Philippine Journal of Science
132 (1): 39-46, June 2003
ISSN 0031 - 7683

Antifertility Activity of Various Extracts of *Crotalaria juncea* Linn., Seeds in Male Mice

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Petroleum ether, benzene and ethanolic extracts of Crotalaria juncea Linn. seeds were administered intraperitoneally at the dose level of 25 mg/100 g body weight to albino male mice for 30 days. The results show decrease in the number of spermatogonia, spermatocytes and spermatids in testis along with reduced caudal spermatozoa. Biochemical observations indicate increased level of cholesterol and significant reduction in protein and glycogen content. The increased cholesterol content along with degeneration of Leydig cells indicate inhibited steroidogenesis. The decrease in the weight of accessory reproductive organs further attributes lowered availability of androgens due likely to inhibited steroidogenesis. Out of three extracts tested, ethanolic extract seems to be more potent in antispermatogenic and antisteroidogenic activities. When ethanolic extract was tested in immature mice for androgenic activity, it showed its antiandrogenic potency as the weight of accessory sex organs were reduced.

Plants have served as a natural source of antifertility substances. Henshaw (1953) listed many plants used by primitive people in different countries to control fertility. Though many indigenous plants have been shown to prevent births, only a few plants have so far been investigated for antispermatogenic activity (Kholkute, 1977; Segal, 1985; Rao, 1988; Murugavel and Akbarshah, 1991; Chatterjee et al, 1994; Raji and Bolarinwa, 1997; Madhusudan Reddy et al, 1997; Naseem et al, 1998; Purohit, 1999).

Crotalaria juncea Linn. (Papilionaceae), commonly known as Sunn hemp, is used Indian Ayurvedic Medicine states where in its various parts have properties like analgesic, astringent, emmenagogue, abortifacient and also treatment of skin diseases. It is also mentioned that the seeds are known for various medicinal properties (Kirtikar and Basu, 1935; Wealth of India, 1952; Chopra et al, 1956). The seeds of this plant have been reported to possess antifertility activity (Chaudhury, 1966; Bala and Garg, 1973). The alcoholic extract of *C. juncea* seeds has shown antiimplantation activity in mice (Prakash et al., 1993). In the present work an attempt has been made to find if the plant could be used as a male antifertility agent using albino mice as the experimental system.

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Materials and Methods

Plant material

Fresh seeds of *C. juncea* were collected from the fields of North Karnataka region, India, during the month of October and November 2001. A voucher specimen was deposited at the Herbarium of the Department of Botany, Gulbarga University, Gulbarga, India.

Preparation of the extract

The seeds were shade dried, powdered and subjected to soxhlet extraction using successively and separately nonpolar to polar solvents i.e., petroleum ether (B. P. 60-80° C), benzene and ethanol (95%). The decoction obtained each time was evaporated under

reduced pressure below 45°C. The dried mass was considered as the extract and individually screened for antifertility activity in mice. For administration to test animals the extracts were macerated in Tween-80 (1%) (S. D. Fine-Chem. Ltd. Mumbai), and resuspended in normal saline for their complete dissolution.

Animals

Adult, healthy, virgin Swiss strain male albino mice *(Mus musculus*) of 60-70 days old and weighing 35-40 g, selected from the inbred animal colony were used for the experiment. The animals were maintained under uniform husbandry conditions of light and temperature and given pellet diet as prescribed by Central Food and Technological Research Institute (CFTRI), Mysore, India and water *ad libitum*. The animals were divided into 4 groups of 6 each.

Treatment

After preliminary trials, 25mg/100g body weight dose was selected for evaluating the effect of the crude drug. The animals were divided into five groups consisting of six in each and treated intraperitonially everyday for 30 days as shown below.

Group I: Treated with 0.1 ml Tween-80 as vehicle.

Group II: Treated with 25mg/100g body weight petroleum ether extract in 0.1 ml Tween-80 (1%).

Group III: Treated with 25mg/100g body weight benzene extract in 0.1ml Tween-80 (1%).

Group IV: Treated with 25mg/100g body weight ethanol extract in 0.1ml Tween-80 (1%).

Antifertility testing

The control and drug treated animals were sacrificed on day 31 by cervical dislocation, 24h after the last treatment. The testes, epididymis, seminal vesicle, vas deferens, prostate gland, bulbourethral gland, and levator ani were excised, blotted free of blood, carefully made free from surrounding fat and connective tissue and weighed up to the nearest milligram on an electronic balance. Fresh tissues from testis, epididymis and vas deferens were processed for the estimation of glycogen (Carrol et al., 1956), protein (Lowry et al., 1951) and cholesterol (Peters and Van Slyke, 1946). The tissues were also fixed in Bouin's fluid and processed for histological preparation. These tissues were fixed in paraffin, sectioned at 5μ and stained with haematoxylin-eosin (Gurr, 1962). The micrometric measurements such as diameter of testis and somniferous tubules were calculated by the method described by Deb et al., (1964). Spermatogenic elements count (Abercrombie, 1946) was made from randomly chosen twenty round cross sections taken from the middle part of the testis. The cauda epididymal sperm suspension was prepared in normal saline and count of cauda epididymal spermatozoa of control and drug treated mice were determined by the method described by Kempinas and Lamano Carvalho (1987).

Androgenic/ antiandrogenic activity

Among the three extracts of *C. juncea* seeds, the ethanolic extract showed maximum antifertility activity. Therefore the ethanolic extract at the dose level of 25mg /100 g body weight was used alone to test androgenic /antiandrogenic activity. Swiss strain immature mice of 25 days old were injected intraperitoneally for 5 days as follows:

Group I- 0.1 ml Tween – 80 as vehicle (control).

Group II- 20μg / animal of testosterone in 0.1ml groundnut oil.

Group III- 25 mg / 100 g body weight of ethanol extract in 0.1 ml Tween-80.

All the three groups of animals were sacrificed on the 6th day by cervical dislocation. The testes, epididymis, seminal vesicle, vas deferens, prostate gland, bulbourethral gland and levator ani were excised, blotted free of blood and carefully made free from surrounding fat and connective tissue then weighed up to the nearest milligram on an electronic balance and adjudged for androgenic /antiandrogenic activity.

Statistical analysis

The data were statistically analyzed by using Student's 't' test (Snedecor and Cochran, 1974). 'P' values <0.05 were considered significant.

Results

Changes in the testis

Gravimetric and histometric changes (Table-1)

Highly significant (P<0.001) reduction in the weight of testis was observed due to the administration of ethanolic extract, whereas significant (P<0.01) reduction was observed after the administration of petroleum ether and benzene extracts. Histometric data were in parallel with weight, as reduction in the diameter of testis and seminiferous tubules were highly significant (P<0.001) with ethanolic extract treatment and significant (P<0.01) with petroleum ether and

benzene extract treatment.

Histological changes (Table 1., Figs. 1&2)

The number of spermatogonia, spermatocytes and spermatids are reduced highly significantly (P<0.001) due to the treatment of ethanolic extract of *C. juncea* seeds. Petroleum ether extract treatment reduced the number of spermatogonia and spermatocytes significantly (P<0.01) and that of spermatids nonsignificantly. Benzene extract showed highly significant (P<0.001), significant (P<0.01) and nonsignificant reduction in the number of spermatogonia, spermatocytes and spermatids, respectively. Pyknosis in the primary and secondary spermatocytes and degeneration of Leydig cells in the interstitium were observed. Spermatozoa were completely absent in the lumen of seminiferous tubules in all treated groups. Though sperms were available in the cauda epididymal preparation, their number was highly significantly (P<0.001) reduced.

Biochemical changes (Table 1)

The cholesterol content of the testis was increased highly significantly (P<0.001), significantly (P<0.01) and nonsignificantly with the respective treatment of ethanol, benzene and petroleum ether extracts as compared to that of control. Highly significant (P<0.001) reduction in the protein content of testis was observed in ethanol extract treated group but significant reduction in the petroleum ether and benzene extract treated groups. Whereas glycogen content was reduced highly significantly (P<0.001) after the administration of benzene and ethanol extracts, it was reduced nonsignificantly with the petroleum ether extract treatment.

Changes in accessory reproductive organs Epididymis (Tables 2&3)

The weight of epididymis was reduced highly significantly P<0.001), significantly (P<0.01) and nonsignificantly with respective treatments of ethanol, benzene and petroleum ether extracts. The cholesterol content of the epididymis was increased significantly (P<0.01) with the treatment of all three extracts of *C. juncea* seeds. The protein and glycogen content of epididymis was reduced highly significantly (P<0.001) with ethanol extract and significantly (P<0.01) with petroleum ether and benzene extracts.

Vas deferens (Tables 2&3)

Significant reduction in the weight of vas deferens (P<0.01) was observed in ethanol extract treatment, whereas change was non significant with petroleum ether and benzene extract treatment. The protein content of vas deferens was reduced significantly (P<0.01) after the administration of ethanolic extract, nonsignificantly with petroleum ether and benzene extracts. Though all three extracts of *C. juncea* seeds reduced the glycogen content of vas deferens, significant reduction (P<0.01) was only with that of ethanol extract.

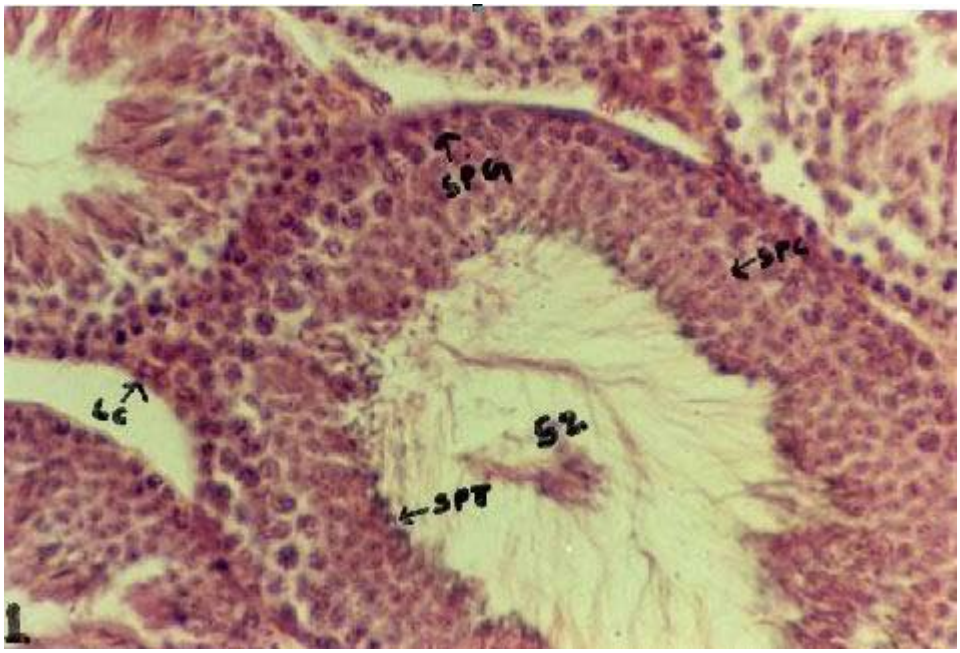

Figure 1. T.S. of control mice testis showing normal seminiferous tubules with all types of spermatogenic elements and spermatozoa in the lumen. Note the healthy Leydig cells x400.

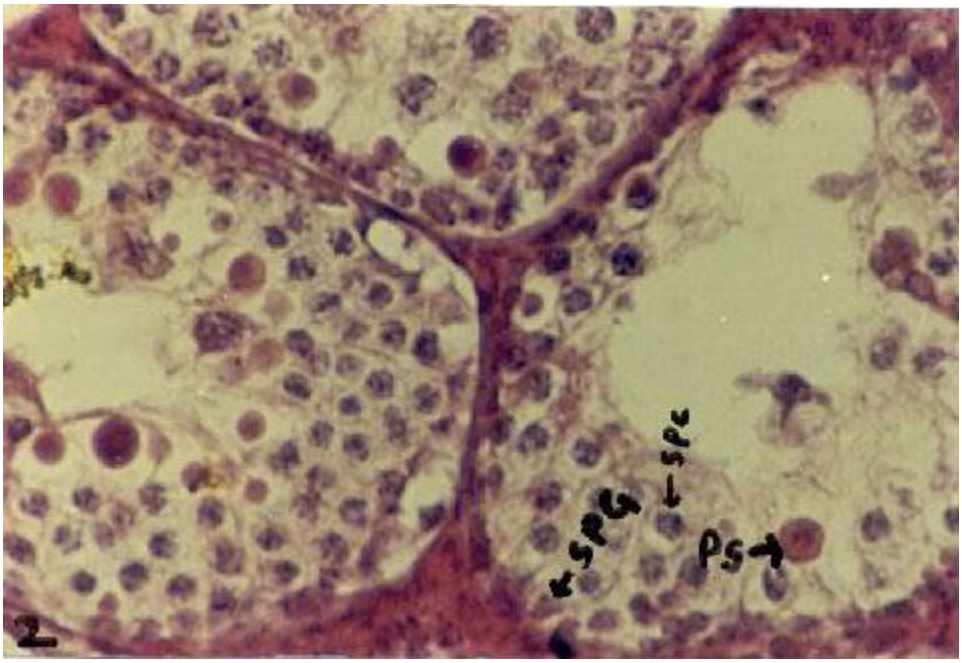

Figure 2. T.S. of testis of ethanolic extract treated mice showing shrinkage of seminiferous tubules, decreased interstitium and Leydig cells. Note the significant decrease in the spermatogonia, spermatocytes and spermatids and absence of spermatozoa. In spermatocytes nuclear pyknosis is seen x400.

Seminal vesicle and prostate gland (Table 2)

The weight of seminal vesicle showed highly significant (P<0.001) reduction with the ethanol extract and significant (P<0.01) reduction with both petroleum ether and benzene extract treatments. The weight of prostate gland (P<0.001) was reduced highly significantly with ethanolic extract, but nonsignificant by petroleum ether and benzene extract treatments.

Bulbourethral gland and levator ani (Table 2)

The weight of bulbourethral gland and levator ani showed highly significant (P<0.001) reduction in the benzene and ethanolic extract treated groups, and significant (P<0.01) reduction in petroleum ether extract treated group.

Androgenic/antiandrogenic activity (Table 4)

The administration of testosterone to immature mice caused highly significant (P<0.001) increase in wet weight of epididymis , vas deferens, seminal vesicle, prostate gland, bulbourethral gland and levator ani but nonsignificant weight increase in testis. Administration of ethanolic extract of *C. juncea* seeds at the dose of 25mg/100 g body weight decreased the wet weight of epididymis, vas deferens, seminal vesicle, prostate gland, bulbourethral gland and levator ani highly significantly (P<0.001), but nonsignificantly in the testis when compared to that of respective control.

Discussion

Administration of petroleum ether, benzene and ethanolic extract of *C. juncea* has caused reduction in the weight of testes. This reduction may be due to the decreased production of seminiferous tubular fluid, which contributes to the weight of testis (Ghosh et al., 1992). The reduced protein content may also be another reason as the growth rate of any organ is proportional to its protein content. Since evidently FSH stimulates the development of spermatogonia to spermatocytes and also maintains the spermatogenic process (Connel and Eik-Nes, 1968; Johnson and Ewing, 1971; Holt et al., 1973; Dorrington and Armstrong, 1975) and both FSH and LH/ICSH are necessary for meiosis and development of spermatids (Lostroch, 1963) as well as the androgens are necessary to induce meiosis, formation and development of spermatids in response to FSH (Chemes et al., 1979; Haneji et al., 1984; Russell et al., 1987; Hall, 1994), the observed reduction in the number of spermatogonia, spermatocytes and spermatids may indicate lowered availability of FSH, the hormones which are essential for initiation and maintenance of spermatogenesis. Also because of this reason seminiferous tubular lumen has became

Table 1. Changes in the testis due to administration of various extracts of *C. juncea* seeds.

Group	Treatment	Weight	Diameter (mm)	Diameter of seminiferous tubules (mm)	Spermatogenic elements			Sperm count (million/ml)	Cholesterol (mg/mg)	Protein (mg/mg)	Glycogen (mg/mg)
					Spermatogonia	Spermatocytes	Spermatids				
I	Control	725	278.4	79.8	66.4	58.4	56.8	20.5	2.32	1.36	2.73
		±8.98	±2.73	±2.29	±2.40	±0.68	±0.58	±0.22	±9.15	±0.09	±0.14
II	Petroleum ether	689.7*	247.2*	71.6*	45.0*	38.0*	42.2	11.5**	2.64	0.86*	1.62
		±1.32	±2.24	±1.64	±0.24	±0.58	±0.37	±0.22	±0.09	±0.02	±0.75
III	Benzene	682.0*	238.2*	70.1*	44.2**	36.8*	40.2	8.7**	3.08*	0.84*	1.58**
		±0.95	±2.62	±2.75	±0.37	±0.44	±0.36	±0.20	±0.14	±0.02	±0.03
IV	Ethanol	644.4**	228.2**	58.0**	35.4**	30.8**	20.6**	3.4**	3.63**	0.82**	1.56**
		±0.20	±2.60	±1.58	±0.23	±0.12	±0.51	±0.23	±0.01	±0.03	±0.04

Dose, 25 mg/100g body weight; duration, 30 days; organ weight, mg/100g body weight.
Values are mean ± S.E.
Six animals were maintained in each group
*<0.01, **P<0.001, when compared with control.

Table 2. Changes in the weight of accessory reproductive organs due to administration of various extracts of *C. juncea* seeds.

Group	Treatment	Epididymis	Vas deferens	Seminal vesicle	Prostate gland	Bulbourethral gland	Levator ani
I	Control	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
II	Petroleum ether	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
III	Benzene	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
IV	Ethanol	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Dose, 25 mg/100g body weight; duration, 30 days; organ weight, mg/100g body weight.
Values are mean ± S.E.
Six animals were maintained in each group
*<0.01, **P<0.001, when compared with control.

Table 3. Biochemical changes in epididymis and vas deferans due to the administration of various extracts of *C. juncea* seeds.

Group	Treatment	Epididymis			Vas deferens	
		Cholesterol (mg/mg)	Protein (mg/mg)	Glycogen (mg/mg)	Protein (mg/mg)	Glycogen (mg/mg)
I	Control	1.76 ±0.24	1.28 ±0.15	1.54 ±0.14	1.01 ±0.09	2.37 ±0.26
II	Petroleum ether	2.36* ±0.20	0.47* ±0.01	0.76* ±0.02	0.76 ±0.04	1.72 ±0.02
III	Benzene	2.08* ±0.15	0.48* ±0.09	0.74* ±0.03	0.7 ±0.03	1.68 ±0.03
IV	Ethanol	2.43* ±0.01	0.46** ±0.02	0.73** ±0.01	0.68* ±0.03	1.66* ±0.22

Dose, 25 mg/100g body weight; duration, 30 days
Values are mean ± S.E.
Six animals were maintained in each group
*<0.01, **P<0.001, when compared with control.

Table 4. Effect of ethanolic extract of C. juncea seeds on the weights of male reproductive organs of immature male mice.

Treatment	Testis	Epididymis	Vas deferens	Seminal vesicle	Prostate gland	Bulbourethral gland	Levator ani
0.1ml Tween – 80	623.6 ± 1.80	137.8 ± 1.20	26.4 ±0.51	211 ±0.36	62.4 ±0.51	68.4 ±0.24	74.7 ±0.18
20 µg testosterone	635.6** ±1.29	208.2** ±0.91	31.6** ±0.51	312.0** ±0.70	96.2** ±0.52	101.4** ±0.24	99.2** ±0.10
25 mg ethanol extract	619.5 ±0.22	113.9** ±0.24	18.2** ±0.37	170.4** ±0.24	41.5** ±0.22	44.5** ±0.22	51.0** ±0.27

Dose, 25 mg/100g body weight; duration, 30 days; organ weight, mg/100g body weight.
Values are mean ± S.E.
Six animals were maintained in each group
*<0.01, **P<0.001, when compared with control.

devoid of spermatozoa. Though spermatozoa are observed in the cauda epididymis the number is significantly lowered. Presence of spermatozoa in the cauda epididymis, may have been contributed by their production before the experimentation.

The glycogen content in cells indicates energy storage. Sertoli cells and spermatogonia often contain glycogen, secrete substrates from the blood and provide source of reserve carbohydrates for seminiferous tubular cells, and the glycogen level has been found to be directly proportional to the steroid hormones (Gregoire et al., 1967). The decreased glycogen content of the testis after the administration of *C. juncea* seed extracts might be correlated with decreased spermatogenic number due to reduced energy source for spermatogenic activity.

The increased cholesterol content of testis after the administration of *C. juncea* seed extracts indicates reduced conversion of cholesterol to androgens which is dependent on the availability of LH/ ICSH (Catt et al., 1974; Rommerts et al., 1974; Hall, 1994). The reduced or non-availability of androgens is further supported by the reduction in the weight of accessory organs like epididymis, seminal vesicle, vas deferens, prostate gland, bulbourethral gland and levator ani. All these organs play important roles in the maturation and mobility of the sperm and formation of semen (Orgebin-Crist, 1969; Hamilton, 1975). Moreover, testosterone plays a pivotal role in sexual maturation, behavior and maintenance of accessory sex organs (Mann and Ludwak- Mann, 1981; Jean–Faucher et al., 1984; Luke and Coffey, 1994; Ojeda and Urbanski, 1994). As the administration of seed extracts has caused reduction in the spermatogenesis, steroidogenesis and androgen production, it may alter the sexual behavior and may cause antifertility. Out of the three extracts tested, ethanolic extract is more effective in causing antispermatogenic and antisteroidogenic activities. The ethanolic extract when tested in immature mice has shown antiandrogenic effects. This antiandrogenic effect may also add to the antifertility potency of the seed extract.

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