

1 RESEARCH ARTICLE

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

5 VIJAYKUMAR B. MALASHETTY and SARASWATI B. PATIL

6 For author affiliations, see end of text.

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

8 This paper is available online at <http://ijpt.iums.ac.ir>

9 ABSTRACT

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

24 **Keywords:** *Crotalaria juncea*; Antioviulatory; Estrous cycle; Antifertility; Rat

25 *Crotalaria juncea* (L.), commonly called as Sunn 45 authenticated at the Herbarium, Department of Botany,
 26 hemp, belongs to the family Papilionaceae. The medici- 46 Gulbarga University, Gulbarga (HGUG No. 141), India;
 27 nal properties have been described in Ayurveda, by Su- 47 where voucher specimens were deposited.
 28 shruta as well as in ancient books like Sarangadhara and
 29 Bhavaprakasha. In Ayurveda, the leaves are used as an
 30 emetic, laxative, abortifacient and analgesic, and for
 31 treating diarrhea, dysentery and bleeding disorders. The
 32 seeds are used as abortifacient and in the treatment of
 33 impetigo, psoriasis and as an emmenagogue [1-2]. Pre-
 34 vious works on the ethanol extract of *C. juncea* demon-
 35 strated their anti-implantation [3], antioviulatory [4] and
 36 anti-spermatogenic activities [5-7] in rats and mice. In
 37 the present work, we have undertaken the investigation
 38 of the antioviulatory activity of chromatographic frac-
 39 tions of crude ethanol extract of *C. juncea* to elucidate
 40 its active ingredient.

41 MATERIALS AND METHODS

42 Plant material

43 The fresh seeds of *C. juncea* were obtained from a
 44 local source during October and November 2003, and

48 Preparation of test material

49 Fresh dried seeds of *C. juncea* were powdered and
 50 Soxhleted with ethanol (95%) for 24 hours. The filtrate
 51 was dried under reduced pressure and chromatographed
 52 by thin layer chromatography over silica gel 'G' as ab-
 53 sorbent. The extract was loaded on the preparative plates
 54 and developed with solvent system benzene: methanol
 55 (80:20). Two major bands were observed by Iodine vapors.
 56 The compounds having high retention power (R_f) was des-
 57 ignated as fraction I and the compounds with low R_f value
 58 was designated as fraction II. The fraction I of the ethanol
 59 extract yielded brownish gummy material and the fraction
 60 II yielded yellow gummy material when the silica gel was
 61 washed with methanol. Both the fractions were used after
 62 making required doses in Tween-80 (1%).

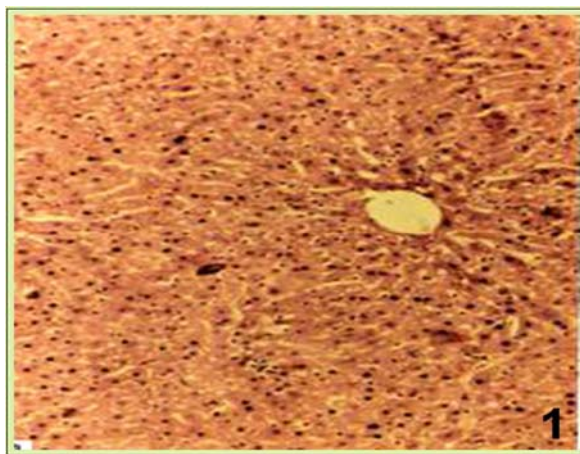


Fig 1. C. S. of liver of control mice showing normal well organized hepatic chords with parenchymatous hepatocytes and blood vessels ($\times 125$).

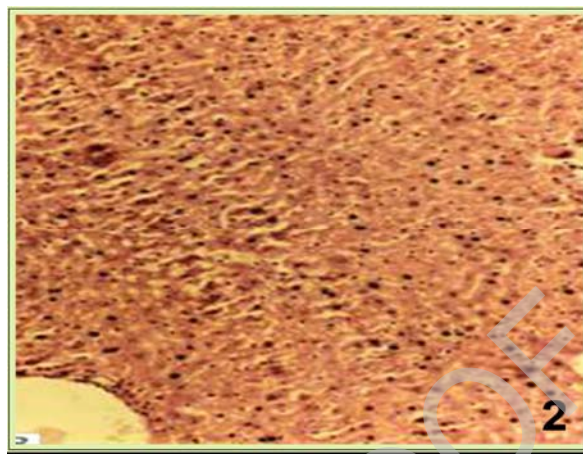


Fig 2. C. S. of liver of crude ethanol extract of *C. juncea* treated mice showing normal histological picture similar to that of control ($\times 125$).

63 Acute toxicity studies

64 The acute toxicity study was performed according to
65 the method described by Kattan and colleagues [8].
66 Adult albino mice of either sex were divided into five
67 groups containing ten animals in each. Graded doses
68 (100 and 200 mg/kg b.w.) of fraction I and II of ethanol
69 extract of *C. juncea* seeds in tween-80(1%) were admin-
70 istered orally by mean of intragastric catheter to healthy
71 adult mice (30-35g). Following administration of the
72 extracts, the animals were observed continuously for 2
73 hours and then frequently for further 4 hours. Mortality
74 was recorded. After 7th day, the detection of hematotox-
75 icity, haemoglobin concentration, total RBC and WBC
76 counts were examined. The histological changes in the
77 liver were also studied.

78 Animals

79 Sexually matured, healthy, colony-bred virgin fe-
80 male rats of Wistar strain (*Rattus norvegicus*), aged 3
81 months and weighing 150-200g were used for the ex-
82 periments. The rats were housed in polypropylene cages
83 measuring 12''x10''x8'', under well-ventilated animal
84 house conditions (ambient temperature: 28-31°C, pho-
85 toperiod: 12h natural light and 12h dark; relative humid-
86 ity: 50-55%). The rats were given pelleted feed (Hindu-
87 stan Lever Ltd., India) and tap water *ad libitum*. They
88 were maintained as per the principles of Laboratory
89 Animal Care [9]. The experimental protocol was ap-
90 proved by the Institutional Animal Ethics Committee.

91 Experimental design

92 The animals were divided into 5 groups consisting
93 of six animals in each group.
94 Group I: Control, received 0.2ml Tween-80 (1%)
95 Group II: Received 100mg /kg b.w. fraction I in 0.2ml
96 tween-80 (1%)
97 Group III: Received 200mg /kg b.w. fraction I in 0.2ml
98 tween-80 (1%)
99 Group IV: Received 100mg /kg b.w. fraction II in 0.2ml
100 tween-80 (1%)

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

103 All the above treatments were given orally by using
104 intragastric catheter for 30 days to cover six regular
105 estrous cycles. The treatment was started from estrous
106 phase (by observing cornified cells in the vaginal
107 smear), as the ovarian activities change markedly from
108 one phase to another phase of estrous cycle. The treat-
109 ment was given orally everyday between 10.00 and
110 11.00 am. The stages of estrous cycle were recorded
111 daily by observing vaginal smears.

112 Autopsy and organ weight

113 On 31st day, 24 hours after last dosing, all the ani-
114 mals were weighed and sacrificed by cervical disloca-
115 tion. The ovaries were dissected out immediately and
116 separated out from the adherent tissue and weighed us-
117 ing an electronic balance.

118 Histopathological studies

119 The ovary from one side of each animal was fixed in
120 Bouin's fluid, embedded in paraffin wax, sectioned at
121 5 μ m, stained with haematoxylin-eosin for follicular
122 studies.

123 Morphometric analysis

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

- 127 Class I: Small preantral follicles (SPAF) (<90 μ m)
- 128 Class II: Large preantral follicles (LPAF) (91-260 μ m)
- 129 Class III: Small antral follicles (SAF) (261-350 μ m)
- 130 Class IV: Medium sized antral follicles (MSAF) (351-
131 430 μ m)
- 132 Class V: Large sized antral follicles (LSAF) (431-
133 490 μ m)
- 134 Class VI: Graafian follicles (GF) (>491 μ m)

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

137 Stage IA: Pyknosis in some granulose cells.

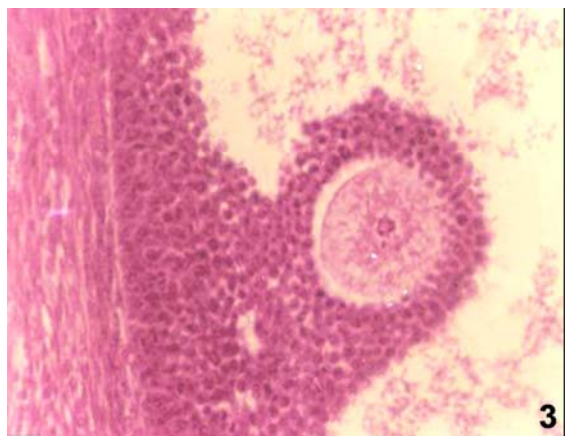


Fig 3. Control rat with normal ovarian follicle with antrum, corona radiata, cumulus oophorus and granulosa membrane ($\times 400$).

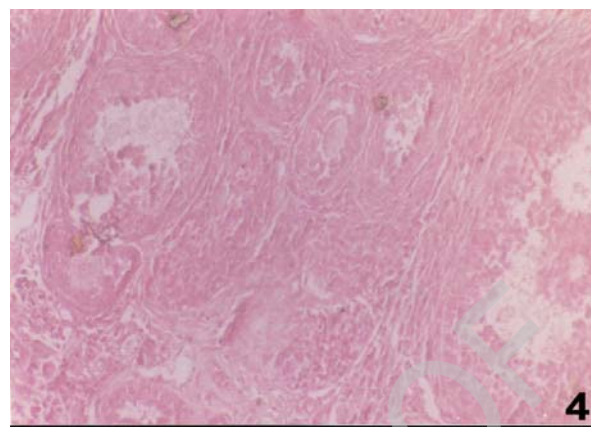


Fig 4. Rat treated with fraction I of ethanol extract of *C juncea* seeds showing greater follicular atresia ($\times 400$).

138 Stage IB: Degenerative changes in the entire granulosa
139 layer.

140 Stage IIA: Oocytes with pyknotic nuclei, blocked meio-
141 sis in metaphase I (pseudomaturation) and degenerating
142 cumulus cells.

143 Stage IIB: Characterized by oocytes floating in the an-
144 trum with few pyknotic bodies

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

147 Biochemical studies

148 Ovary from the other side of each animal was used
149 for biochemical estimations like protein [12], glycogen
150 [13], cholesterol [14], acid and alkaline phosphatase
151 [15].

152 Data processing

153 The statistical analysis was done to determine sig-
154 nificant difference of values between treated and control
155 groups using Student's *t*-test.

156 RESULTS

157 Acute toxicity studies

158 In acute toxicity tests, no change in the behaviour or
159 body weight of animals was observed. The mortality
160 was nil. Blood variables, i.e., RBC, WBC and haemo-
161 globin were within the normal range. There was no his-
162 tological change in the liver of treated mice with frac-
163 tion I and II of ethanol extract of *C. juncea* seeds when
164 compared to that of control (Table 1 & 2; Figs 1 & 2).

165 Changes in body weight

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

173 Changes in estrous cycle

174 Administration of fraction I at both doses levels sig-
175 nificantly increased the estrous and metestrous phases
176 ($p < 0.001$) and decreased the diestrous and proestrous
177 phases. The administration of fraction II increased the
178 estrous phase nonsignificantly but metestrous phase
179 significantly ($p < 0.001$) and decreased proestrus phase
180 nonsignificantly (Table 4).

181 Changes in the ovary

182 Gravimetric changes

183 Administration of fraction I at both dose levels de-
184 creased the ovarian weight significantly ($p < 0.001$);
185 whereas nonsignificant reduction was obtained with
186 both doses of fraction II (Table 5).

187 Biochemical changes

188 Protein content was reduced significantly ($p < 0.001$)
189 with both doses of fraction I and II. Glycogen content
190 was reduced and cholesterol content was increased sig-
191 nificantly ($p < 0.001$) with both low and high dose of
192 fraction I and high dose of fraction II. Acid and alkaline
193 phosphatase activity showed significant ($p < 0.001$) in-
194 crease with both the doses of fraction I and high dose of
195 fraction II. However, fraction II at low dose is less sig-
196 nificant ($p < 0.01$) in increasing their enzyme activities
197 (Table 5).

198 Changes in follicular kinetics

199 Healthy follicles: Fraction I and II at both dose lev-
200 els decreased the number of follicles of class I to class
201 V significantly ($p < 0.001$). The class VI or Graafian fol-
202 licles were totally absent. Fraction II administration at
203 low dose level reduced all the classes of healthy folli-
204 cles, but it was significant with class III ($p < 0.001$),
205 class V ($p < 0.001$) and class VI ($p < 0.01$) follicles (Table
206 6; Figs 3 & 4).

207 Regressing follicles: Fraction I at low dose level
208 caused significant ($p < 0.001$) increase in regressing fol-
209 licles. 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. In spite of the influence of

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 antiovarian 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 antiovarian property.

REFERENCES

- Kirtikar KR, Basu AD. In: Indian Medicinal Plants. Vol. I. Lalit Mohan Basu, Allahabad. 1935; p 792.
- Wealth of India. Raw Materials. Vol. IV F-G, Published by C. S. I. R., New Delhi. 1952.
- 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.
- 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.

3275. 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.
3306. Vijaykumar BM, Sangamma I, Saharanabasappa A, Saraswati BP. Antispermatic and hormonal effect of *Crotalaria juncea* Linn. seed extracts in male mice. *Asian J Androl* 2004; 6: 67-71.
3337. 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.
3378. 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-arabinofuranosyl). *J Med Chem* 1994; 37: 2583.
3419. Hariharan S. Laboratory animals information service center news ICMR, Hyderabad. 1980.
34310. Hirshfield AN. Development of follicles in the mammalian ovary. *Int Rev Cytol* 1991; 124: 43-100.
34511. 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.
34912. Lowry OHN, Rosenbrough J, Farr, NL, Randall RJ. Protein measurement with folic phenol reagent. *J Biol Chem* 1951; 193: 265-75.
35213. Carrol NV, Langelly RW, Row RH. Glycogen determination in liver and muscle by use of anthrone reagent. *J Biol Chem* 1956; 20: 583-93.
35514. Peters JP, Vanslyke DD. Quantitative Clinical Chemistry, Vol. I. Williams and Wilkins, Baltimore. 1946.
35715. 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.
36016. Lerner LJ. The biology of non-steroidal antifertility. In: Contraception, chemical control of fertility. ed. by Lednicer D, Marcel Dekker Inc, New York. 1969; p. 161.
36317. Mandel AM. Cyclical changes in the vaginal smear of adult ovariectomized rats. *J Exp Biol* 1951; 28: 585-92.
36518. Boettiger EG. Changes in the glycogen and water contents of the rat uterus. *J Cell Comp Physiol* 1946; 27: 9-13.
36719. Hsueh AJW, Adashi EY, Jones PBC, Welsh THJ. Hormonal regulation of the differentiation of cultured ovarian granulosa cells. *Endocrine Rev* 1984; 5: 76-127.
- Zuckerman L, Weir BJ. The ovary-1. 2nd ed. General aspects. Academic Press. Inc. New York and London. 1977: 277-279.
- Hodgon GD. Neuroendocrinology of the normal menstrual cycle. *J Reprod Med* 1989; 34: 68-75.
- Scott RT, Anderson GD. The ovarian follicle: life cycle of the pelvic clock. *Obstet Gynecol* 1990; 33: 551-61.
- 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.
- 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.
- Sharanabasappa A, Vijaykumar B, Patil SB. Effect of *Momordica harantia* seed extracts on ovarian and uterine activities in albino rats. *Harmcetic Biol* 2002; 40: 501-7.
- Richards JS. Maturation of ovarian follicles: actions and interactions of ituitary and ovarian hormone on follicular cell differentiation. *Physiol Rev* 980; 60: 51-89.
- 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.
- 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.
- 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.
- Means Ar. Biochemical effects of follicle stimulating hormone on the testis. *Endocrinology* 1975; 5: 203-18.
- 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