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Inhibition of spermatogenesis by Triterpenes of *Albizia lebbeck* (L.) Benth pods in male albino rats.

Rakesh Chaudhary[1]*, R. S. Gupta[1] , J. B. S. Kachhaw[1], Dharmendra Singh[1], S. K. Verma[2]

1. Reproductive Physiology Section, Department of Zoology, University of Rajasthan, Jaipur - 302 004. India
2. Department of Chemistry, University of Rajasthan, Jaipur - 302 004. India

Abstract

Aim : To evaluate the antifertility activity of triterpenes isolated from *Albizia lebbeck* (L.) Benth pods in male albino rats. Methods : Oral administration of triterpenes isolated from *Albizia lebbeck* pods at the dose level of 50 mg/rat/day for 60 days. Testicular sperm count, epididymal sperm count and motility were assessed. Biochemical and histological analysis were performed in blood samples and reproductive organs. Results : Oral administration of triterpenes did not cause any significant change in the body weights but a significant reduction in the weight of reproductive organs i.e. testis, epididymides, seminal vesicle and ventral prostate were observed. Testicular sperm count, epididymal sperm count and motility were significantly reduced ($p<0.001$), when compared to controls. Arrest of spermatogenesis was noted as various stages of spermatogenesis showed depression. Production of primary spermatocytes (preleptotene and pachytene), secondary spermatocytes and step-19 spermatids declined by 57.80%, 57.46%, 58.14% and 55.89% respectively. The size of seminiferous tubules reduced by 20.09%. Cross sectional surface area of Sertoli cell as well as its counts were found to be reduced significantly ($p<0.001$). Leydig cell nuclear area and number of mature Leydig cells decreased by 51.66% and 52.48% respectively. Serum testosterone level showed significant reduction after triterpenes feeding ($p<0.001$). Biochemical parameters of tissues i.e. protein, sialic acid, glycogen, cholesterol content of testis and seminal vesicular fructose showed significant reduction ($p<0.001$). There were no significant changes in RBC and WBC count, haemoglobin, haematocrit, blood glucose, cholesterol, protein, triglycerides, phospolipids and HDL-cholesterol. Conclusion : Oral administration of triterpenes isolated from *Albizia lebbeck* pods causes spermatogenesis arrest in male albino rats.

Key Words : Albizia lebbeck, triterpenes, sperm motility, primary spermatocytes, Sertoli cells, Leydig cells.

1. Introduction

A number of plants have been identified and evaluated by various researchers for fertility regulation in males [1, 2, 3, 4]. The present study is influencing fertility patterns to investigate substances of plant origin. *Albizia lebbeck* (L.) Benth (Mimosoideae) is commonly called Indian Siris of East Indian Walnut. It is indigenous to South-East Asia and Australia. The tree is used in folk remedies for abdominal tumours, boils, cough, eye ailments, flu and lung ailments. It is also reported to be astringent, pectoral, rejuvenant and tonic [5]. The seed oil is used for leprosy and the powdered seed in scrofulous swellings. Indians

* Corresponding author
Email : chaudharybabloo_77@yahoo.co.in

use the flowers for spermatorrhea [6]. The ethanolic extracts of *Albizia lebback* leaves exhibited anticonvulsant activity [7]. Saponins of *Albizia lebbeck* leaves are reported to be nootropic and anxiolytic activity in albino mice [8]. *Albzia julibrissin* Durazz is reported to have sedative activity [9]. Anti-fertility effects of various triterpenes isolated from various medicinal plants have also been reported [10, 11, 12]. There is no documented evidence referring to male anti-fertility activity of triterpenes of *Albizia lebbeck*. It was therefore of interest to investigate the male anti-fertility activity of the plant.

2. Materials and Methods

2.1 Animal Model

Colony-bred, healthy adult (4-5 months old) male albino rats (Rattus norvegicus) of the Wistar strain, weighing between 150 and 200 g were used. The animals were housed in polypropylene cages, measuring 430 x 270 x 150 mm, under controlled enviornmental conditions with provision 12 h light : 12 h dark regimen. The animals were fed a standard rat chow supplemented with soaked gram and wheat. Water was provided *ad libitum*.

2.2 Test Material

The pods of *Albizia lebbeck* were collected in the month of may, 2003 from campus of University of Rajasthan, Jaipur. Plant material was identified (Voucher no – RUBL 19894) and authenticated by Dr. N.J. Sarana, Associate Professor, Department of Botany, University of Rajasthan, Jaipur-302 004, India.

2.3 Extraction of the Plant Material

The pods of *Albizia lebbeck* were shade dried and ground to powder. 2.5 kg of powdered bark was extracted with methanol for approximately (48-50 h). The extract was concentrated under reduced pressure that yielded 42 g (16.80%) of dark brown semi solid-mass. This mass was washed with petroleum ether (40-60°C) to remove the fatty components. This fat free part of the extract was dried, approximately 36 g mass was finaly obtained which was subjected to silica gel (60-120 mesh) column chromatography. For this purpose a column with a height of 1.5 meter with diameter of 3.5 cm with 800 g silica gel (60-120 mesh) was taken. The column was eluted with various solvents in increasing order of their polarity.

In $CHCl_3$: CH_3OH (1 : 1) three triterpenes – Vitalboside-A, Lupeol and acacic acid lactone, were isolated from the pods of *Albizia lebbeck*. The yield of these three compounds were 5.32 g out of 36 g crude methanolic extract (14.77%). Their structures were established through spectral analysis [13].

2.4 Study protocol

Male rats of proven fertility were divided into two groups of 10 each. One group was treated with *Albizia lebbeck* pods fraction (50 mg/rat/day) for 60 days. The control group received vehicle (distilled water 0.5 ml/day) for 60 days. On day 61, animals were sacrificed under ether anaesthesia testis, epididymides, seminal vesicle, ventral prostate, liver and adrenal glands were removed, cleared off fat and connective tissues and weighed.

2.5 Fertility test

The mating tests were performed from day 55 to day 60 (6 days) and also before commencement of the treatment. The male rats were cohabited with proestrous females at a ratio of 1 : 3. The presence of vaginal plug and sperm in the vaginal smear the next morning were considered for positive matings. The mated females were separated to note the implantation site on day 16 of pregnancy through laparotomy.

2.6 Sperm motility and density

The motility of cauda epididymal spermatozoa was recorded. Percentage of motile sperms was calculated per unit area. Cauda epididymal and testicular sperm counts were assessed using Neubaur's counting chamber of hemocytometer.

2.6 Tissue Biochemistry

Testicular tissues were assayed for protein, sialic acid, glycogen and cholesterol. Fructose in seminal vesicle was estimated.

2.7 Blood and serum biochemistry

Blood was collected from heart. The blood was analyzed for RBC, WBC count, haemoglobin, haematocrit and blood sugar. Serum protein, cholesterol, triglycerides, phospholipids and HDL-cholesterol were estimated.

2.8 Hormonal Assay

Serum testosterone levels were assayed from samples using radio immuno assay method [14]. The sensitivity of the assay was 10 pg/ml and intra assay error was 4.5%.

2.9 Histologiocal preparation

Tissues were fixed in Bouin's fluid. Paraffin sections were made and stained with hematoxylin and eosin or Periodic Acid Shiff reagent (PAS) for the discrimination of the stages of spermatogenesis [15].

2.10 Cell Population Dynamics

Testicular cell population dynamics was performed by using Camera lucida drawings. Germinal cell population i.e. Sertoli cells, spermatogonia, primary spermatocytes (preleptotene and pachytene), secondary spermatocytes, rounded spermatids and step-19 spermatids were counted. Testicular cell counts were based on the calculation made for each cell type per cross tubular section. Atleast 20 round tubular cross sections were counted for each stage of spermatogenesis. These crude counts were corrected by using Abercrombie's correcting factor [16]. Interstitial cell types such as mature, degenerating and fibroblast Leydig cells were estimated applying a differential count which were statistically verified by the binomial distribution. Mean seminiferous tubular diameters were determined by measuring and tracing an average of 100 selected seminiferous tubules.

2.11 Ethical Aspects

The study was approved by the ethical committee of the department of Zoology, University of Rajasthan, Jaipur-302004 (India). Indian National Science Academy guidelines were followed for maintenance and use of experimental animals [17].

2.12 Statistical calculation

All the values of body and organs weights, biochemical estimation, histometry and testicular dynamics were expressed in terms of mean ± standard error. The treated groups were compared to controls using the Student's "*t*" test.

3. Results

3.1 Weight response

The oral administration of *Albizia lebbeck* pods fraction (50 mg/rat/day) for 60 days did not cause any significant change in the body weights of treated rats but the weights of testis ($p<0.001$), epididymides ($p<0.001$), seminal vesicle ($p<0.001$) and ventral prostate ($p<0.001$) were reduced in a significant manner. (Table-1)

3.2 Sperm concentration, motility and fertility

Administration of *Albizia lebbeck* pods fraction significantly reduced sperm count of testis and epididymides ($p<0.001$). The motility of the cauda epididymal sperm was also reduced significantly ($p<0.001$). The extract reduced the fertility of male rats by 100%. (Table-1)

Table 1. Effect of triterpenes of *A. lebbeck* pods on the body weight, organ weights, sperm motility, density, fertility and serum testosterone in male rats.

Treatment	**Body weight (g)**	Organ weight (mg/100 g b.wt.)				Sperm motility % (Cauda epididymides)	Sperm density (million/ml)		Fertility %		**Serum testosterone (ng/ml)**
		Testis	**Epididymides**	**Seminal vesicle**	**Ventral prostate**		**Testis**	**Cauda epididymides**	**Pre Fertility test**	**Post fertility test**	
Control	218.20 ± 7.39	1324.04 ± 10.04	495.06 ±7.49	638.86 ± 4.99	395.45 ± 3.98	72.15 ± 1.39	11.17 ± 1.17	68.34 ± 4.17	100 (+) ve	100 (+) ve	3.39 ± 0.02
A. lebbeck 50 mg/rat/d	220.15 ± 7.91^{ns}	895.32 *p* 12.76**	325.25 *p* 10.45**	492.32 *p* 13.42**	204.79 *p* 2.87**	18.05 ± 1.09**	1.99 *p* 0.48**	9.45 *p* 0.77**	100 (+) ve	100 (+) ve	2.04 ± 0.01**

Values are mean ± SEM (n = 10)
ns = non significant; ** p<0.001 vs Control

Table 2. Effect of triterpenes of *A. lebbeck* pods on biochemical parameters of male rats.

Treatment	Protein (mg/g)				Sialic acid (mg/g)				Glycogen **(mg/g)**	Cholesterol **(mg/g)**	Fructose **(mg/g)**
	Testis	**Cauda epididymides**	**Seminal vesicle**	**Ventral prostate**	**Testis**	**Cauda epididymides**	**Seminal vesicle**	**Ventral Prostate**	**Testis**	**Testis**	**Seminal Vesicle**
Control	232.15 ± 6.24	261.42 ± 6.34	208.13 ± 4.85	199.54 ± 3.17	5.49 ± 0.12	6.24 ± 0.13	5.21 ± 0.09	5.48 ± 0.17	2.81 ± 0.12	13.05 ± 0.54	5.67 ± 6.24
A. lebbeck 50 mg/rat/d	188.63 ± 4.97**	199.54 ± 5.21**	179.12 ± 3.53**	155.52 ± 2.98**	3.72 ± 0.09**	3.97 ± 0.08**	3.71 ± 0.07**	3.82 ± 0.12**	1.77 ± 0.07**	6.03 ± 0.34**	4.10 ± 0.13**

Values are mean ± SEM (n = 10)
** p<0.001 vs Control

Table 3. Effect of triterpenes of *A. lebbeck* pods in serum and bloods in rats.

Treatment	Protein	Cholesterol	Triglycerides	Phospholipid	HDL Cholesterol	Blood Sugar	RBC (million/mm^3)	WBC (-/mm^3)	Haematocrit (%)	Haemoglobin (g%)
	(mg/dl)									
Control	13333.32 ± 135.93	104.13 ± 11.05	110.32 ± 7.39	113.47 ± 5.67	50.04 ± 3.19	90.34 ± 4.12	5.26 ± 0.19	72.90 ± 54.32	36.43 ± 1.84	14.73 ± 0.47
A. lebbeck 50 mg/rat/d	13111.11 ± 120.03^{ns}	107.45 ± 10.17^{ns}	112.42 ± 6.99^{ns}	111.13 ± 6.03^{ns}	53.12 ± 2.84^{ns}	92.42 ± 3.97^{ns}	5.17 ± 0.12^{ns}	71.72 ± 49.34^{ns}	35.93 ± 1.72^{ns}	14.20 ± 0.51^{ns}

Values are mean ± SEM (n = 10)

ns = non significant; ** p<0.001 vs Control

Table 4. Effect of triterpenes of *A. lebbeck* pods on Testicular Cell Population Dynamics

Treatment	Testicular Cell Counts (Number/10 Cross - Section)							Leydig cell				Sertoli cell are (μm^2)	Semini ferus tubule diameter (μm^2)
	Sertoli cell	Sperma togonia	Prelepto-tene	Pachy tene	Secon dary sperma tocyte	Roun ded sperm atid	Step-19 sperm atid	Nuclear are (μm^2)	Differential counts(%)				
									Fibro blast	Degen rating	Mat ure		
Control	2.93 ± 0.05	6.84 ± 0.71	21.52 ± 2.10	33.27 ± 3.31	62.84 ± 5.82	25.24 ± 2.11	27.89 ± 0.03	18.02 ± 0.92	22.17 ± 1.19	21.15 ± 1.17	56.68 ± 2.11	43.49 2.05	253.34 4.36
A. lebbeck 50 mg/rat/d	2.09 ± 0.04**	3.52 ± 0.49**	9.03 ± 0.59**	14.15 ± 0.92**	26.30 ± 2.04**	7.39 ± 0.58**	12.30 ± 0.67**	8.71 ± 0.57**	42.13 ± 1.49**	31.94 ± 2.39**	25.93 ± 1.89**	23.41 ± 1.97**	202.43 ± 3.07**

Values are mean ± SEM (n = 10)

** p<0.001 vs Control

3.3 Tissue biochemistry

Protein contents of testis ($p<0.001$), cauda epididymides ($p<0.001$), seminal vesicle ($p<0.001$) and ventral prostate ($p<0.001$) were reduced significantly. Content of sialic acid showed significant decrease in testis ($p<0.001$), cauda epididymides ($p<0.001$), seminal vesicle ($p<0.001$) and ventral prostate ($p<0.001$). Testicular glycogen and testicular cholesterol showed significant reduction ($p<0.001$). Fructose level in the seminal vesicle was also reduced significantly ($p<0.001$). (Table-2)

3.4 Blood and serum biochemistry

RBC, WBC, haemoglobin, haematocrit and blood sugar were found to be with in the normal range. Serum protein, cholesterol, triglycerides, phospholipids and HDL-cholesterol were within normal range throughout the study period. (Table-3)

3.5 Hormonal Assay

Serum testosterone level of *A. lebbeck* pods fraction treated animals was decreased significantly ($p<0.001$) in comparison to controls. (Table-1)

3.6 Cell population dynamics

Administration of *A. lebbeck* pods fraction resulted in significant reduction in most of the cell types in seminiferous tubules. Total counts of spermatogonia, primary spermatocytes (preleptotene and pachytene), secondary spermatocytes and rounded spermatids declined by 48.53%, 57.80%, 57.46%, 58.14% and 70.72% respectively. The number of mature Leydig cell declined by 51.66% and Leydig cell nuclear area was also decreased significantly. Seminiferous tubular diameter and Sertoli cell area as well as its counts were also significantly reduced. (Table-4++)

Discussion

The result showed that triterpenes of *A. lebbeck* pods significantly affected male reproductive functions. It is a well known fact that the presence of -OH group and unsaturation in triterpenes enhances its antifertility activity [18,19]. Statistically significant reduction in weights of the testis and accessory sex organs of the albino rats following ingestion of the triterpenes of *Albizia lebbeck* pods again indicates low level of androgen which was not enough to maintain the weight of the gonads and accessories [20, 21, 22], as observed in the present study also. The ability of the triterpenes–fed rats to mate, was due to low levels of circulating plasma testosterone, which was probably sufficient for normal mating behavior but was insufficient for the maintenance of fertilizing ability of the epididymal spermatozoa [23, 24]. All these factors brought about functional sterility in the triterpenes-fed rats.

Triterpenes of *A. lebbeck* pods might possibly inhibit the activity of adenosine triphasphate (ATP) in the spermatozoa by uncoupling of oxidative phosphorylation from the respiratory chain and prevent phsophorylation of adenosine diphosphate to adenosine triphosphate and thus, renders the spermatozoa immobile [25] and thus, renders spermatozoa immobile.

In the present investigation, protein content of testis decreased significantly ($p<0.001$) after treatment with triterpenes of *A. lebbeck* pods, probably due to the absence of the stages of spermatogenesis in seminiferous tubules [26]. The term sialic acid refers to a group of lipids, proteins or polysaccharides and forming glycolipids, glucoproteins and glucopolysaccharides. The concentration of sialic acid is regulated by androgen. Triterpenes of *A. lebbeck* significantly reduced the sialic acid content of the testis due to inhibition of

spermatogenesis and suppressed Leydig cell funtion [27]. The decrease in fructose level in the seminal vesicle of drug treated animal was another important observation. Because the function of fructose in seminal plasma is to induce the glycolytic metabolism of spermatozoa, it can be suggested that the depletion of fructose content due to triterpenes treatment hampers the glycolytic metabolism of spermatozoa. This resulted in abnormal sperm functions which ultimately gave rise to complete male sterility.

Leydig cells influence the seminiferous tubules by maintaining a high concentration of testosterone in the peritubular compartments of the testis [28]. A reduction in the seminiferous tubule diameter along with alterations in the number of different germ cells probably correspond to the decrease in testosterone production and/or inhibition of pituitary gonadotropin secretion, disrupting spermatogenesis [29]. This is further confirmed by low serum levels of testosterone. Sertoli cells play an important role in germ cell maturation, but are highly susceptible to extraneous damage [30, 31]. The triterpenes of *A. lebbeck* pods induced cytoplasmic morphological damages in the sertoli cells. Thus, degeneration and maturational arrest of germ cell i.e. primary spermatocytes, secondary spermatocytes and spermatids could be due to the sertoli cell damage [4].

In conclusion , the triterpenes of *A. lebbeck* pods brought about the infertile state in male rats due to interference in the testicular androgen levels altering the process of spermatogenesis.

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