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Original Research Article

Evaluation of antispermatogenic and antifertility properties of *Terminalia chebula* (Retz.) in albino mice

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ABSTRACT

Background: The development of an orally active, safe, reversible, and effective male contraceptive of plant origin has always been a matter of great interest among researchers due to its ready availability, cost-effectiveness, and most importantly protection of privacy. The present study was conducted to evaluate the effect of oral feeding of *Terminalia chebula* Retz. (*T. chebula*; family: Combretaceae) on male reproductive organs and fertility.
Methods: The albino mice were administered orally acetone, methanol, 50% ethanol, and aqueous bark extracts of *T. chebula* (300 mg/kg body weight daily) for 35 days, and the effect of the treatments on testis, epididymis, seminal vesicle, sperm parameters, biochemical, and fertility indices was investigated. Toxicological studies were also carried out.
Results: Treatment with Terminalia extracts brought non-uniform but detectable histologic alterations in the testis, epididymis, and seminal vesicle; the alterations caused in the reproductive organs were, however, severe in mice treated with the aqueous extract of Terminalia compared to those in other treated groups and controls. Further, the level of fructose in the seminal vesicle and that of sialic acid in the epididymis reduced significantly in the above treated mice. Sperm parameters were adversely affected in extracts-treated mice. Libido was not affected, but fertility reduced significantly in aqueous extract-treated males as compared to controls. Further, histoarchitecture of the liver and kidney, serum levels of ALT, AST, creatinine, and haematological parameters remained unaltered in Terminalia-treated mice compared to controls.
Conclusions: The results of the present study, therefore, suggested that the aqueous bark extract of *T. chebula* causes suppression of spermatogenesis and fertility in albino mice, and therefore, might be valuable in male fertility regulation.

Keywords: Creatinine, Fertility, Libido, Terminalia, Testis

INTRODUCTION

The development of an orally active, safe, reversible, and effective male contraceptive has always been a matter of great interest among researchers. As far as control of male fertility is concerned, none of the currently available approaches fulfill the requirements of an ideal male contraceptive. In India, as well as across the world, plants and plant preparations have been used frequently for fertility regulation in humans; this may be because of their ready availability, cost-effectiveness, and most importantly protection of privacy.[1-2] Further, a plant-based male contraceptive is generally considered to be free from unwanted side effects. Therefore, there is a growing interest in developing a plant-based male contraceptive across the world.

Terminalia chebula (*T. chebula*) Retz. (family: Combretaceae) is a flowering evergreen tree native to India and Southeast Asia. It carries several local names such as Black Myrobalan (English), Haritaki (Sanskrit and Bengali), Harad (Hindi), Karkchettu (Telugu), Kadukkaya

(Tamil), and Harada (Marathi and Gujarati) *etc*. It is used as a popular folk medicine in homeostatic, antitussive, laxative, diuretic, and cardio-tonic treatments, and has become a cynosure of modern medicines.[3-6] Further, this plant has been studied extensively for a variety of biological properties like antibacterial, antifungal, antiviral, anticancer, antioxidant, antidiabetic activities.[7-19] In addition, neuroprotective, hepatoprotective, cardioprotective, cytoprotective, radioprotective and immunomodulatory effects of *T. chebula* have also been reported.[20-25] The available literature on the effect of *T. chebula* on the male reproductive organs and fertility is limited to a few reports and preliminary findings in our laboratory.[26-28] The present study was, therefore, undertaken to investigate the possible effects of crude extracts from the bark of *T. chebula* on reproductive organs and fertility in the male mouse. Various male reproductive endpoints such as histopathology, sperm parameters, biochemical analyses, and fertility indices were assessed; toxicological studies were also performed.

METHODS

Study type and study place

This was a randomized controlled trial study that was conducted in the Department of Zoology, at K. N. Government P. G. College, Gyanpur, Bhadohi (India) during the period from November 2021 to December 2022.

Selection, identification, and preparation of plant extract

The bark of *T. chebula* was selected for the trial study since it has not been investigated earlier for its antifertility potential in males as revealed after a thorough literature survey. The sample was collected from the campus of Banaras Hindu University (B.H.U.), Varanasi in June 2021, after proper taxonomic identification (Voucher specimen no. Combreta. 2023/03) of the plant by Professor N. K. Dubey at Department of Botany, B.H.U., Varanasi (India). Plant extracts were prepared strictly according to WHO protocol.[29] In brief, the bark of the plant was washed properly with distilled water, shade-dried for one week, and then ground into fine powder with an electric grinder. The bark powder (100 gm) of *T. chebula* was subjected to extraction each with acetone, methanol, 50% ethanol, and aqueous (distilled water) respectively (1000 ml, w/v 1:10) in a soxhlet apparatus for 12 hours. The filtrates, obtained by using different solvents, were evaporated to dryness and lyophilized to get brownish-blackish extracts. All the extracts were stored at 4^0C. The lyophilized extracts were suspended in sterile distilled water, and the doses were expressed as the dry weight of the extract.

Ethical approval and animal treatments

A total of 36 adult (age 12-14 weeks; weight 28-38 gm) male laboratory mice of proven fertility belonging to the Parkes (P) strain were used in the investigation after ethical approval of the Institutional Animal Ethics Committee of Mahatma Gandhi Kashi Vidyapith, Varanasi (India). Animals were obtained from a randomly bred colony maintained in our animal room under standard conditions (temperature 23±2 ^{0}C, photoperiod 12 hours, and relative humidity 50±20% with proper ventilation) in polypropylene cages (450 mm X 270 mm X 150 mm) having dry rice husk as the bedding material, following the guidelines of CPCSEA, New Delhi.[30] Animals were given standard pellet feed (Mona Laboratory Animal Feeds, Varanasi) and fresh drinking tap water *ad libitum*. Animals were randomly allocated into six (I-VI) groups, each comprising six animals.

Mice in experimental groups (III-VI) were treated with extracts (300 mg/kg body weight/day) of *T. chebula* for 35 days and were sacrificed 24 hours after the last treatment. The dose and duration for the treatment with *T. chebula* were determined based on a series of preliminary studies conducted on albino mice in our laboratory. Bark extracts were suspended in sterile distilled water and administered orally, using separate and properly sterilized oral feeding needles. Mice in Group II (vehicle control) received only sterile distilled water (5.0 ml/kg body weight).

Table 1: Experimental design.

Groups	Treatments	Duration (Days)	Autopsy (after last treatment
I	Untreated controls	35	24 hours
II	Distilled water-treated controls	35	24 hours
III	Acetone bark extract	35	24 hours
IV	Methanol bark extract	35	24 hours
V	50% Ethanol bark extract	35	24 hours
VI	Aqueous bark extract	35	24 hours

Animal autopsy and sample collection

On day 36, animals in groups I-VI were sacrificed together by decapitation after recording their final body weights. Trunk blood was collected, and serum was separated and stored at -20^0C for determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine levels. Haematological tests were performed with fresh blood. The testis, epididymis, vas deferens, seminal vesicle, brain, liver, kidney, adrenal gland, and spleen were excised, cleared off fat and connective tissue, and weighed. The epididymes and seminal vesicles from five animals in each group were stored at -20^0C for assays of sialic acid and fructose respectively.

Histological techniques

To study histologic alterations, testis, epididymis, and seminal vesicle, a portion of the liver and kidney were fixed in aqueous Bouin's fluid overnight, dehydrated in graded ethanol series, cleared in benzene, and embedded in paraffin wax (60-62^{0}C). Tissues were sectioned at 6 µm, and sections were stained with periodic acid-Schiff (PAS) and counter-stained with Harris haematoxylin. The stained tissue sections were examined under a Leitz/Leica (Germany) light microscope. Quantitative alterations in the testes were determined by measuring the height of the germinal epithelium, diameter of round or slightly oblique stage VII seminiferous tubules, percent frequency of stages of spermatogenesis and that of affected seminiferous tubules from randomly selected testis sections. Histologic alterations in mouse testis were studied according to criteria described by Russell, Ettlin, Sinha Hikim, and Clegg; for histological study, the epididymis was divided into five (I-V) segments.[31-32]

Analyses of sperm parameters

At autopsy, cauda epididymidis was taken out randomly from the left or right sides of each of the five animals in each group, and placed in a watch glass containing 0.5 ml of 0.9 % normal saline maintained at 37^{0}C on a hot plate.[33] The tissue was minced properly and the sperm suspension was used for analyses of percent motility, viability, abnormality, and number of spermatozoa according to WHO protocol.[34] The criteria of Wyrobek and Bruce, as well as Zaneveld and Polakoski were followed to evaluate morphological abnormalities in spermatozoa.[35-36]

Assays of sialic acid and fructose levels

The level of sialic acid in the epididymis was determined according to thiobarbituric acid method and calculated using Warren's equation 2 with minor modifications.[37] In brief, the whole epididymis was homogenized in ice-cold 0.1 N suphuric acid, and kept in a water bath at 80^{0}C for 1 hour to liberate bound sialic acid. The homogenate was then cooled for 2 hours followed by oxidization with 25 mM periodate solution for exactly 30 minutes, and subsequent reduction with 2% sodium arsenite solution. This was followed by mixing with 0.1 M thiobarbituric acid solution and subsequent boiling in a water bath for exactly 7.5 minutes. The chromophore, thus obtained, was extracted in acid butanol and O.D. was recorded at 549 nm and 532 nm respectively in a spectrophotometer against a blank of distilled water.

The level of fructose in the seminal vesicle was assessed with minor modifications.[38] In brief, the whole seminal vesicle was homogenized in 80% ethanol and centrifuged at 5,000 rpm for 10 minutes. The supernatant was deproteinized with 0.3 N barium hydroxide and 5% zinc sulphate (1:1) followed by cooling for 2 hours. The homogenate was then centrifuged at 5,000 rpm for 10 minutes, and the supernatant was used for fructose assay. The diluted sample solution was mixed thoroughly with 0.1% ethanolic resorcinol followed by the addition of 30% hydrochloric acid. The samples were kept at 80^{0}C in a water bath for exactly 10 minutes followed by rapid cooling in an ice bath. The O.D. of the sample solution was measured within 30 minutes at 410 nm in a spectrophotometer against a blank of distilled water.

Toxicological investigations

Haematology and serum biochemistry: Trunk blood was analyzed for mean counts of blood cells (RBC and WBC), haemoglobin (Hb), and haematocrit (Hct) according to standard laboratory procedures.[39] The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were also calculated. Serum levels of ALT, as well as AST, were estimated to determine the functional status of the liver.[40] The serum creatinine was estimated using a diagnostic kit (Span Diagnostics Limited, India) to determine the functional status of the kidney.

Fertility tests

A total of 25 adult males (five animals per group) and 25 females of proven fertility were employed in the fertility tests. The fertility of males from groups II (distilled water-treated) and III-VI (extracts-treated) was tested after the last treatment (on 35th day) by allowing each male to cohabit overnight with one coeval, virgin female in proestrus showing regular cycles. Positive mating was confirmed by the presence of a vaginal plug in a mated female. After 12/13 days of gestation, pregnant females were autopsied to record the total number of implants in both the uteri and the total number of corpora lutea in both ovaries. The resorption sites were counted after treating the uteri with a 10% ammonium sulphide solution.[41] The males were considered fertile if impregnated females showed live implants.

Statistical analyses

All data, except those for body weight, were analyzed by one-way analysis of variance (ANOVA), followed by Neuman-Keuls' multiple range test. Data on body weight were analyzed by Student's t-test. Values were expressed as mean ± S.E.M. Results were considered significant at $p<0.05$ level.

RESULTS

Body and organs weight

Treatment with Terminalia extracts had no significant impact on the body weight and the general behavior or appearance of the treated animals. Terminalia-treated mice showed no alterations in the weights of the testis, epididymis, and vas deferens, though, the weight of seminal vesicles decreased significantly in mice in all treated groups compared to controls (Table 2). The treatment, however, did not affect the weights of the brain, liver, kidney, adrenal gland, and spleen (Table 3).

Table 2: Effect of *T. chebula* treatment (300 mg/kg body weight/day for 35 days) on body weight and weights of reproductive organs.

Groups/ treatments	Initial	Final	Testis		Epididymis		Vas deferens		Seminal vesicle	
			Absolute	Relative	Absolute	Relative	Absolute	Relative	Absolute	Relative
I, Control (untreated)	35.83 ±0.75	36.67 ±1.03	96.66 ±2.85	246.83 ±16.43	39.81 ±3.08	103.06 ±8.11	16.31 ±1.04	41.43 ± 5.66	118.61 ±14.19	309.10 ±41.26
II, Control (distilled water)	34.33 ±0.88	34.67 ±0.67	89.25 ±4.04	257.48 ±10.39	35.51 ±0.98	101.63 ±3.53	12.21 ±1.69	35.56 ±4.90	98.01 ±5.26	280.59 ±16.34
III, Acetone extract	30.50 ±0.56	31.17 ±0.54	84.41 ±4.48	256.47 ±9.77	30.01 ±1.78	99.47 ±3.73	12.43 ±0.43	38.67 ±1.23	64.30* ±9.43	195.27* ±25.84
IV, Methanol extract	32.67 ±0.67	33.33 ±0.42	90.58 ±4.74	285.22 ±11.10	35.16 ±1.27	114.2 ±4.78	13.91 ±2.07	44.61 ±6.93	66.88* ±9.27	204.12* ±22.69
V, Ethanol extract	34.17 ±0.65	33.67 ±0.56	82.6 ±3.80	256.98 ±11.78	33.2 ±2.99	101.31 ±9.20	14.65 ±0.91	45.62 ±2.94	65.21* ±6.07	201.81* ±15.81
VI, Aqueous extract	36.33 ± 0.49	36.00 ±0.76	81.11 ±6.87	226.87 ±12.10	37.08 ±2.33	105.77 ±5.91	14.05 ±1.66	39.49 ±4.16	65.40* ±10.08	186.16* ±29.30

Organ weight refers to the weight of the unpaired organ; Values are mean ± S.E.M. for six animals; *significantly different from controls ($p<0.05$) by ANOVA followed by Newman-Keuls' multiple range test

Table 3: Effect of *T. chebula* treatment (300 mg/kg body weight/day for 35 days) on weights of vital body organs.

Groups/ treatments	Brain		Liver		Kidney		Adrenal		Spleen	
	Absolute	Relative	Absolute	Relative	Absolute	Relative	Absolute	Relative	Absolute	Relative
I, Control (untreated)	349.98 ±10.30	912.38 ±29.87	1371.72± 69.12	4010.54± 217.15	269.04 ±14.70	699.48 ±30.41	4.22 ±0.15	10.99 ±0.37	89.72 ±7.78	232.74 ±16.83
II, Control (distilled wate	366.28 ±9.38	1054.76 ±34.63	1197.62 ±38.92	3449.53 ±139.56	268.62 ±20.95	762.09 ±55.48	4.94 ±0.55	14.04 ±1.62	85.10 ±1.94	242.02 ±7.07
III, Acetone extract	324.78 ±15.37	1023.44 ±53.88	1162.78 ±116.89	3641.58 ±330.30	238.02 ±7.56	749.78 ±27.96	3.56 ±0.68	11.23 ±2.25	95.24 ±7.86	299.39 ±23.66
IV, Methanol extract	346.98 ±19.34	1018.88 ±56.42	1276.80 ±22.15	4015.88 ±233.15	252.28 ±12.62	708.09 ±32.95	3.16 ±0.17	10.10 ±1.29	99.04 ±4.58	313.12 ±28.01
V, Ethanol extract	336.92 ±21.61	1018.18 ±47.44	1497.36 ±58.48	3438.51 ±179.75	265.92 ±16.58	803.98 ±36.78	4.12 ±0.48	12.50 ±1.48	94.46 ±6.66	286.72 ±21.18
VI, Aqueous extract	319.64 ±11.60	904.55 ±36.14	1289.62 ±104.05	3634.92 ±255.90	231.80 ±14.20	652.09 ±28.30	3.98 ±3.98	11.24 ±0.68	91.32 ±10.04	258.02 ±30.65

Organ weight refers to the weight of the unpaired organ; Values are mean ± S.E.M. for six animals; *significantly different from controls ($p<0.05$) by ANOVA followed by Newman-Keuls' multiple range test

Histologic observations

Testis

Histologically, testes in distilled water-treated (Figure 1A) controls showed normal spermatogenesis in nearly all the seminiferous tubules except in a few (see Table 4). In contrast, noticeable histologic alterations were observed in testes of Terminalia-treated mice (Figure 1B-H). Out of 24 extracts-treated mice examined for histologic alterations, 19 (5 in group III, 4 each in groups IV and V, and 6 each in group VI) showed obvious degenerative histologic alterations in the seminiferous tubules, while the remaining 5 (1 in group III, 2 each in groups IV and V) showed almost normal histologic features in nearly all tubules of their testes. Testes in 12 out of the above 19 extracts-treated mice showed non-uniform histologic alterations as both affected and normal tubules were observed in the same testis section (Figure 1G). On the other hand, testes in 7 out of 19 extracts-treated mice (1 in group III, 2 in group V, and 4 in group VI) showed uniform histologic alterations with almost all the tubules in degenerating and atrophic conditions (Figure 1 H). The histologic alterations in testes were more pronounced in mice treated with aqueous extract compared to those treated with other extracts of Terminalia suggesting that the effect was dependent on the type of plant extract administered (Table 4). The frequency of affected tubules was significantly high (76.18 %) in the testes of above treated mice compared to the testes of mice in other treated groups (Groups III-V; Table 4). The affected tubules in the testes of Terminalia-treated mice showed some common histologic alterations which are described together. In general, the affected tubules in testes of extracts-treated mice showed loosening of germinal epithelium, intraepithelial vacuolation, exfoliation of germ cells, mixing of spermatids of different stages of the spermatogenic cycle in the same tubule, failure of spermiation, phagocytosis of elongated spermatids, the occurrence of apoptotic and giant cells, and thinning and

disorganization of germinal epithelium due to marked depletion of germ cells (Figure 1B-H). In aqueous extract-treated mice, testes frequently contained atrophic and severely degenerating tubules (Figure 1G-H) with a very thin and disorganized germinal epithelium consisting of Sertoli cells and a few spermatogonia with rare spermatocytes and/or round spermatids (Figure 1G-H). Quantitative assessment of alterations in testes of treated mice showed a significant decrease in the height of germinal epithelium in stage VII tubules with no differences among the treated groups (Table 4); the diameter of stage VII tubules, however, was not found to be affected in testes of Terminalia-treated mice compared to controls (Table 4). Treatment with Terminalia had no effect on the frequency of stages V-VI, IX-X, and XI-XII of the spermatogenic cycle, though, a significant decrease in the frequency of tubules in stages VII-VIII (20.31%), and an increase in the number of tubules with unidentifiable stages (48.33%) were noticed in testes of aqueous extract-treated mice (Table 4); the frequency of tubules in stages IX-X increased significantly in testes of ethanol extract-treated mice compared to controls (Table 4).

Table 4: Effect of *T. chebula* treatment (300 mg/kg body weight/day for 35 days) on the height of germinal epithelium, diameter of stage VII tubules, percent frequency of affected tubules, and on the frequency of stages of the spermatogenic cycle in the testis.

Groups/ treatments	Height of germinal Epithelium (µm)	Diameter of stage VII tubules (µm)	Affected seminiferous tubules (%)	Stages I-IV	Stages V-VI	Stages VII-VIII	Stages IX-X	Stages XI-XII	Unidenti-fiable stages
I, Control (untreated)	58.83 ±2.91	195.07 ±7.07	12.77 ±2.93	19.03 ±1.95	9.51 ±1.45	37.59 ±1.04	11.24 ±1.73	20.56 ±1.72	3.02 ±1.3
II, Control (distilled water)	55.31 ±3.79	194.75 ±8.35	17.19 ±1.59	20.65 ±2.08	7.95 ±1.44	41.44 ±2.16	9.01 ±1.80	15.36 ±2.22	5.69 ±0.99
III, Acetone extract	45.19* ±2.11	196.01 ±5.82	45.15* ±8.11	18.24 ±4.01	11.28 ±2.02	32.42 ±6.72	10.12 ±2.15	15.46 ±3.49	12.50 ±12.48
IV, Methanol extract	44.28* ±1.6	195.70 ±7.06	36.54* ±2.78	14.09 ±2.01	14.66 ±2.67	34.54 ±2.39	10.93 ±0.86	21.88 ±2.44	0.56 ±0.55
V, Ethanol extract	44.81* ±2.44	175.01 ±7.53	34.72* ±3.93	15.38 ±1.96	10.72 ±1.55	34.85 ±3.41	16.54* ±1.46	20.02 ±2.23	2.50 ±0.63
VI, Aqueous extract	38.27* ±4.27	175.33 ±4.88	76.18[a] ±7.27	13.42 ±3.70	5.16 ±0.78	20.31* ±5.52	7.43 ±2.20	17.52 ±4.03	48.33* ±17.38

Values are mean ± S.E.M. for six animals; *Significantly different from controls ($p<0.05$); a significantly different from controls and those in groups III, IV, V and VI ($p<0.05$) by ANOVA followed by Newman-Keuls' multiple range test

Epididymis

Epididymis in distilled water-treated (Figure 2 A-C, G & L) controls exhibited normal histologic features. Treatment with Terminalia brought degenerative alterations in the epididymis (Figure 2 H-K, M-Q); similar to testes, the alterations were severe in mice treated with aqueous extract compared to those treated with other extracts of the plant. In aqueous extract-treated mice, segments I-III of the epididymes showed nearly normal histologic features, except that the lumen of the tubules in these segments was empty and contained PAS-positive materials (Figure 2 D-F); epithelial cells in segments IV (Figure 2 K) and V (Figure 2 P-Q) showed vacuolization; lumen was filled with relatively less sperm or sperm fragments and PAS-positive materials; stroma was also increased (Figure 2 P-Q); epithelial cells in segment IV in aqueous extract-treated mice showed distinct PAS-positive inclusions (Figure 2 K).

Seminal vesicle

Histologically, seminal vesicles in control mice (Figure 3 A) presented normal features; on the other hand, those in Terminalia-treated mice exhibited non-uniform but detectable histologic alterations (Figures not presented), which were severe in aqueous extract-treated mice with reduced height of the secretory epithelium, fewer epithelial mucosal folds and increased calcareous secretion in the lumen (Figure 3 B).

Liver and kidney

No histopathological alterations were noticed in the liver and kidney in mice in all treated groups compared to controls (Figure 3 C-F).

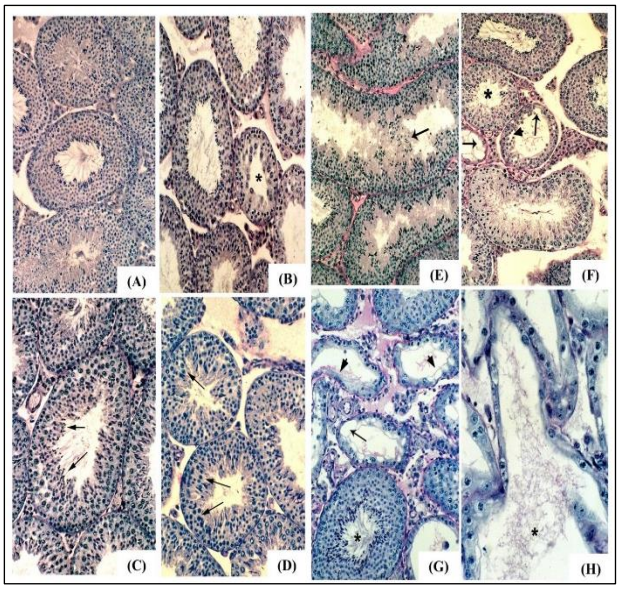

Figure 1 (A-H): Photomicrographs of PAS-H stained sections of mouse testis. (Original magnification: A, E, F: X 160; B-D & G: X 200; H X 252). (A) Distilled water-treated control showing normal spermatogenesis in the seminiferous tubules; (B) After treatment with acetone extract of *T. chebula* (300 mg/kg body weight/day for 35 days) showing the presence of a degenerating tubule (asterisk) among normal ones; (C) After treatment with methanol extract (same dose and duration). Note the failure of spermiation (arrows) in the middle tubule; (D) After the same treatment, as shown in (C), showing the presence of giant round spermatids (arrows) and loosening of germinal epithelium in tubules; (E) After treatment with 50% ethanol extract (same dose and duration) showing exfoliation of germ cells (arrow) in the middle tubule; (F) After treatment with aqueous extract (same dose and duration). Note mixing of spermatids of different stages (asterisk), intraepithelial vacuolization (arrows), and loosening of germinal epithelium (arrowheads) in tubules; (G) After the same treatment as shown in (F) showing non-uniform histologic alterations; (H) After the same treatment as shown in (F) showing a seminiferous tubule (asterisk) with germinal epithelium containing only sertoli cells, a few spermatogonia and spermatocytes.

Note disorganization of germinal epithelium (arrowheads), intraepithelial vacuolization (arrow) in affected tubules, and the presence of a normal tubule (asterisk).

Motility, viability, number, and morphology of spermatozoa

Significant reductions were noticed in motility, viability, and number of sperm (Figure 4 A); on the other hand, the percent frequency of morphologically abnormal spermatozoa was significantly high in cauda epididymidis of Terminalia-treated mice compared to controls, though, no significant differences were present for any of sperm parameters among the treated groups (Figure 4 A).

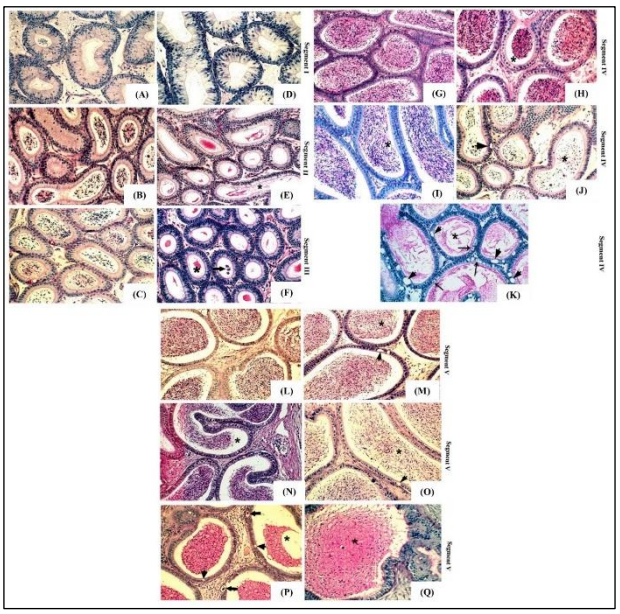

Figure 2 (A-Q): Photomicrographs of PAS-H stained sections of mouse epididymis. (Original magnification: A-C, E-F, G-H: X 160; D & I-K: X 200). Segments I-III (A-C) of a distilled water-treated control show normal histologic features of epithelium. Note the presence of sperm in segments II (B) and III (C); Segments I-III (D-F) of mice after treatment with aqueous extract of *T. chebula* (300 mg/kg body weight/day for 35 days) showing normal appearance of the segments, except that the lumen is devoid of sperm, and contains PAS-positive materials (asterisk). Note the presence of exfoliated germ cells in the tubular lumen of segment III (arrow); Segments IV (G) and V (L) of a distilled water-treated control showing normal histologic features of epithelium, and lumen distended with sperm; Segments IV-V of mice after treatment with acetone extract (H & M), methanol extract (I & N), 50% ethanol extract (J & O) and aqueous extract (K & P-Q) of *T. chebula* (300 mg/kg body weight/day for 35 days) respectively showing the intraepithelial vacuolation (arrowheads) and relatively less number of sperm (asterisk) in the tubular lumen. Segment IV (K) of mice treated with an aqueous extract of *T. chebula* (same dose and duration) showed extensive vacuolization, the presence of PAS-positive inclusions in the epithelial cells (arrows), and complete absence of sperm with accumulation of PAS-positive materials in the tubular lumen (asterisk). Segment V (P-Q) of above treated mice showed the presence of sperm fragments, active phagocytosis of sperm by the epithelial cells (arrows), and accumulation of PAS-positive materials in the lumen (asterisk).

Note the reduced diameter of the lumen and an increase in stroma in segment V of the epididymis.

Sialic acid and fructose levels

Significant reductions were noticed in the level of sialic acid in the epididymis and that of fructose in seminal vesicle in mice treated with aqueous extract of Terminalia compared to controls, though, these parameters remained unaltered in mice in other treated groups (see Figure 4 B).

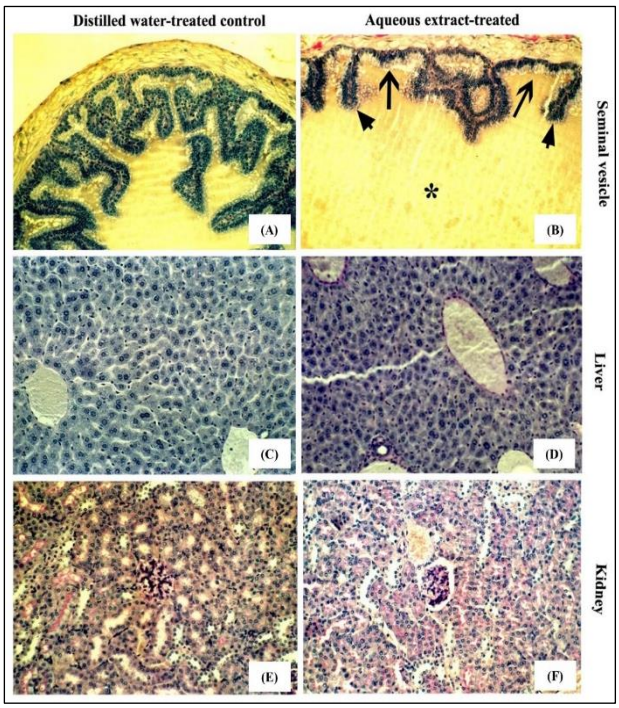

Figure 3 (A-F): Photomicrographs of PAS-H stained sections of the seminal vesicle, liver, and kidney of the mouse. (Original magnification: A-F: X 200). Seminal vesicle (A) of a distilled water-treated control showing normal histologic features of epithelium, and that (B) of mice treated with aqueous extract of *T. chebula* (300 mg/kg body weight/day for 35 days) showing the reduced height of the secretory epithelium (arrows), fewer mucosal folds (arrowheads), and increased calcareous secretion in the lumen (asterisk); Livers (C-D respectively) and kidneys (E-F respectively) of a distilled water-treated control and an aqueous extract-treated mice (same dose and duration) showing normal histologic features.

Haematology

The mean counts of RBC and WBC, and indices Hb, Hct, MCV, MCH, and MCHC remained unaltered in Terminalia-treated mice compared to controls (Table 5).

Serum biochemistry

No significant differences were found in serum levels of ALT, AST, and creatinine in Terminalia-treated mice compared to controls (Table 5).

Fertility test and pregnancy outcome

Libido was not affected in Terminalia-treated males when caged with virgin females after the last treatment; there was, however, a significant reduction in the number of live implants in females impregnated by males treated with methanol and aqueous extract of the plant compared to controls (see Table 6). The fertility, however, reduced significantly in aqueous extract-treated mice compared to controls (Table 6). Treatment with Terminalia caused a significant increase in post-implantation loss in females impregnated by males treated with aqueous extract, while other extracts had no such effect (Table 6); no significant increase in pre-implantation loss was, however, noticed in females impregnated by Terminalia-treated males (Table 6).

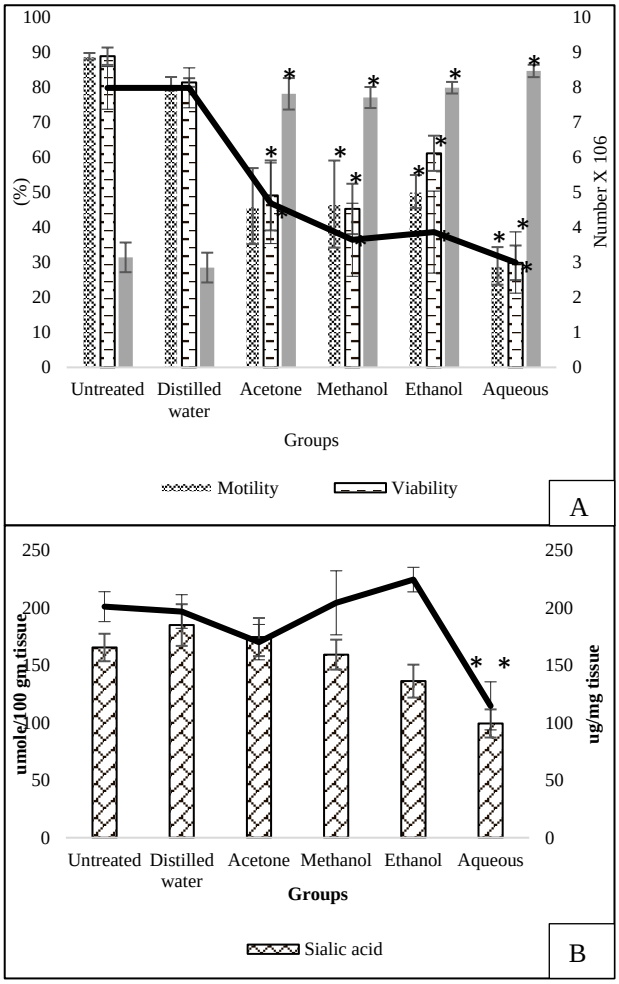

Figure 4 (A-B): Effect of *T. chebula* treatment (300 mg/kg body weight/day for 35 days) on (A) sperm parameters; (B) on the level of sialic acid in the epididymis and that of fructose in the seminal vesicle.
Values are mean±S.E.M. for five animals. *Significantly different from controls ($p<0.05$) by ANOVA followed by Newman-Keuls' multiple range test.

Table 5: Effect of *T. chebula* treatment (300 mg/kg body weight/day for 35 days) on haematological and toxicological parameters.

Groups/ treatments	Haematological parameters							Toxicological parameters		
	Hb (g/ 100 ml)	RBC (X 10^6/ mm^3)	WBC (X 10^3/ mm^3)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (%)	ALT (U/L)	AST (U/L)	Creatinine (mg/ 100ml)
I, Control (untreated)	12.86 ±0.79	6.65 ±0.32	3.80 ±0.38	52.35 ±4.51	82.19 ±9.35	19.54 ±1.56	25.18 ±3.73	24.61 ±2.24	26.73 ±1.75	1.56 ±0.19
II, Control (Distilled water)	13.04 ±0.25	6.00 ±0.26	3.90 ±0.38	46.90 ±2.22	78.82 ±5.09	21.93 ±1.20	28.01 ±1.19	16.48 ±3.17	19.00 ±3.49	1.59 ±0.07
III, Acetone extract	14.28 ±0.45	6.01 ±0.55	4.01 ±0.83	48.58 ±2.23	83.72 ±9.34	24.46 ±2.22	29.49 ±0.71	48.04 ±7.43	9.36 ±3.12	1.99 ±0.11
IV, Methanol extract	14.70 ±0.73	7.41 ±0.33	5.55 ±0.32	45.19 ±2.87	61.70 ±6.03	19.89 ±1.02	33.10 ±2.82	25.32 ±3.46	27.52 ±5.79	1.74 ±0.20
V, Ethanol extract	14.52 ±0.59	6.23 ±0.36	4.44 ±0.99	56.01 ±3.34	90.26 ±4.23	23.50 ±1.12	26.22 ±1.56	29.01 ±5.71	21.05 ±1.60	2.03 ±0.15
VI, Aqueous extract	13.40 ±0.26	6.92 ±0.40	4.70 ±0.17	56.62 ±1.76	85.75 ±6.83	19.60 ±1.04	23.04 ±0.65	21.71 ±1.39	22.30 ±1.39	1.81 ±0.15

Values are mean±S.E.M. for five animals

Table 6: Effect of *T. chebula* treatment (300 mg/kg body weight/day for 35 days) on libido and fertility of males, and pregnancy outcome in impregnated females.

Groups/ treatments	Number of males			Number of females			Index of libido (%)	Pre-implantation loss (%)	Post-implantation loss (%)	Number of live implants	Index of fertility (%)
	T	M	F	T	M	P					
II, Distilled water	5	5	5	5	5	5	100	2.00 ±2.00	4.00 ±2.45	10.60 ±0.60	100
III, Acetone extract	5	5	5	5	5	5	100	26.09 ±2.60	10.67 ±6.86	8.00 ±0.55	100
IV, Methanol extract	5	5	3	5	5	4	100	36.00 ±18.05	27.22 ±18.77	4.80* ±2.06	60 ±24.49
V, Ethanol extract	5	5	4	5	5	4	100	35.30 ±16.84	4.00 ±4.00	7.20 ±1.83	80 ±20.0
VI, Aqueous extract	5	5	0	5	5	5	100	14.00 ±9.80	100.00* ±0.0	**Nil***	**Nil***

T- Tested; M- Mated; F- Fertile; and P- Pregnant, Index of libido= (number mated/number paired) x 100; Index of fertility = (number of males siring live implants/ number mated) x 100; Values are mean ± S.E.M. for five animals; *significantly different from controls ($p<0.05$) by ANOVA followed by Newman-Keuls' multiple range test

DISCUSSION

The results of the study in albino mice indicate that treatment with bark extracts (acetone, methanol, 50% ethanol, and aqueous) of Terminalia at the dose of 300 mg/kg body weight/day for 35 days did not affect the weight of testis, though, non-uniform histologic alterations were noticed in the seminiferous tubules as both affected and normal tubules were observed in the same testis sections. The affected tubules, in general, showed exfoliation of germ cells, intraepithelial vacuolation, loosening of germinal epithelium, presence of spermatids of different stages of spermatogenic cycle in the same tubule, failure of spermiation, and frequent occurrence of apoptotic and giant cells. It is important to mention here that non-uniform histologic alterations in the testis as described above with or without a weight change have also been reported in mice after treatment with bark extract of *Albizia lebbeck (L) Benth*, rhizome extract of *Curcuma longa* L., *Bacopa monnieri*, leaf extract of *Citrus limon*, and leaf extract of *Coccinia indica*.[42-46] It has been suggested that non-uniform focal damages in the testis occur because tubules in certain stages of spermatogenesis are more prone to damage by various treatments than others.[31] This is known that stages VII-VIII of spermatogenesis are highly androgen-dependent, and therefore, a noticeable decrease in the frequency of tubules in stages VII-VIII of the spermatogenic cycle as observed in testes of aqueous extract-treated mice indicate that treatment-induced non-uniform histologic alterations

might be the result of androgen-dependent adverse effect on kinetics of spermatogenesis.[47-48] The present study also indicates that mice treated with aqueous extract of Terminalia showed more severe alterations in their testes compared to those treated with other extracts of the plant; the percent frequency of affected tubules was significantly high (76.18 %) in testes of above treated mice compared to testes of mice in other treated groups. Further, the testes in aqueous extract-treated mice frequently showed atrophied seminiferous tubules consisting of Sertoli cells and a few spermatogonia with rare spermatocytes, and occurrence of giant cells in the germinal epithelium, as observed in other studies.[49,50,45] The frequency of tubules in unidentifiable stages (48.33%) was significantly high in testes of above treated mice. Significant reduction was noticed in the height of germinal epithelium in stage VII tubules in testes of Terminalia-treated mice which could be due to mass depletion of germ cells suggesting that treatment with Terminalia interferes with normal spermatogenesis.[45] The diameter of stage VII tubules, however, remained unaffected in Terminalia-treated mice. Terminalia treatment had no effect on weights of epididymis and vas deferens in albino mice, though, detectable histologic alterations were observed in epididymes of extracts-treated mice; similar to the testis, histologic changes in epididymis were severe in mice treated with aqueous extract; segment IV of epididymis in above treated mice frequently showed PAS-positive inclusions in epithelial cells as reported after treatment with rhizome extract of *Curcuma longa* L., leaf extract of *Citrus limon*, and leaf extract of *Coccinia indica*.[43,45,46] It has been reported that principal cells in segment II (caput) secrete PAS-positive material in the lumen which is utilized by sperm during maturation in subsequent segments, and that in absence of sperm, such material is reabsorbed by the principal cells of segment IV (corpus) of epididymis.[51,32] Thus, presence of PAS-positive inclusions in epithelial cells of segment IV might be because of absence or presence of a few sperm in epididymal duct which is evident from photomicrographs of different segments (I-V) of epididymis in aqueous extract-treated mice. Treatment with Terminalia caused significant reduction in the number of cauda spermatozoa, which might be because of suppressive effects of the treatment on spermatogenesis; on the other hand, alterations in sperm motility, viability, and morphology could have resulted from disturbance in the secretory function of epididymis.[52-54] This is indicated by a marked reduction in the level of sialic acid in epididymis in mice after treatment with aqueous extract. Significant reduction in weight of the seminal vesicle accompanied with severe histologic alterations and reduced secretory activity were noticed in mice after treatment with aqueous extract of Terminalia. The epididymis and seminal vesicle are two important reproductive organs largely dependent on androgen for the maintenance of structure and function, and therefore, histologic alterations in the above organs accompanied with significant reductions in the levels of sialic acid and fructose respectively indicate disturbance in the intratesticular androgen level.[55] However, more studies are needed to reach a conclusion regarding the mechanism of action behind the antispermatogenic effect of Terminalia.

The present investigation in albino mice indicate that treatment with Terminalia had no effect on libido of extracts-treated males. The fertility however, was adversely affected in mice treated with aqueous extract of the plant as revealed by the complete absence of live implants in impregnated females because of significant increase in post-implantation loss. The antifertility effect of Terminalia in albino mice as observed in the present study might be due to treatment-associated adverse effect on sperm parameters in treated males. The observations that there were no alterations in mean body weight and in weights of the brain, liver, kidney, adrenal gland and spleen, in histological features of liver and kidney accompanied with normal levels of serum ALT, AST and creatinine respectively, as well as in haematological indices suggest that treatment with Terminalia does not produce toxic effects.

CONCLUSION

The results of the present investigation suggest that treatment with aqueous bark extract of T. chebula results in suppression of spermatogenesis and fertility in albino mice without any apparent toxic effects and, therefore, Terminalia might be useful for male fertility regulation.

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