IJPT
1735-2657/07/62-159-163
IRANIAN JOURNAL OF PHARMACOLOGY & THERAPEUTICS

IJPT 6:159-163, 2007
RIDR

RESEARCH ARTICLE

# Effect of Chromatographic Fractions of Ethanolic Extract of *Crotalaria Juncea* (L.) Seeds on Ovarian Follicular Kinetics and Estrous Cycle in Albino Rats

VIJAYKUMAR B. MALASHETTY and SARASWATI B. PATIL

*For author affiliations, see end of text.*

Received November 7, 2006; Revised May 5, 2007; Accepted August 14, 2007

This paper is available online at *http://ijpt.iums.ac.ir*

## ABSTRACT

In spite of considerable development in contraceptive technology, search for female antifertility agent in plants continues to be a potential area of investigation. The ethanol extract of *Crotalaria juncea* seeds which showed promising antiovulatory activity in female albino rats was examined for the isolation of its active fractions. Two fractions were obtained using Thin Layer Chromatography (TLC) of the extract. Both fractions were subjected for testing their anti-ovulation activity and estrous cycle in rats. After preliminary trials, the fraction I (200mg/kg body weights) showed maximum antiovulatory activity when administered orally to the rats for 30 days. Decreased number of healthy follicles (Class I – ClassVI) and corpora lutea and increased number of regressing follicles (Stage IA, Stage IB, Stage IIA, Stage IIB) were observed in the ovary after 30 days treatment. The treatment caused an increase in the cholesterol level and acid/alkaline phosphatase activity and a decrease in protein and glycogen contents of the ovary. Estrous cycle was affected as a significant increase in estrus and metaestrus phases and a decrease in diestrus and proestrus phases in the treated groups during experimental period of 30 days were observed. These results suggest that a fraction of ethanolic extract of *crotalaria juncea* might be used as a contraceptive in the females.

**Keywords:** *Crotalaria juncea; Antiovulatory; Estrous cycle; Antifertility; Rat*



*Crotalaria juncea* (L.), commonly called as Sunn hemp, belongs to the family Papilionaceae. The medicinal properties have been described in Ayurveda, by Sushruta as well as in ancient books like Sarangadhara and Bhavaprakasha. In Ayurveda, the leaves are used as an emetic, laxative, abortifacient and analgesic, and for treating diarrhea, dysentery and bleeding disorders. The seeds are used as abortifacient and in the treatment of impetigo, psoriasis and as an emmenogogue [1-2]. Previous works on the ethanol extract of *C. juncea* demonstrated their anti-implantation [3], antiovulatory [4] and anti-spermatogenic activities [5-7] in rats and mice. In the present work, we have undertaken the investigation of the antiovulatory activity of chromatographic fractions of crude ethanol extract of *C. juncea* to elucidate its active ingredient.

## MATERIALS AND METHODS

### *Plant material*

The fresh seeds of *C. juncea* were obtained from a local source during October and November 2003, and authenticated at the Herbarium, Department of Botany, Gulbarga University, Gulbarga (HGUG No. 141), India; where voucher specimens were deposited.

### *Preparation of test material*

Fresh dried seeds of *C. juncea* were powdered and soxhleted with ethanol (95%) for 24 hours. The filtrate was dried under reduced pressure and chromatographed by thin layer chromatography over silica gel 'G' as absorbent. The extract was loaded on the preparative plates and developed with solvent system benzene: methanol (80:20). Two major bands were observed by Iodine vapors. The compounds having high retention power ($R_f$) was designated as fraction I and the compounds with low $R_f$ value was designated as fraction II. The fraction I of the ethanol extract yielded brownish gummy material and the fraction II yielded yellow gummy material when the silica gel was washed with methanol. Both the fractions were used after making required doses in Tween-80 (1%).

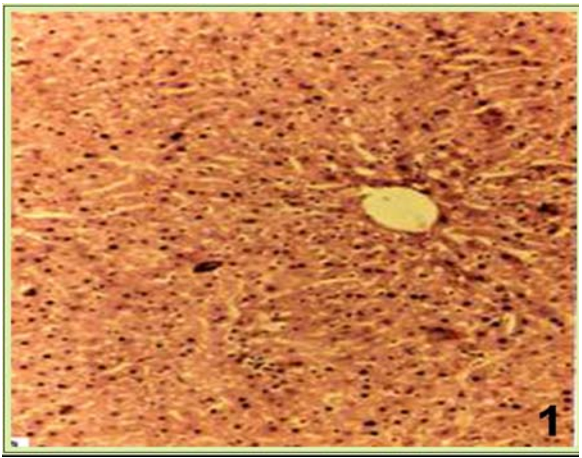

**Fig 1**. C. S. of liver of control mice showing normal well organized hepatic chords with parenchymatous hepatocytes and blood vessels (×125).

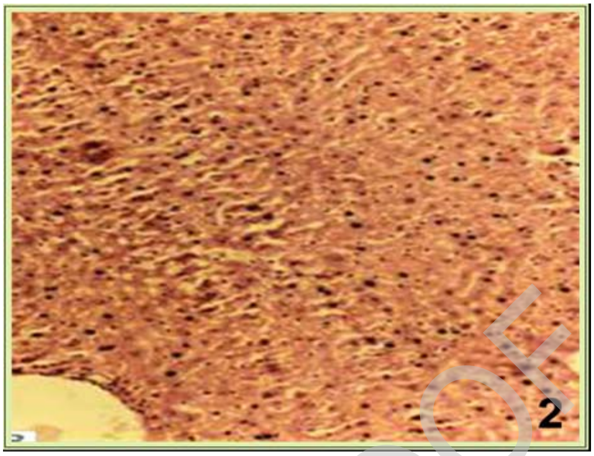

**Fig 2.** C. S. of liver of crude ethanol extract of *C. juncea* treated mice showing normal histological picture similar to that of control (×125).

### *Acute toxicity studies*

The acute toxicity study was performed according to the method described by Kattan and colleagues [8]. Adult albino mice of either sex were divided into five groups containing ten animals in each. Graded doses (100 and 200 mg/kg b.w.) of fraction I and II of ethanol extract of *C. juncea* seeds in tween-80(1%) were administered orally by mean of intragastric catheter to healthy adult mice (30-35g). Following administration of the extracts, the animals were observed continuously for 2 horrs and then frequently for further 4 hours. Mortality was recorded. After 7$^{th}$ day, the detection of hematotoxicity, haemoglobin concentration, total RBC and WBC counts were examined. The histological changes in the liver were also studied.

### *Animals*

Sexually matured, healthy, colony-bred virgin female rats of Wistar strain (*Rattus norvegicus*), aged 3 months and weighing 150-200g were used for the experiments. The rats were housed in polypropylene cages measuring 12''x10''x8'', under well-ventilated animal house conditions (ambient temperature: 28-31$^{0}$C, photoperiod: 12h natural light and 12h dark; relative humidity: 50-55%). The rats were given pelleted feed (Hindustan Lever Ltd., India) and tap water *ad labium*. They were maintained as per the principles of Laboratory Animal Care [9]. The experimental protocol was approved by the Institutional Animal Ethics Committee.

### *Experimental design*

The animals were divided into 5 groups consisting of six animals in each group.

Group I: Control, received 0.2ml Tween-80 (1%)

Group II: Received 100mg /kg b.w. fraction I in 0.2ml tween-80 (1%)

Group III: Received 200mg /kg b.w. fraction I in 0.2ml tween-80 (1%)

Group IV: Received 100mg /kg b.w. fraction II in 0.2ml tween-80 (1%)

Group V: Received 200mg /kg b.w. fraction II in 0.2ml tween-80 (1%)

All the above treatments were given orally by using intragastric catheter for 30 days to cover six regular estrous cycles. The treatment was started from estrous phase (by observing cornified cells in the vaginal smear), as the ovarian activities change markedly from one phase to another phase of estrous cycle. The treatment was given orally everyday between 10.00 and 11.00 am. The stages of estrous cycle were recorded daily by observing vaginal smears.

### *Autopsy and organ weight*

On 31$^{st}$ day, 24 hours after last dosing, all the animals were weighed and sacrificed by cervical dislocation. The ovaries were dissected out immediately and separated out from the adherent tissue and weighed using an electronic balance.

### *Histopathological studies*

The ovary from one side of each animal was fixed in Bouin's fluid, embedded in paraffin wax, sectioned at 5µm, stained with haematoxylin–eosin for follicular studies.

### *Morphometric analysis*

Follicular diameter and morphologies were used to classify follicles using established methods [10-11] as follows:

Class I: Small preantral follicles (SPAF) (<90µm)

Class II: Large preantral follicles (LPAF) (91-260µm)

Class III: Small antral follicles (SAF) (261-350µm)

Class IV: Medium sized antral follicles (MSAF) (351-430µm)

Class V: Large sized antral follicles (LSAF) (431-490µm)

Class VI: Graafian follicles (GF) (>491µm)

Follicles under regression were classified depending on the degree of regression as:

Stage IA: Pyknosis in some granulose cells.

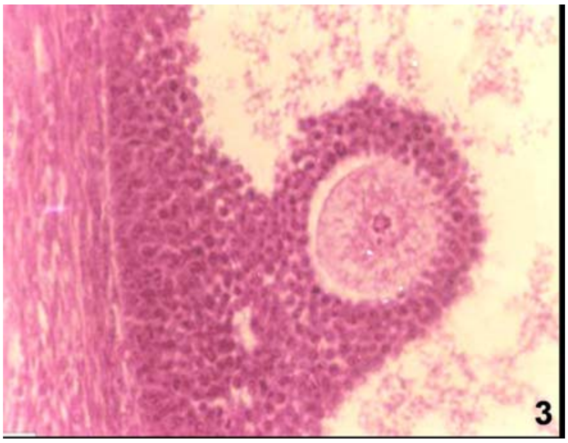

**Fig 3**. Control rat with normal ovarian follicle with antrum, corona radiata, cumulus oophorus and granulosa membrane (×400).

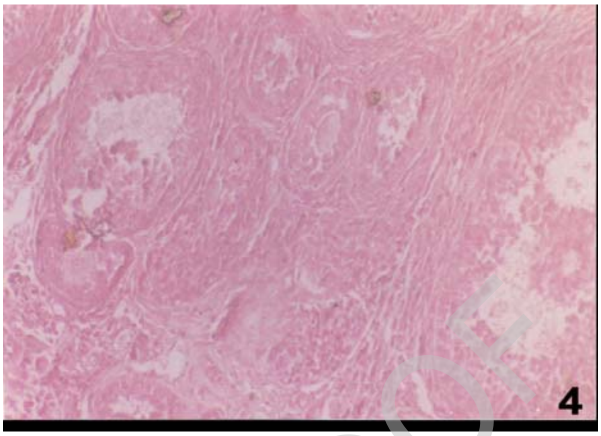

**Fig 4.** Rat treated with fraction I of ethanol extract of *C juncea* seeds showing greater follicular atresia (x 400).

Stage IB: Degenerative changes in the entire granulose layer.
Stage IIA: Oocytes with pyknotic nuclei, blocked meiosis in metaphase I (pseudomaturation) and degenerating cumulus cells.
Stage IIB: Characterized by oocytes floating in the antrum with few pyknotic bodies

Morphometric studies of the ovary were calculated by using stage and ocular micrometers.

### *Biochemical studies*

Ovary from the other side of each animal was used for biochemical estimations like protein [12], glycogen [13], cholesterol [14], acid and alkaline phosphatase [15].

### *Data processing*

The statistical analysis was done to determine significant difference of values between treated and control groups using Students *t*- test.

## RESULTS

### *Acute toxicity studies*

In acute toxicity tests, no change in the behaviour or body weight of animals was observed. The mortality was nil. Blood variables, i.e., RBC, WBC and haemoglobin were within the normal range. There was no histological change in the liver of treated mice with fraction I and II of ethanol extract of *C. juncea* seeds when compared to that of control (Table 1 & 2; Figs 1 & 2).

### *Changes in body weight*

Administration of fraction I and II at both dose levels to female rats revealed no change in the body weight. The treated rats were healthy and maintained normal growth rate throughout the experiment. Though the body weight of the animals in treated groups showed slight fluctuation when compared to control group, this was negligible (Table 3).

### *Changes in estrous cycle*

Administration of fraction I at both doses levels significantly increased the estrous and metestrous phases ($p<0.001$) and decreased the diestrous and proestrous phases. The administration of fraction II increased the estrous phase nonsignificantly but metestrous phase significantly ($p<0.001$) and decreased proestrus phase nonsignificantly (Table 4).

### *Changes in the ovary*

### *Gravimetric changes*

Administration of fraction I at both dose levels decreased the ovarian weight significantly ($p<0.001$); whereas nonsignificant reduction was obtained with both doses of fraction II (Table 5).

### *Biochemical changes*

Protein content was reduced significantly ($p<0.001$) with both doses of fraction I and II. Glycogen content was reduced and cholesterol content was increased significantly ($p<0.001$) with both low and high dose of fraction I and high dose of fraction II. Acid and alkaline phosphatase activity showed significant ($p<0.001$) increase with both the doses of fraction I and high dose of fraction II. However, fraction II at low dose is less significant ($p<0.01$) in increasing their enzyme activities (Table 5).

### *Changes in follicular kinetics*

Healthy follicles: Fraction I and II at both dose levels decreased the number of follicles of class I to class V significantly ($p<0.001$). The class VI or Graafian follicles were totally absent. Fraction II administration at low dose level reduced all the classes of healthy follicles, but it was significant with class III ($p< 0.001$), class V ($p<0.001$) and class VI ($p<0.01$) follicles (Table 6; Figs 3 & 4).

Regressing follicles: Fraction I at low dose level caused significant ($p<0.001$) increase in regressing follicles. Fraction II at low dose level increased the number of stage IB ($p<0.001$) and stage IIB ($p<0.05$) regressing follicles. Fraction II at high dose level caused significant reduction in stage IA ($p<0.001$), stage IB ($p<0.001$), stage IIA ($p<0.05$) and stage IIB ($p<0.001$) regressing follicles (Table 7; Figs. 3 & 4).

### *Changes in corpora lutea*

The number of corpora lutea were reduced highly significantly ($p<0.001$) with both doses of fraction I and high dose of fraction II (Table 6).

## Discussion

Cyclic changes in the vaginal smear observed in the estrous cycle gives a reasonable index of the ovarian activity and its hormonal synthesis of estrogen and progesterone. The levels of these hormones are controlled by hypothalamic releasing hormones and pituitary gonadotrophins [16]. A feedback mechanism also operates where the pituitary gonadotrophins secretion in turn is controlled by estrogen and progesterone. The cornification in the vaginal epithelial cells is mainly due to high levels of estrogens secreted by the ovarian matured follicles. It is also known that exogenous administration of estrogen consistently stimulates the proliferation of the vaginal epithelium in adult spayed animals [17-18]. The basic functional unit of reproduction within the ovary is the follicle [19]. Follicles start to grow at all times and as they develop, they produce large number of granulosa and thecal cells. The conversion of follicles to atretic state is functional rather than a degenerative process and is considered to be integral part of ovarian function [20-10]. Most of the follicles undergo atresia and very few mature to ovulate among the new crop of recruited follicles during every cycle. After the early stage of gonadotrophin independence, the entire process of follicle growth becomes dependent on the continuous presence of gonadotrophins [21-22]. Evans et al. [23] have shown that the ovarian androgen and inhibin secretion by follicles may play an important part in the regulation of FSH secretion and follicular dynamics. The integral role in the control of ovarian function is played by the hypothalamo-pituitary unit. Functioning in a coordinated manner with appropriate signals provided by ovary via pituitary gland is responsible for the synthesis and storage of gonadotrophins LH and FSH. These glycoprotein hormones in turn play a key role as regulators of folliculogenesis.

The data obtained in the present study reveal that the control rats exhibited regular estrous cycle of 4-5 days. Treatment with extracts of *Crotalaria juncea* seeds caused a significant increase in the estrous and metestrous with concomitant decrease in the duration of diestrous and proestrous phases. Similar results have been obtained with *Hibiscus rosa sinensis* [24] and *Momordica charantia* [25] in mice and rats respectively. This may be attributed to the fact that the increased estrogen production at regular intervals which is influenced by the crude extracts of *C. juncea* is responsible for vaginal cornification. Inspite of the influence of crude extracts of *C. juncea*, the rats have not lost the rhythmic cyclicity. These findings on the estrous cycle clearly corroborate the potent estrogenic nature of the extract.

In the present study, the decrease in the number of healthy follicles from class I to class VI and increase in the number of regressing follicles and atretic follicles attributes to the non-availability of pituitary gonadotrophins due to the treatment with *C. juncea* seed extracts. The recruitment of SPAF (class I) from primary follicles depends on availability of FSH and further folliculogenesis from SPAF (class I) to GF (class VI) requires both FSH and LH [26-29]. The observed estrogenic nature of the extracts might have brought the inhibition in the gonadotrophins secretion and release that is responsible for follicular regression rather than maturation. The reduced number or total absence of corpora lutea in extracts-treated ovaries of rats indicates the blockade of ovulation which depicts the antiovulatory property of the extracts.

Pituitary FSH, LH and prolactin are essential for utilization of cholesterol for steroidogenesis in two cell compartments of theca and granulosa cells in the ovary. The nonavailability of these gonadotrophins increases the cholesterol depot in the ovary of extracts-treated rats. Protein is considered to be the building material and is involved in the alteration of almost every physiological function. In the present study the low protein content of the ovary indicates the retarded ovarian growth. It is well understood that FSH is essential for protein synthesis in gonads [30]. The blockade of pituitary FSH releases in extracts-treated rats might have resulted in low protein content.

The presence of glycogen plays very important role in reproduction. It is involved in providing energy to various processes like ovulation, transportation and survival of eggs and implantation. All these changes are hormone-dependent [31]. The decreased ovarian glycogen content in extracts of *C. juncea* seeds treated rats may be due to lowered steroidogenesis, which attributed to nonavailability of gonadotrophins.

In conclusion, the fraction I reduced the number of healthy follicles and corpora lutea but increased the number of regressing follicles. This indicates nonavailability of gonadotrophins for follicular development and ovulation. Hence, fraction I of ethanol extract of *C. juncea* has strong antiovulatory property.

## References

1. Kirtikar KR, Basu AD. In: Indian Medicinal Plants. Vol. I. Lalit Mohan Basu, Allahabad. 1935; p 792.
2. Wealth of India. Raw Materials.Vol. IV F-G, Published by C. S. I. R., New Delhi. 1952.
3. Vijaykumar BM, Sharanabasappa A, Saraswati BP. Post-coital antiimplantation and pregnancy interruption potency of the seed extracts of Crotalaria juncea. Orient Pharm Exp Med 2004; 4: 70-6.
4. Vijaykumar BM, Sangamma I, Sharanabasappa A, Saraswati BP. Effect of Crotalaria juncea seed extracts on the estrous cycle and ovarian activity in albino mice. Orient Pharm Exp Med 2004; 4: 77-81.

5. Vijaykumar BM, Sangamma I, Sharanabasappa A, Saraswati BP. Antifertility activity of various extracts of Crotalaria juncea Linn. seeds in male mice. Philip J Sci 2003; 132: 39-46.
6. Vijaykumar BM, Sangamma I, Saharanabasappa A, Saraswati BP. Antispermatogenic and hormonal effect of Crotalaria juncea Linn. seed extracts in male mice. Asian J Androl 2004; 6: 67-71.
7. Vijaykumar BM, Saraswati BP. Spermatogenic index and hormonal profile in the rats received chromatographic fractions of ethanol extract of Crotalaria juncea L. seeds. Orient Pharm Exp Med 2006; 6: 86-95.
8. Kattan GFE, Goudgaon NM, Ilksvo N, Huang JT, Watanabe KA, Sommadossi JP, Schinazi RF. Synthesis and biological properties of 5-O-Carboranyl-1-(2-deoxy-2-fluoro-β-D-arabinofuranosy). J Med Chem 1994; 37: 2583.
9. Hariharan S. Laboratory animals information service center news ICMR, Hyderabad. 1980.
10. Hirshfield AN. Development of follicles in the mammalian ovary. Int Rev Cytol 1991; 124: 43-100.
11. Sanjay VS, Joshi BN. Melatonin and exposure to constant light/darkness affects ovarian follicular kinetics and estrous cycle in Indian desert gerbil Meriones hurrianae (Jerdon). Gen Comp Endocrinol 1997; 180: 352-7.
12. Lowry OHN, Rosenbrough J, Farr, NL, Randall RJ. Protein measurement with folic phenol reagent. J Biol Chem 1951; 193: 265-75.
13. Carrol NV, Langelly RW, Row RH. Glycogen determination in liver and muscle by use of anthrone reagent. J Biol Chem 1956; 20: 583-93.
14. Peters JP, Vanslyke DD. Quantitative Clinical Chemistry, Vol. I. Williams and Wilkins, Baltimore. 1946.
15. Bessey OA, Lowry OH, Brick NJ. A method for rapid determination of acid and alkaline phosphatase with 5 cu. mm. of serum. J Biol Chem 1946; 164: 321.
16. Lerner LJ. The biology of non-steroidal antifertility. In: Contraception, chemical control of fertility. ed. by Lednicer D, Marcel Derker Inc, New Yark. 1969; p. 161.
17. Mandel AM. Cyclical changes in the vaginal smear of adult ovariectomized rats. J Exp Biol 1951; 28: 585-92.
18. Boettiger EG. Changes in the glycogen and water contents of the rat uterus. J Cell Comp Physiol 1946; 27: 9-13.
19. Hsuch AJW, Adashi EY, Jones PBC, Welsh THJ. Hormonal regulation of the differentiation of cultured ovarian granulosa cells. Endocrine Rev 1984; 5: 76-127.
20. Zuckerman L, Weir BJ. The ovary-1. 2nd ed. General aspects. Academic Press. Inc. New York and London. 1977: 277-279.
21. Hodgon GD. Neuroendocrinology of the normal menstrual cycle. J Reprod Med 1989; 34: 68-75.
22. Scott RT, Anderson GD. The ovarian follicle: life cycle of the pelvic clock. Obstet Gynecol 1990; 33: 551-61.
23. Evans ACO, Komar CM, Wandji SA, Fortune JE. Changes in androgen evretion and lutenizing hormone pulse amplitude are associated with the ecruitment and growth of ovarian follicles during the luteal phase of the ovine estrous cycle. Biol Reprod 1997; 57: 394-401.
24. Murthy DRK, Reddy CM, Patil SB. Effect of benzene extract of H. rosa inensis on the estrous cycle and ovarian activity in albino mice. Biol harm Bull 1997; 20: 756-8.
25. Sharanabasappa A, Vijaykumar B, Patil SB. Effect of Momordica harantia seed extracts on ovarian and uterine activities in albino rats. Harmceutic Biol 2002; 40: 501-7.
26. Richards JS. Maturation of ovarian follicles: actions and interactions of ituitary and ovarian hormone on follicular cell differentiation. Physiol Rev 980; 60: 51-89.
27. Young EL, Biard DT, Hillier SG. Melatonin gonadotrophin stimulated rowth and differentiation on human granulosa cells by 31-51 -onophosphate: One molecule, two genes. Clinic Endocrinol 1992; 73: 51-5.
28. Wang X, Greenwald GS. Hypophysectomy of the cyclic mouse. I. Effects on folliculogenesis, oocyte growth, follicle stimulating hormone and human chorionic gonadotrophin receptors. Biol Reprod 1993; 48: 585-94.
29. Greenwald GS, Roy SK. Follicular development and its control. In: The Physiology of Reproduction, second edition, Knobil E, Neill JD (Eds), Raven Press Ltd, New York, 1994; 629-724.
30. Means Ar. Biochemical effects of follicle stimulating hormone on the testis. Endocrinology 1975; 5: 203-18.
31. Walaas O. Effect of oestrogens on the glycogen contents of the rat uterus. Acta Endocrinol 1952; 10: 175-92.


## CURRENT AUTHOR ADDRESSES


Vijaykumar B. Malashetty, Dept. of MRDG, Indian Institute of Science, Bangalore- 560 012, India. E-mail:viju_yes@rediffmail.com, (Corresponding author)

Saraswati B. Patil, Department of Zoology, Gulbarga University, Gulbarga-585 106, India