the antifertility effects of *E. rheedii* seed kernel extract in female Wistar rats.

2. Materials and Methods

Ethics committee approval was taken before starting the study. (BVDUMC/723/2022/003/025) Wistar rats (female) were obtained from the central animal house, BVUMC, Pune. Rats weighing between 150-200 g were included in the study. Housing was done in standard cages with bedding of rice husk and the environment was maintained as 10% air exhausts in the air conditioning unit, relative humidity of 60 ± 5 %, temperature 25 ± 3 °C and cycle divided of 12 hours of light and dark each. A rodent diet in the form of pellets was given at10 am. Aqua guard purified water in the sterile bottles was given *ad libitum*. All experimental procedures were performed as per the CCSEA guidelines *E. rheedii* seeds were obtained from the authentic *Ayurveda* shop. Seed kernels were separated, powdered and extract prepared.

2.1 Extraction Procedure

The *E. rheedii* seed of the plants was cleaned with water and kept for drying at room temperature. The seed kernels were separated and pulverized to become fine powder. white coloured fine powdered material of ERS in 100 gms soaked in 500 ml of 70% ethanol and stirred intermittently. After 48 hours the material was filtered through Whatman (No. 1) filter paper. The same procedure was repeated for the residue. The filtrate was dried at room temperature and kept in air-tight containers for use. Since this extract was insoluble in water, a suspension was prepared with 3% gum acacia and used for the study.

Groups of animals (female Wistar rats n=10) and treatment

Group 1- Control-saline treated

Group 2- ERS seed kernel extract 200mg/kg

Group 3- ERS seed kernel extract 400mg/kg

Three separate sets of animals were used to test the different outcomes, 30 animals were there in each activity, and they were divided into three groups such as control, ERS seed kernel extract low dose and high dose. Drug treatment is given orally as per the group and as per the type of activity for the specified period.

2.2 Monitoring of Estrous Cycle

On glass slides, vaginal fluid was applied and evaluated for the oestrous phase. Vaginal smears were observed under a microscope for the cellular changes to find out the phase of the oestrous cycle. Rats with regular cycles were included in the study. The antifertility activity was assessed with reproductive outcomes, anti-implantation, and abortifacient effect as well as hormonal analysis was done.

2.3 Reproductive Outcome

Female rats were treated with the drugs as per the groups for 8 days. On day 8 they were kept overnight with a proven fertile male in the ratio of 3:1 (female: male), in the pro-oestrous phase and examined evidence of copulation the following morning. Those rats, which show thick clumps of spermatozoa, sperms, and vaginal plugs on vaginal smears or by direct observation of the mating behaviour were selected. They

were included in the study and day 8 was considered as day 1 of pregnancy. These pregnant female rats were divided into three groups comprising eight rats in each group. The extracts were given orally for 21 days. The study's animals were examined for vaginal bleeding. On parturition, the litter size was measured, and litters were observed for the congenital abnormality. After the washout period, animals were observed for the regain of fertility as well as foetuses for the abnormality. Percentage inhibition of fertility was calculated. The cut-off value was 60% inhibition of fertility.

2.4 Anti-implantation Activity

Healthy males and females were kept for mating in the ratio of (3:1). Vaginal smear was taken and checked for presence of sperm. Drug treatment was given for 7 days as per the groups. On day 10 Laparotomy was done under ketamine anaesthesia, with all aseptic precautions and several implants were measured. Pregnancy continued and after the parturition litter size was measured and compared with the control. The following formula was used for measuring ant-implantation activity.

$$\text{Anti-implantation activity} = \frac{\text{No. of implants in control} - \text{No. of implants in test group}}{\text{No. of implants in control}} \times 100$$

2.5 Abortifacient Effect

Healthy males and females were kept for mating in the ratio of (3:1). On day 10 laparotomy was done under ketamine anaesthesia with all aseptic precautions. Both horns were evaluated for the number of implants. The incision was closed with sutures and gently the animal was kept back in the cage. Post-operative care was taken. Drug treatment was given from day 10-21. Vaginal bleeding was checked daily. On day 21 again laparotomy was done. Several litters were observed. The resorption of implants was compared with the initial number of implants.

2.6 Hormonal Analysis

Blood was collected in EDTA-coated vacutainers from the retro-orbital puncture under ketamine anaesthesia and processed. Serum was used for FSH, LH, 17β-estradiol and prolactin and 17OH progesterone estimation using enzyme-linked immunoassay (ELISA) technique.

2.7 Statistical Analysis

Statistical analysis was performed with ANOVA followed by Tukey's test. Results are expressed as mean±SD, P value $p<0.05$ was considered as significant. For analysis graph pad prism version 6. (Graph Pad Software, San Diego, California) was used.

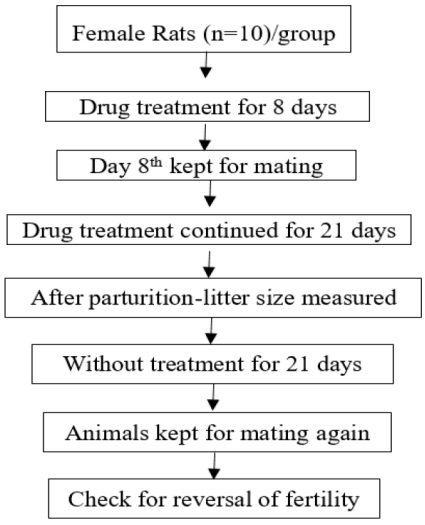

Figure 1. Methodology for the reproductive outcome.

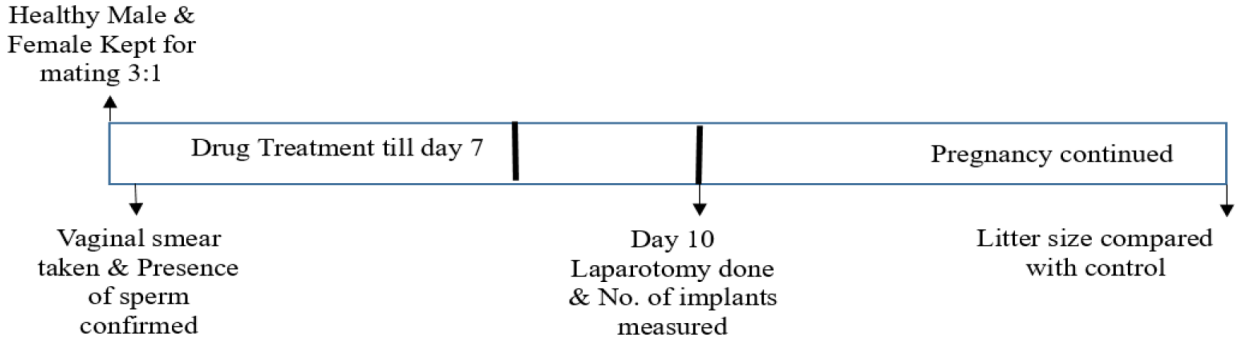

Figure 2. Methodology for anti-implantation activity.

3. Results

The control rats showed a significantly high number of litters and then the drug-treated animal in both the doses of ERS low ($P < 0.001$) and high ($P < 0.001$). In high doses, a 100% reduction in fertility was observed. High dose was most effective causing complete inhibition of fertility. Not only fertility was regained after the washout period, but there was also an increase in the litter size was observed. Normal growth was seen in these pubs till adulthood showing no teratogenic effect.

Anti-implantation and abortifacient activities were calculated based on several implants and several litters. Both effects were significantly high in the ERS extract-treated groups in comparison with the control group. The treatment with ERS extract increased the percentage of resorption index ($P < 0.001$). Indicates the failure in the development of the embryo. The mean percentage of anti-implantation and abortifacient activity was found to be highest for both doses.

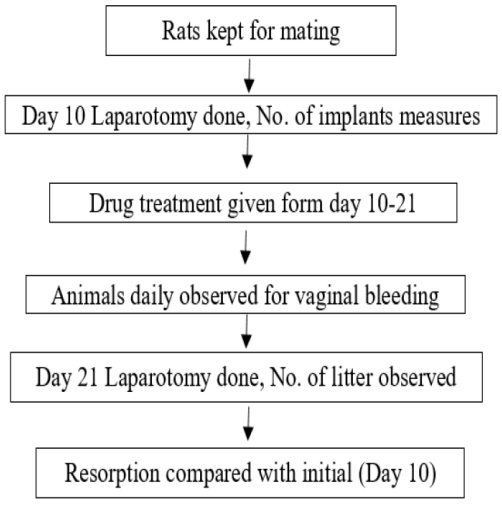

Figure 3. Methodology for the abortifacient effect.

Significant decreases in the levels of LH ($p<0.001$), FSH ($p<0.001$) with high doses, Prolactin ($p<0.01$) and 17β estradiol ($p<0.001$) were observed in the drug-treated animals when compared with control. Decreased LH and FSH levels could also be indicative of ovarian dysfunction, which may lead to irregular menstrual cycles, infertility, or premature ovarian failure.

4. Discussion

Currently available contraceptives have many limitations due to adverse effects and a major concern is about the risk of developing breast and cervical cancer[17]. Safe oral contraceptive is the need of the time. The antifertility effect of *E. rheedii* seed kernel was evaluated in the present study. The established animal model was used for the study.

Female Wistar rats were used in the study, which shows similarity to the human reproductive system.

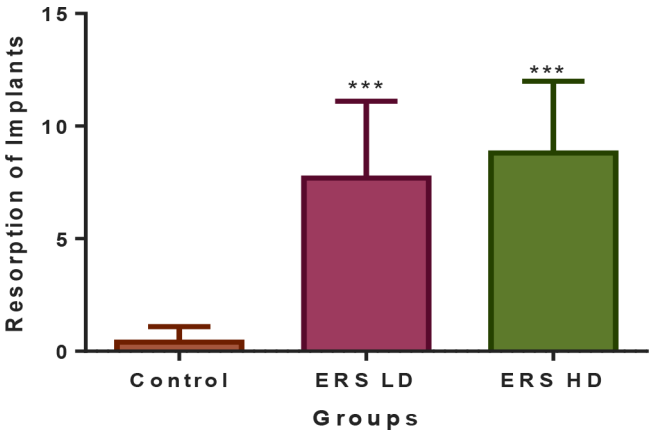

Figure 4. Effect of ERS extract on abortifacient activity in female Wistar rats.
Values expressed with mean±SD, ANOVA followed by Tukey's multiple comparisons test was used.
P Value ***p<0.001 when compared with normal control.

Table 1. Reproductive outcome - effect of ERS extract on reproductive outcome in female rats

Groups	Treatment before mating For 8 days	Treatment After mating till 21 days	No pregnant Rats	No pubs delivered	Percentage reduction in pregnancy	Percentage Fertility
1	Distilled water		10/10	10,11,9,11,12, 13,10,8,9.9	0	100
2	ERS extract LD		1/10	3	90	10***
3	ERS extract HD		0/10	0	100	0***

Values are expressed as Numbers and percentages, ***p<0.001 in comparison with normal control.

There are four phases of the estrus cycle in rats - proestrus, estrus, metestrus and diestrus and lasts for 4 to 5 days[18]. The proestrus phase in the rats is like the follicular stage in women which is characterised by the raised circulating estradiol leading to Luteinising Hormone (LH) rise and Follicle Stimulating Hormone (FSH) release[19]. FSH concentration reaches the peak and a fall in the estradiol levels along with the LH surge results in ovulation and similar things are observed in rats during the estrus phase. The phases of the oestrus cycle match with the human menstrual cycle and show similar hormonal changes[20]. The levels of progesterone are raised during this phase.

Oestrous cycles were checked with the help of vaginal smears for the normal fertility of the female Wistar rats and those with regular cycles were included in the study. During the study period in all the study components, after the oral administration of ERS in both doses change in the pattern of the oestrus cycle was observed. In the control rats, estrus cycles were normal. The irregular cycles in ERS extract-treated groups may be because of the oestrogen and progesterone levels. The oestrous cycle is controlled by the ovarian and extra-ovarian hormones (Table 3).

In the assessment of reproductive outcome, (Figure 1) in the control rats number of litters was observed normal. Treatment with the ERS extract produced a significant reduction in litter size. ERS extract was effective in both doses showing the antifertility effect. High dose was most effective causing complete inhibition of fertility (Table 1). Fertility was regained after the washout period, rather there was an increase in the litter size observed. No abnormality was seen in the litters of the extract-treated rats. Normal growth was seen in these pubs till adulthood showing no teratogenic effect. To date, many herbal preparations are tested for the antifertility effect with minimal adverse effects and reversible fertility[21,22]. Plants with a high content of flavonoids, alkaloids and terpenoids show contraceptive effects[23]. ERS contains high concentrations of these including saponins and phenols[24-26].

There are many mechanisms involved in the antifertility effect. Disturbances in the estrogen and progesterone cause inhibition of the LH surge as a result, follicles are unable to grow, mature and rupture. Progestin effect in the form of making cervical mucus secretion thick and unfavourable for sperm penetration. The blastocyst may fail to implant because of the changes in the endometrium like hyperproliferation or hypersecretion or atrophic and not suitable for nidation. Mismatch or synchronization in uterine and tubal contractions may disfavour fertilization and dislodge just implanted blastocyst or may interfere with fertilization/implantation[27].

In the present study anti-implantation, (Figure 2) and abortifacient activities (Figure 3) were evaluated in several implants and litters. Both effects were significantly high in the ERS extract-treated groups

Table 2. Anti-implantation effect of ERS extract on fertility in female rats

Groups	Treatment	No of implants (n=10)	No pubs delivered	Mean Percentage Anti-Implantation
1	Distilled water	91	87	Nil
2	ERS extract LD	21	11	87.35***
3	ERS extract HD	12	9	89.65***

Values are expressed as Numbers and percentages, ***p<0.001 in comparison with normal control.
The mean percentage of anti-implantation activity was significant (***p<0.001)in ERS-treated groups in comparison with the control group.

Table 3. Effect of ERS extract on hormonal levels in female rats

	Hormonal levels in different experimental groups				
	LH ng/ml	**FSH ng/ml**	**Prolactinpg/ml**	**17β estradiolpg/ml**	**17-OH progesterone pmol/L**
Control	11.61±2.71	10.12±1.43	44.44±8.78	759.88±67.42	15.75±1.71
ERS LD	6.85±1.30***	7.06±1.76**	34.92±9.46	548.65±86.62***	26.25±6.31***
ERS HD	3.72±1.55***	5.45±2.51***	28.58±9.70**	454.93±55.82***	28.35±3.36***

Values expressed with mean±SD, n=10, ANOVA followed by Tukey's multiple comparisons test was used. **p<0.01, ***p<0.001 with comparison with normal control. LH: Luteinizing hormone, FSH: Follicle-stimulating hormone.

in comparison with the control group. (Figure 4) Percentage inhibition (Table 2) of implantation may be due to blastocytotoxic activity or expulsion of the embryo.

Hormone estimation showed that there is a decrease in the LH and FSH levels (Table 1). A decrease in the FSH level results in anovulatory cycles and can be seen from the disturbances in the estrus phases. The FSH concentration decline results in failure of ovulation at the same time disturbances in the oestrus cycle[28]. LH surge is required for the ovulation, decreased levels of LH result in failure of ovulation and is necessary for the corpora lutea to continue developing and function normally[29]. This could be the reduced fertility reason. But at the same time, abortifacient and anti-implantation effects were also significant which may have resulted from the decreased levels of progesterone which is needed for maintenance of the pregnancy. Normal physiological balance of estrogen and progesterone levels is an integral component for the implantation which showed disturbed in present study rats. (Table 3) At the same time, it may have resulted from the increased uterine contraction and a synchronization of the uterus and fallopian tubes.

Entada rheedii seed kernel showed an antifertility effect because of abortifacient and anti-implantation activity. Seed kernel has been evaluated for various activities like antioxidant and antiproliferative and showed medicinal utility through its active ingredient *E. rheedii* nosides A and B[30]. The mechanism behind antifertility effects is complex, further studies are required to confirm the mechanism behind this action.

5. Limitations

The antifertility potential of *E. rheedii* seed kernel extract showed promising results however no standard comparator was used. Further studies with comparative groups are planned.

6. Conclusion

Entada rheedii seed kernel showed significant abortifacient and anti-implantation potential and possesses a reversible antifertility effect in female Wistar rats.

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