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Spermatogenic index and hormonal profile in the rats received chromatographic fractions of ethanol extract of *Crotalaria juncea* L. seeds

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SUMMARY

The ethanol extract of the *Crotalaria juncea* seeds, which showed promising antispermatogenic and antiandrogenic activities in albino mice, was taken up further for the isolation of the active fractions present in it. Two fractions that were obtained from thin layer chromatography were subjected for testing to know their antispermatogenic and antiandrogenic activities. After preliminary trials the fraction I showed maximum antifertility activity at the dose level of 200 mg/kg body weight when administered orally to the rats for 50 days. The fraction I was found to affect spermatogenesis as well as the endocrine functions of the testis as indicated by gravimetric, histopathological and biochemical changes. Further this fraction has caused degenerative changes in the seminiferous tubules and Leydig cells of the testis. The accessory reproductive organs like epididymis, seminal vesicles, vas deferens, prostrate, Cowper's gland and Levator Ani muscle showed significant malfunction. Cauda epididymal sperm count and sperm motility were reduced significantly. The treatment has also resulted in increase in the cholesterol level and alkaline phosphatase activity, and decrease in protein, glycogen, sialic acid contents and acid phosphatase activity in testis. It is noteworthy that RIA studies have shown significant reduction in serum FSH, LH and testosterone. Scanning electron microscopic observations revealed abnormalities in sperm structure.

Key words: *Crotalaria juncea*; Rats; Testis; Sperm count; FSH; LH; Testosterone

INTRODUCTION

Given the risks and benefits of presently available methods of male contraception, a method which is of indigenous plant origin may have particular merits such as cost effectiveness, less or non toxic and orally bioactive. Though the combined efforts by investigators during the last five decades to develop an ideal male contraceptive agent of plant origin, have met with limited success and the recent findings in this area are encouraging (Kamboj and Dhawan, 1989). As earlier studies in our laboratory have reported (Vijaykumar *et al.*, 2004) complete suppression of spermatogenesis in mice with ethanol extract of *Crotalaria (C.) juncea* seeds, a lot of interest has been generated to determine the active principle in this extract of *C. juncea* seeds that has antifertility effects. In this article we are presenting the data of the antifertility characteristics and associated effects of chromatographic fractions of crude ethanol extract to elucidate the active principle.

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MATERIALS AND METHODS

Preparation of test material

Fresh dried seeds of *C. juncea* were powdered and soxhleted with ethanol (95%) for 24 h. The filtrate was dried under reduced pressure and subjected to thin layer chromatography over silica gel 'G' as absorbent. The extract was loaded on the preparative plates, developed with solvent system benzene : methanol (80 : 20). Two major bands were observed by Iodine vapors. The compounds having high retention power (R_f) has designated as fraction I and the compound having 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%).

Animals

Sexually matured, healthy, colony bred virgin male rats of Wistar strain (*Rattus norvegicus*), aged 3 months and weighing 150 - 200 g were used for the experiments. The rats were housed in polypropylene cages measuring 12"× 10"× 8", under standard animal house conditions (temperature: 28 - 31°C, photoperiod: 12 h natural light and 12 h dark; humidity: 50 - 55%). The rats were given pelleted feed (Hindustan Lever Ltd., India) and tap water *ad libitum*. They were maintained as per the principles of Laboratory Animal Care (Hariharan, 1980).

Study protocol

The animals were divided into five groups of six in each group

Group I : Control, received 0.2 ml tween-80 (1%)
Group II : Received 100 mg fraction I/kg b.w. in 0.2 ml tween-80 (1%)
Group III : Received 200 mg fraction I/kg b.w. in 0.2 ml tween-80 (1%)
Group IV : Received 100 mg fraction II/kg b.w. in 0.2 ml tween-80 (1%)
Group V : Received 200 mg fraction II/kg b.w. in 0.2 ml tween-80 (1%).

All the above treatments were given orally by using intragastric catheter for 50 days as to cover one complete spermatogenic cycle. On day 51 all rats were sacrificed by cervical dislocation. Testes, epididymides, seminal vesicles, ventral prostate, vas deferens, Cowper's gland and Levator Ani muscle were dissected out, surrounding blood vessels and tissues were removed and blotted free of blood and mucous. Caput and cauda regions of the epididymis were separated. The tissues were weighed quickly to the nearest mg on an electronic balance. The organ weights were expressed in terms of 100 g body weight. The tissues were subjected for biochemical and histological studies. The testis obtained from one side from each animal was used for the estimation of protein (Lowry *et al.*, 1951), glycogen (Carrol *et al.*, 1956), cholesterol (Peters and Vanslyke, 1946), sialic acid (Warren, 1959), total ascorbic acid (Roe and Kuether, 1943) and acid and alkaline phosphatase activity (Bessey *et al.*, 1946).

The testis from the other side was fixed in Bouin's fluid, embedded in paraffin, sectioned at 5 μm and stained in haematoxylin and eosin (Gurr, 1962) and processed for histological studies. For histometry, the prepared slides were placed under high power objective in a microscope and with the help of the ocular micrometer the testis diameter (TD), seminiferous tubular diameter (STD) and Leydig cell nuclear diameter (LCND) were measured by the method described by Deb *et al.* (1964) from randomly chosen 20 round sections of each group. The spermatogenic elements like spermatogonia (SPG), spermatocytes (SPC) and round spermatids (RSPT) were counted (Abercrombie, 1946) from randomly chosen 20 round sections. Mature and degenerating Leydig cells were counted. Mature Leydig cells are those containing agranular clear cytoplasm and spherical nucleus and degenerating

Leydig cells, containing residual bodies or dense bodies in abundance (Christensen, 1975). The sperm count and percentage of motility from cauda epididymis were calculated by the method described by Kempinas and Lemano Carvalho (1987).

Serum testosterone, FSH and LH levels were assayed by RIA using Count-A-Count Kit (Los Angeles, US). The morphological alterations of sperm were studied by Scanning Electron Microscopy (SEM) (Chinoy *et al.*, 1979).

Statistical analysis

The mean and standard error of mean (M ± SE) were calculated and the significance of difference was analyzed by applying student's '*t*' test.

RESULTS

Changes in the testis

Gravimetric changes: The administration of both low and high dose of fraction I has caused highly significant ($P < 0.001$) reduction in the weight of testes when compared with that of control rats. Significant ($P < 0.01$) reduction in the weight of testes is observed only with high dose of fraction II (Table 1).

Biochemical changes (Table 2)

Protein: Administration of both the doses of fraction I and II has caused significant ($P < 0.001$) reduction in the protein content.

Cholesterol: Highly significant increase in the cholesterol level is observed due to the treatment of both the doses of fraction I ($P < 0.001$) and high dose of fraction II ($P < 0.001$), but it is less significant with low dose of fraction II.

Glycogen: The administration of both the doses of fraction I has caused significant ($P < 0.001$) reduction in the glycogen content, whereas, fraction II at both the dose level is less effective.

Sialic acid: Sialic acid content is reduced significantly ($P < 0.001$) with both the doses of fraction I. Significant reduction in the sialic acid content is observed only with that of high dose ($P < 0.01$) of fraction II treatment.

Ascorbic acid: Significant reduction ($P < 0.001$) in the ascorbic acid content is observed with both doses of fraction I and high dose of fraction II treatment. The treatment of low dose of fraction II is not much effective.

Acid phosphatase: The administration of both the doses of fraction I and II has caused significant ($P < 0.001$) decrease in the acid phosphatase activity.

Alkaline phosphatase: The alkaline phosphatase activity is increased significantly ($P < 0.001$) with both low and high dose of fraction I and II when compared with that of control.

Changes in micrometry and Leydig cells

Diameter of testis and seminiferous tubules is decreased significantly ($P < 0.001$) with the treatment of both the doses of fraction I and II. The administration of both the doses of fraction I has caused significant ($P < 0.001$) reduction in the diameter of Leydig cell nucleus. Treatment of both the doses of fraction II seems to be ineffective. Significant ($P < 0.001$) decrease in the number of mature Leydig cells is seen with both the doses of fraction I and high dose of fraction II. However, fraction II at low dose is less effective ($P < 0.01$). Degenerating Leydig cell number is increased significantly ($P < 0.001$) with both the doses of fraction I and II in comparison with that of control animals (Table 3).

Changes in spermatogenic elements and spermatozoa

The number of spermatogonia, spermatocytes and round spermatids is decreased significantly ($P < 0.001$) with both the doses of fraction I and high dose of fraction II treatment. The administration of low dose of fraction II has caused less significant reduction in the number of spermatogonia, spermatocytes and round spermatids ($P < 0.001$) in comparison with that of control animals (Table 4, Fig. 1 and 2)

The cauda epididymal sperm count is decreased highly significantly ($P < 0.001$) due to the treatment of both the doses of fraction I. Low ($P < 0.05$) and high ($P < 0.01$) doses of fraction II are less effective.

Talble 1. Changes in reproductive organ weights due to treatment of Fraction I and II of ethanol extract of *C. juncea* seed in rats

Treatment	Dose (mg/kg)	Testes	Caput epididymis	Cauda epididymis	Vas deferens	Seminal vesicle	Ventral prostate	Cowper's gland	Levator ani
Control	Tween-80 (1%)	2046.50 ± 17.12	329.80 ± 2.86	193.30 ± 1.70	96.10 ± 1.83	269.50 ± 2.07	95.80 ± 2.83	72.16 ± 3.09	205.60 ± 3.71
Fraction I	100	1417.00 ± 9.82***	227.80 ± 7.55***	112.30 ± 3.57***	54.50 ± 2.32***	150.66 ± 3.07***	53.50 ± 3.22***	38.30 ± 2.53***	101.60 ± 3.48***
	200	997.50 ± 4.31***	212.16 ± 2.35***	94.00 ± 2.25***	45.80 ± 2.21***	99.10 ± 3.54***	43.60 ± 1.92***	32.60 ± 1.92***	84.60 ± 2.26***
Fraction II	100	2006.10 ± 7.57	325.60 ± 2.62	179.30 ± 3.05***	82.60 ± 1.92***	241.30 ± 2.06***	82.00 ± 1.89***	63.00 ± 1.71***	175.00 ± 2.80***
	200	1975.50 ± 8.59**	309.30 ± 4.03***	166.00 ± 3.23***	76.60 ± 2.73***	323.30 ± 2.47***	77.16 ± 2.41***	59.00 ± 2.69***	169.10 ± 2.77***

Organ weight: mg/kg body weight. M ± S.E. = Mean ± Standard error. Duration of treatment: 50 days. Six animals were maintained in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with control.

Table 2. Effect of Fraction I and II of ethanol extract of *C.juncea* seeds on some biochemical markers in the testis of rats

Treatment	Dose (µg/kg)	Protein (µg/mg)	Cholesterol (µg/mg)	Glycogen (µg/mg)	Sialic acid (µg/mg)	Ascorbic acid (µg/mg)	Acid phosphatase (µM of P-nitrophenol released/ 100 mg/30min)	Alkaline phosphatase (µM of P-nitrophenol released/100 mg/ 30 min)
Control	Tween-80(1%)	13.60 ± 0.24	1.10 ± 0.17	2.60 ± 0.05	6.10 ± 0.12	3.00 ± 0.08	5.40 ± 0.19	1.00 ± 0.17
Fraction I	100	54.0 ± 0.21***	9.10 ± 0.23***	1.20 ± 0.14***	1.70 ± 0.18***	1.90 ± 0.08***	1.90 ± 0.10***	2.90 ± 0.11***
	200	3.10 ± 0.34***	18.90 ± 0.30***	0.70 ± 0.14***	0.8 ± 0.09***	1.0 ± 0.12***	1.2 ± 0.14***	3.8 ± 0.17***
Fraction II	100	10.9 ± 0.18***	1.70 ± 0.11**	2.5 ± 0.17	5.8 ± 0.11	2.8 ± 0.14	4.6 ± 0.19***	1.2 ± 0.15***
	200	10.5 ± 0.22***	2.0 ± 0.07***	2.3 ± 0.24	5.5 ± 0.16**	2.6 ± 0.13**	4.6 ± 0.17***	1.2 ± 0.13***

M ± S.E = Mean ± Standard error. Six animals were maintained in each group. Duration: 50 days. $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with control.

Table 3. Micrometric and Leydig cell changes in the test is due to administration of fraction I and II of ethanol extract of *C. juncea* seeds in rats

Treatment	Dose (mg/kg)	Diameter of testis (μm)	Diameter of somniferous tubules (μm)	Diameter of Leydig cell nucleus (μm)	Mature Leydig cells	Degenerating Leydig cells
Control	Tween-80 (1%)	4985.0 ± 7.68.	250.0 ± 3.87	18.20 ± 1.19	73.10 ± 2.65	26.50 ± 2.21
Fraction I	100	3170.1 ± 17.08***	125.0 ± 3.16***	10.1 ± 1.49***	41.1 ± 3.06***	44.3 ± 3.08***
	200	2987.5 ± 6.97***	45.0 ± 4.08***	7.6 ± 0.66***	25.0 ± 1.86***	63.6 ± 3.31***
Fraction II	100	4895.3 ± 6.90**	237.1 ± 3.47***	16.6 ± 1.25	62.5 ± 2.01**	36.1 ± 3.47***
	200	4802.6 ± 8.59***	225.1 ± 3.54***	14.3 ± 1.92	53.1 ± 2.06***	39.3 ± 4.03***

M ± S.E.= Mean ± Standard error. Duration: 50 days. Six animals were maintained in each group. $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with control.

Table 4. Changes in the spermatogenic elements and spermatozoa due to administration of fraction I and II of ethanol extract of *C juncea* seeds in rats

Treatment	Dose (ms/Kg)	Spermatogonia	Spermatids	Round spermatids	Cauda epididymis	
					Sperm count (million/ml)	Sperm motility (%)
Control	Tween-80 (1%)	143.00 ± 3.45	233.10 ± 4.34	190.60 ± 3.66	71.83 ± 3.87	81.5 ± 3.85
Fraction I	100	75.0 ± 3.79***	65.1 ± 3.75***	96.6 ± 3.06***	21.1 ± 1.64***	17.8 ± 2.00***
	200	28.3 ± 2.14***	19.8 ± 1.66***	44.8 ± 3.21***	9.3 ± 1.08***	10.1 ± 1.49***
Fraction II	100	131.8 ± 3.13**	221.2 ± 4.22*	179.0 ± 3.54*	62.0 ± 2.20*	72.0 ± 2.53***
	200	122.0 ± 2.53***	209.6 ± 3.71***	161.5 ± 3.23***	59.5 ± 2.81**	66.1 ± 2.49***

M ± S.E.= Mean ± Standard error. Duration; 50 days. Six animals were maintained in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with control.

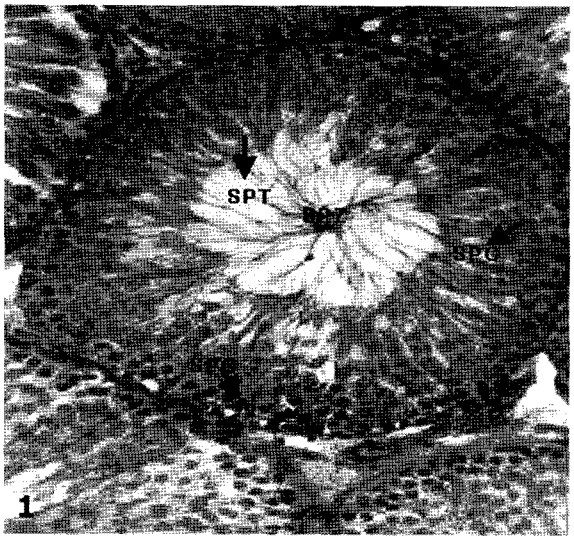

Fig. 1. Control rat showing normal seminiferous tubules with active spermatogenesis and normal Leydig cells (× 400). SPG, spermatogonia; SPC, spermatocytes; SPT, spermatids, SPZ, spermatozoa.

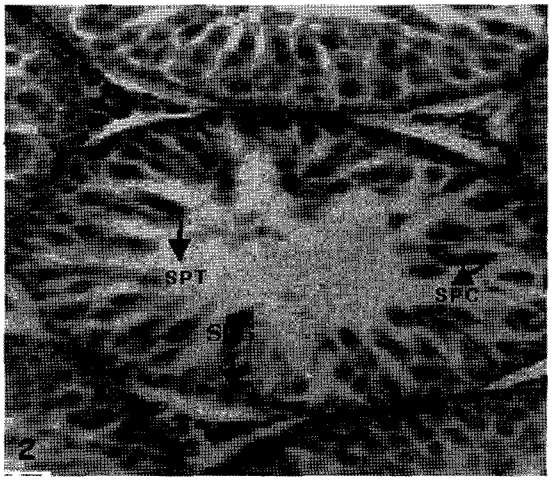

Fig. 2. Rat treated with fraction I 400 mg/kg. Note the shrinkage of seminiferous tubules, significant decrease in the number of spermatogonia, spermatocytes and spermatids and complete absence of spermatozoa. The germinal epithelium also showed degenerative changes (× 400). SPG, spermatogonia; SPC, spermatocytes; SPT, spermatids, SPZ, spermatozoa.

Table 5. Effect of fraction I of ethanol extract of *C. juncea* seeds on serum Testosterone, FSH and LH levels in rat.

Treatment (Dose)	Testosterone (ng/dl)	FSH (mIu/ml)	LH (mIu/ml)
Control (Tween-80, 1%)	30.60 ± 0.80	3.60 ± 0.05	3.80 ± 0.04
Fraction I (400 mg/kg)	14.60 ± 0.80***	1.20 ± 0.01***	1.40 ± 0.02***

M ± S.E. Mean ± Standard error. Six animals were maintained in each group. Duration of treatment: 50 days.
***$P < 0.001$ when compared with control.

The caudal sperm motility is decreased highly significantly ($P < 0.001$) with both the doses of fraction I and high dose of fraction II. But treatment of fraction I is less effective ($P < 0.05$) in reducing the cauda epididymal sperm motility (Table 4).

Changes in accessory reproductive organs

Gravimetric changes: The weight of caput epididymis is reduced significantly ($P < 0.001$) with both the doses of fraction I and high dose of fraction II. The effect of low dose of fraction II is nonsignificant. The administration of both the doses of fraction I and II has caused significant ($P < 0.001$) reduction in weight of cauda epididymis, vas deferens, seminal vesicle, ventral prostate, Cowper's gland and Levator Ani (Table 1).

Changes in serum Testosterone, FSH and LH levels (Table 5)

Testosterone: The weight of all accessory reproductive organs in male is dependent on androgens produced by the testis. The weight of accessory organs in all the different extract treated rats is reduced. This reduction is 2 to 3 folds more with high dose of fraction I of ethanol extract indicating the fraction I is highly effective. Therefore only high dose of fraction I treated group was subjected to find out the serum testosterone level. The serum testosterone level is reduced highly significantly ($P < 0.001$) from 30.6 ± 0.80 to 14.60 ± 0.80 with fraction I treated group when subjected to RIA.

FSH and LH: The level of FSH which is 3.6 ± 0.05 mIu/ml in control rats is reduced to 1.2 ± 0.01 mIu/ml ($P < 0.001$) and LH level which is 3.8 ± 0.04 mIu/ml in control is reduced to 1.4 ± 0.02 mIu/ml due to treatment of fraction I of ethanol extract of *C. juncea* seeds. As FSH is essential for maintaining the normal spermatogenic process and LH is for androgen production by Leydig cell in the testis. The observed reduction in the spermatogenic elements in the seminiferous tubules and serum level of testosterone is due to lower availability of FSH and LH.

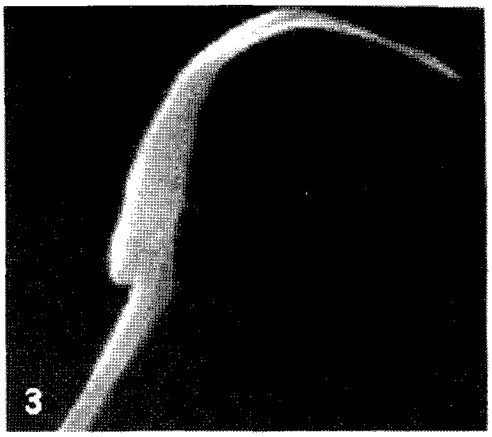

Fig. 3. Control rat showing normal head (× 1400).

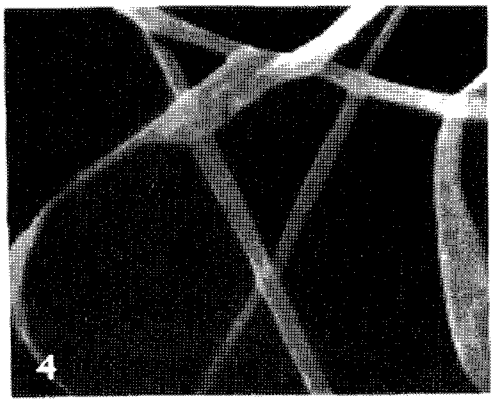

Fig. 4. Rat treated with ethanol extract of *C. juncea* seeds showing degeneration in head portion (× 1400).

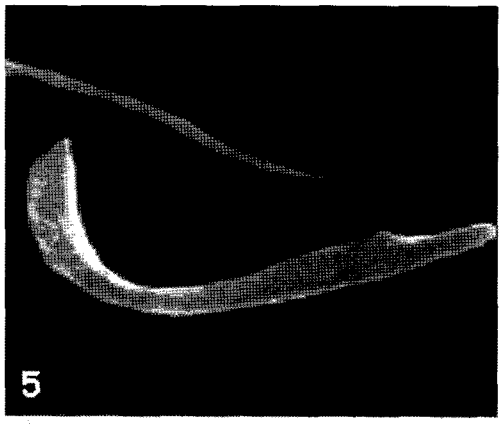

Fig. 5. Rat treated with ethanol extract of *C. juncea* seeds showing decapitation (× 1400).

Sperm morphology

The SEM study of morphology of spermatozoa of normal rat showed characteristic sickle shaped head; whereas that of fraction I treated rat exhibited deformed head. Many spermatozoa of cauda epididymis were decapitated (Fig. 3 - 5).

DISCUSSION

In the present investigation, the observed reduction in the weight of testis due to the treatment of fractions of *C. juncea* seed extracts may be due to the decreased production of seminiferous tubular fluid, which contributes to the weight of testis (Ghosh *et al.*, 1992). The reduced spermatogenic elements and protein content might have been added factors.

It is evident that pituitary FSH stimulates the conversion of spermatogonia to spermatocytes and also maintains the spermatogenic process (Connel and Eik-Nes, 1968; Johnson, 1971; Holt *et al.*, 1973; Dorrington and Armstrong, 1975). Both FSH and LH/ICSH are necessary for meiosis, formation and development of spermatids (Chemes *et al.*, 1979; Haneji *et al.*, 1984; Russel *et al.*, 1987; Hall, 1994). The observed reduction in the number of spermatogonia, spermatocytes and spermatids in the testis and cauda epididymal spermatozoa in the present study may be due to reduction in the availability and FSH and LH. This is further evidenced by the lowered FSH, LH and Testosterone levels estimated by radioimmunoassay technique.

It is known that sperm production cannot proceed optimally to completion without continuous androgen supply (Mohri *et al.*, 1978). However, the incidence of low sperm count and presence of nonmotile spermatozoa in the experimental rat may be due to the lowered availability of androgens, as testosterone level in these rats is significantly reduced.

The spermatozoa produced in the testis attain further development, motility and physiological maturation in the microenvironment of the epididymis (Gaddam and Glover, 1985; Sarkar, 1996). The decrease in the cauda epididymal sperm count may not be only due to decrease in the testicular spermatogenic process, but also due to altered microenvironment of the epididymis due to fraction I treatment.

Sertoli cells play an important role in germ cell maturation, but are highly susceptible to extraneous damage (Flickinger, 1981; Ritzn *et al.*, 1982; Pudney, 1986; Gerard *et al.*, 1994). The fractions of *C. juncea* seeds induced damage in the Sertoli cells. Thus, degeneration and maturational arrest of germ cells, i.e., spermatocytes and spermatids, could be due to the Sertoli cell damage (Pudney, 1986).

Atrophy of androgen target organs, rarefaction in stage specific spermatogenic cells and decrease of protein contents point out that *C. juncea* exhibited its antiandrogenic and antigonadotrophic effects in rats.

The glycogen content in the cell indicates energy storage. Sertoli cells and spermatogonia often contain glycogen and secrete substrates from the blood and provide source of reserve carbohydrates for seminiferous tubular cells, and the glycogen level is found to be directly proportional to the steroid hormone synthesis (Gregoire *et al.*, 1976). Therefore, the decrease in glycogen content of the testis after the administration of fractions of *C.*

juncea seed extracts may be due to reduced availability of gonadotrophins. Reduced glycogen level may be the reason for low protein content of the testis, because protein synthesis in spermatogenic cells is dependent on glucose (Dixit *et al.*, 1979). Gupta *et al.* (2000) have also observed reduced glycogen and protein levels in testis of *Barleria prionitis* root extract treated rats.

Ascorbic acid is present in the spermatogenic chamber and Leydig cells (Kirk, 1962). The reduction in the ascorbic acid in the present study might have caused oxidative damage to the spermatozoa (Latchoumycandane and Mathur, 1999). A decrease in the concentration of ascorbic acid in the testis may reflect a decrease in testicular steroidogenesis (Dabrawski and Ciereszko, 1996).

Sialic acid is a sialomucoprotein and essential for maintenance of the structural integrity of the sperm membrane and sperm maturation (Chinoy and Sequera, 1989; Chinoy *et al.*, 1994). Decreased number of spermatozoa or reduced androgen production may affect the level of sialic acid in the testis. The reduced sialic acid content might alter the structural integrity of acrosomal membrane which ultimately affects the metabolism, motility and fertilizing capacity of spermatozoa.

The fractions of *C. juncea* seed extract feeding caused impairment of Leydig cell function, which was evidenced by reduced Leydig cell area and nuclear dimensions and fewer number of matured Leydig cells. The atrophic state of Leydig cells in the testis of treated animals may be due to declined LH secretion (Nair *et al.*, 1995; Sarkar *et al.*, 1997). The number of mature Leydig cells has a direct bearing on spermatogenesis (Gupta *et al.*, 2000). Deformation of Leydig cells further indicates the inefficiency of these cells to synthesize testosterone (Reddy *et al.*, 1997).

Acid phosphatase, an enzyme of lysosomal origin is detectable in all germinal cells, and its specific activity increases with the development of spermatocytes (Males and Turkington, 1971). Activities of free lysosomal enzymes have been shown to rise when testicular steroidogenesis is increased (Mathur and Chattopadhyay, 1982). A decrease in the acid phosphatase in free state would thus reflects decreased testicular steroidogenesis in the treated rats and this may be correlated with the reduced secretion of gonadotrophins. Decrease in the alkaline phosphatase activity in the fractions of *C. juncea* seed extracts treated rats indicates that *C. juncea* treatment produce a state of decreased steroidogenesis where the inter- and intracellular transport was reduced as the metabolic reactions to channalize the necessary inputs for steroidogenesis have slowed down (Latchoumycandone *et al.*, 1997).

The initiation of spermatogenesis, recovery of secretary activity of accessory sex organs and other biochemical contents observed after withdrawal of treatment indicate that the effects of drug are transient (Vijaykumar, 2005).

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