Journal of Applied Pharmaceutical Research
Volume 12 Issue 4, Year of Publication 2024, Page 139 – 154
DOI: https://doi.org/10.69857/joapr.v12i4.570

Check for updates

Research Article

JOURNAL OF APPLIED PHARMACEUTICAL RESEARCH | *JOAPR*
www.japtronline.com *ISSN: 2348 – 0335*

TREATMENT WITH TERMINALIA CHEBULA (RETZ.): POSSIBLE MECHANISM OF INHIBITION OF SPERMATOGENESIS AND FERTILITY IN ALBINO MICE

Prakash Chandra Gupta*, Laxmi Yadav

Article Information
Received: 24th April 2024
Revised: 21st June 2024
Accepted: 12th July 2024
Published: 31st August 2024

Keywords
Spermatogenesis, Fertility, Sperm, Recovery, Toxicity

ABSTRACT

Background: There has been a continued effort to develop an effective male contraceptive of plant origin due to its ready availability, cost-effectiveness, and fewer side effects. The present study has evaluated the mechanism of inhibitory action of *Terminalia chebula* Retz. (*T. chebula*; family: Combretaceae) on spermatogenesis and fertility in albino mice after oral administration of the aqueous bark extract (100, 300, and 500 mg/kg BW daily) of *T. chebula* for 35 d. **Methodology:** The effects of the Terminalia treatment on various reproductive endpoints such as sperm parameters, testis histology, activities of 3ß- and 17ß-HSDs, immunoblot expressions of StAR and AR proteins, immunostaining of AR, serum testosterone level, LPO level, activities of SOD and catalase, and fertility indices were investigated. Toxicological and recovery studies have also been performed. **Results:** Testes in Terminalia-treated mice showed nonuniform histologic alterations. Sperm parameters, activities of 3ß- and 17ß-HSDs, immunoblot expressions of StAR and AR proteins, immunostaining of AR, and serum testosterone level were adversely affected, though activities of SOD and catalase were unchanged. Libido remained unaffected, but fertility was inhibited markedly in treated males without signs of toxicity. By 42 d of treatment discontinuation, Terminalia-induced deviations in the reproductive endpoints recovered to control levels. **Conclusion:** The results of the present study indicate that *T. chebula* treatment reversibly inhibits spermatogenesis and fertility without signs of toxicity. Further, antifertility effects result from diminished production of testosterone due to Terminalia-mediated inhibition of testicular steroidogenesis.

INTRODUCTION

Researchers worldwide have become very interested in the search for a plant-derived oral, effective, safe, and reversible male contraceptive with no adverse effect on libido. This interest might have developed due to the easy availability and cost-effectiveness of plant products and the protection of privacy for users. In addition, an antifertility drug from a plant source has generally low toxicity [1-4]. Various phytochemicals from

**Department of Zoology, Keshav Prasad Mishra Rajkiya Mahila Mahavidyalaya, Bhadohi-221 301 (Affiliated to Mahatma Gandhi Kashi Vidyapith, Varanasi) India*

For Correspondence:* **pcgzoology@gmail.com

medicinal plant parts have been identified and investigated in different animal species to define their impact on male fertility [5-6]. A large number of plants like *Barleria prionitis*, *Ricinus communis*, *Curcuma longa*, *Aegle marmelos*, *Albizia lebbeck*, *Hymenocardia acida*, *Caesalpinia sp.*, *Butea monosperma*, *Calendula officinalis*, *Feronia limonia*, *Tabernaemontana divaricata*, *Citrulus Colocynthis*, *Cuminum cyminum*, *Hibiscus rosasinensis*, *Dactyloctenium aegyptium*, *Achyranthes aspera*, *Costus lucanuscianus*, *Andrographis paniculata*, etc [7-24] have been worked out for their antifertility effects in males of different mammalian species. However, only a few plants studied till now have proved efficient in inducing infertility in males across different species [25]. Despite this, there is continued hope among researchers that plants or plant-derived products might play a crucial role in male fertility regulation [26]. Hence, plants hold a sustainable future in developing a plant-derived male contraceptive [27].

Terminalia chebula (*T. chebula* Retz.; Family: Combretaceae) with several local names such as Black Myrobalan (English), Haritaki (Sanskrit and Bengali), Harad (Hindi), etc., is native to South Asia [28]. It has been extensively investigated for various biological and pharmacological properties, viz. antibacterial, antifungal, antiviral, anticancer, antioxidant, and antidiabetic activities [29-38]. In addition, neuroprotective, hepatoprotective, cardioprotective, cytoprotective, radio-protective, and immunomodulatory effects of *T. chebula* have also been described [39-44]. Despite such a wide variety of pharmacological properties, the available literature on the impact of Terminalia on the reproductive organs and fertility in mice is limited to a few reports and those from our laboratory in initial trials [45-47]. Further, the mechanism of *T. chebula* induced inhibition of spermatogenesis and fertility is poorly understood. Therefore, in the present study, we have evaluated various male reproductive endpoints, viz. sperm parameters, testicular histology, activities of steroidogenic enzymes, viz. 3ß- and 17ß-hydroxysteroid dehydrogenases (HSDs), immunoblot expressions of steroidogenic acute regulatory (StAR) as well as androgen receptor (AR) proteins, immunostaining of AR, serum testosterone level, and fertility parameters. Treatment-induced oxidative stress in testis has been determined by measuring the level of lipid peroxidation (LPO) and activities of superoxide dismutase (SOD) as well as catalase. Further, toxicological and recovery studies have also been performed.

MATERIALS AND METHODS

Plant authentication and preparation of the extract

The bark of *T. chebula* was collected after scientific identification and authentication of the plant by Professor N. K. Dubey of the Department of Botany, Banaras Hindu University, Varanasi (India). The plant specimen was conserved in the herbarium (Voucher specimen no. Combreta. 2023/03) and kept in the Department of Botany of the above University for future reference. The preparation of plant extract was performed strictly following WHO guidelines [48]. Briefly, Terminalia bark was shade-dried for at least 1 week (w) and then crushed into a fine powder. The material (100 g) was then extracted with sterile distilled water (DW) (1000 ml, w/v 1:10) for 8 hours (h) using a soxhlet apparatus. After filtration, the extract was cooled to room temperature (RT) and then subjected to lyophilization to get a black-colored extract stored at 4°C in a refrigerator. The extract yield was approximately 20 g per 100 g (20%) of the raw bark powder.

Animal handling and ethical approval

The Parkes strain (age: 14-15 w; weight: 28-38 g) male albino mice (*Mus musculus*) were used in the present study. The animal colonies were maintained in an animal house under standard conditions (photoperiod 12 h; temperature 23±2°C; and relative humidity 50±20%) in polypropylene cages with dry rice husk as the bedding material. At regular intervals, animals were given standard food (Mona Laboratory Animal Feeds, Varanasi) and fresh tap water ad libitum. The maintenance of the animals and experimentations were carried out as per standard guidelines of CPCSEA, New Delhi, after the proper approval of the Ethical Committee of the University [49].

Treatment design

35 adult mice were allocated randomly into seven groups (Groups I-VII), each comprising five animals. The treatment design and autopsy schedule are mentioned in Table 1.

Administration of extract

Terminalia bark extract was suspended in sterile DW and administered orally at the doses of 100, 300, and 500 mg/kg BW daily for 35 days (d) to animals in treated groups (III-VI), using a sterile feeding pipe. The doses, duration, and nature of extract for Terminalia were selected based on trial studies conducted in our laboratory. Animals in groups II and VII (DW-treated controls) received an equal volume of sterile DW (0.5 ml/100 g body weight (BW) daily for 35 d).

Animal autopsy and sample collection

After 35 days of the treatment period, animals in groups I and II were sacrificed together with those in Terminalia-treated groups III-V, while the animals in groups VI (TR: Terminalia recovery) were sacrificed together with those in group VII (CR: Control recovery) after 42 d of the treatment discontinuation. At autopsy, body weight was documented for each animal. The testis, epididymis, seminal vesicle, ventral prostate, brain, liver, spleen, kidney, and adrenal gland were removed and weighed using an electric balance. For histopathological or AR immunostaining studies, testis, parts of the liver, kidney, and spleen were excised randomly from either side of mice and fixed in freshly prepared aqueous Bouin's fluid overnight. Testes were kept at -20°C for enzyme assays and western blot analyses for StAR and AR proteins. The blood was collected by decapitation to separate the sera, which were stored at -20°C for biochemical assays of transaminases viz. alanine aminotransferase (ALT), and aspartate aminotransferase (AST), creatinine, and testosterone.

Table 1: Treatment design and autopsy schedule.

Groups	*Treatments*	*Duration (day)*	*Autopsy (after last treatment)*
I	Untreated controls	35	24 h
II	DW-treated controls	35	24 h
III	Terminalia 100 mg (TC 100)	35	24 h
IV	Terminalia 300 mg (TC 300)	35	24 h
V	Terminalia 500 mg (TC 500)	35	42 d
VI	Terminalia 500 mg (TR*)	35	42 d
VII	DW-treated controls (CR**)	35	42 d
	TR*: Terminalia recovery;		CR**: Control (DW) recovery

Analyses of sperm parameters

The cauda epididymides were taken out randomly from either side of the animals and placed in a watch glass containing 0.5 ml of 0.9% physiological saline maintained at 37°C [50]. The caudal tissue was correctly crushed to get a homogeneous sperm suspension, which was used to determine the percent frequency of motility, viability, abnormality, and number of spermatozoa according to the standard protocol of WHO [52-53]. The morphological anomalies in caudal spermatozoa were studied as per the criteria described by Wyrobek and Bruce and Zaneveld and Polakoski [51-54].

Determination of testicular oxidative stress

The testicular level of LPO and the enzyme activities of SOD and catalase were measured to determine the treatment-induced oxidative stress [55-57]. In brief, the testis was homogenized in 10% (w/v) ice-cold 50 mM sodium phosphate buffer containing 0.25 M sucrose. The tissue homogenate was centrifuged at 2,500 rpm for 10 minutes (min) at 4°C, and the supernatant was used for assays of LPO, SOD, and catalase. The thiobarbituric acid (TBA) reagent (3.3 ml) was mixed with 0.2 ml of tissue supernatant in a test tube, and the mixture was heated at 95°C in a water bath for 60 min. This was followed by cooling to RT and centrifugation at 2,000 rpm for 10 min at 4°C. The supernatant containing the bi-product (MDA-TBA) was used to take optical density (OD) against a blank of double distilled water (DDW) at 532 nm. The LPO level was estimated using the following formula:

$$Lipid\ peroxidation\ \ (nmoles\ TBARS/g\ tissue) = \frac{OD \times 3500\ (dilution\ factor)}{156\ (Extinction\ coefficient) \times\ weight\ of\ tissue\ (g)}$$

For assays of SOD and catalase, the above supernatant was further centrifuged at 12,000 rpm for 25 min at 4°C to obtain the post-mitochondrial fraction (PMS). The PMS (100 µl) in a test tube was treated with 1.4 ml of SOD reaction cocktail. The mixture was pre-incubated for 5 min at RT in a special SOD box. This was followed by the addition of 80 µl of 100 µM riboflavin to the mixture and incubation for 10 min at RT in the SOD box. In a blank test tube, the sample and riboflavin were replaced by DDW, while in a control test tube, only the sample was replaced by DDW. At the end of the exposure time, the reaction was stopped by adding 1.0 ml of Greiss reagent. The OD was recorded against blank at 543 nm. SOD activity was estimated using the following formula:

$$SOD\ activity\ (U/mg\ protein) = \frac{\frac{OD\ of\ control}{OD\ of\ sample} - 1}{mg\ of\ protein}$$

In a test tube, 5 µl of ethanol (95%) was added to 500 µl of PMS and allowed to stand for 30 min in an ice chamber. This mixture

(450 µl) was dispensed in a tube containing 50 µl of triton-X 100 (10% v/v) to make the tissue preparation for catalase assay. Took 2.8 ml of 50 mM phosphate buffer in a cuvette and added 100 µl of the tissue preparation. The OD was recorded before and after adding 100 µl of 60 mom H_2O_2 at 240 nm. Catalase activity was estimated using the following formula:

$$Catalase\ activity\ (Pkat/mg\ protein) = \frac{Change\ in\ absorbance\ (\Delta OD) \times 1250}{mg\ of\ protein}$$

Assays of testicular steroidogenic enzymes

The activities of enzymes 3ß- and 17ß-HSDs were assessed biochemically to determine the steroidogenic activity in the testis [58-59]. In brief, the testis was homogenized in 20% spectroscopic grade glycerol containing 5 mM potassium phosphate and 1 mM ethylenediaminetetraacetic acid (EDTA), and the tissue homogenate was centrifuged at 12,000 rpm for 30 min at 4°C to get the supernatant. For determination of 3ß-HSD activity, the supernatant (1.0 ml) was mixed with 1.0 ml of 100 µM sodium pyrophosphate buffer (pH 8.9), 40 µl of ethanol containing 30 µg dehydroepiandrosterone (DHEA) (Sigma Chemical Company, St. Loius, MO, USA) and 960 µl of 25 mg% bovine serum albumin (BSA) making the incubation mixture to a total of 3.0 ml. The enzyme activity was measured after adding 100 µl of 0.5 µM of nicotinamide adenine dinucleotide (NAD) (Sigma Chemical Company, St. Loius, MO, USA) to the incubation mixture against a blank (without NAD) in a spectrophotometer at 340 nm. For each reading, OD was recorded at 30 seconds (sec) intervals for 3 min. For determination of 17ß-HSD activity, the supernatant (1.0 ml) was mixed with 1.0 ml of 440 µM sodium pyrophosphate buffer (pH 10.2), 40 µl of ethanol containing 0.3 µM of testosterone (Sigma Chemical Company, St. Loius, MO, USA) and 960 µl of 25 mg% BSA making the incubation mixture to a total of 3.0 ml.

The enzyme activity was measured after adding 100 µl of 0.5 µM of NAD to the incubation mixture against a blank (without NAD) in a spectrophotometer at 340 nm. For each reading, OD was recorded at 30 sec intervals for 3 min. One unit (U) of enzyme activity for 3ß- and 17ß-HSDs was the amount causing a change of 0.0001/min in OD at 340 nm.

Enzyme activity was estimated using the following formula:

$$Enzyme\ activity\ (U/mg\ tissue/h) = \frac{Change\ in\ absorbance\ (\Delta OD) \times 60}{mg\ of\ tissue}$$

Serum testosterone level

The testosterone level in sera was analyzed by radioimmunoassay using a commercial radioimmunoassay kit, following the protocol described by the manufacturer.

Toxicological investigation

The mean red blood cell (RBC) and white blood cell (WBC) counts, hemoglobin (Hb), and hematocrit (Hct) were determined in freshly collected trunk blood according to standard procedures [60]. The hematological indices, such as mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular volume (MCV), were also calculated. The serum levels of ALT and AST were determined biochemically [61]. The serum creatinine level was assayed using a commercial kit (Span Diagnostics Limited, India).

Histopathological investigation

The testis, liver, kidney, adrenal gland, and spleen were subjected to dehydration in ordered ethanol series, clearing in benzene, and embedding in paraffin wax. Tissue sections (6 µm) were stained with periodic acid-Schiff (PAS) and Harris hematoxylin (H) for histological examination under a Leitz (Germany) light microscope. The criteria defined by Russell *et al.* were used to describe histological alterations and identify stages of spermatogenesis in seminiferous tubules of mouse testis [62]. Enumeration of germ cell number in stage VII tubules was performed to study the Terminalia-induced alterations in the kinetics of spermatogenesis. The crude count for different germ cells (spermatogonia A, preleptotene spermatocytes, pachytene spermatocytes, and step 7 spermatids) was rectified by Abercrombie's formula taking Sertoli cell nuclei as the reference [63-64]. The tubules' diameter and germinal epithelium's height were also measured in stage VII seminiferous tubules. The percentage frequency of affected tubules in the testis was also determined [46-47, 65].

Fertility tests

The fertility of males (n = 5) from groups VI (Terminalia-treated; TR: 500 mg/kg BW/d for 35 d) and VII (DW-treated; CR: 0.5 ml/100 g BW/d for 35 d) was tested at the intervals 24 h, 2, 4 and 6 w after the treatment discontinuation by allowing each male to mate overnight with a virgin female in proestrus. The occurrence of a vaginal plug in the mated female confirmed successful mating. After 12 d of the gestational period, the

females were autopsied to record the total number of implants in both the uteri and the total number of corpora lutea in both ovaries [66]. The males were assumed to be fertile if the impregnated females presented live implants in their uteri. Fertility parameters such as the index of libido and index of fertility in males, and the number of live implants, pre-and post-implantation losses in the impregnated females were determined [47].

Immunoblot expressions of testicular StAR and AR proteins

The expressions of StAR and AR proteins were studied in mouse testis by the standard protocols of previous works [67-68]. In brief, testis was homogenized in 20% (w/v) ice-cold lysis buffer (1M NaCl, 1M tris Cl, pH 7.6, 0.1M EDTA, pH 8.0, 0.1M 100 µg/ml PMSF prepared in DDW). The sample, after proper mixing with 6X gel loading buffer (1:5), boiling for 10 min followed by centrifugation at 10,000 rpm for 15 min at 4°C, was subjected to protein estimation [69]. An equal quantity of proteins for each sample of StAR (100 µg) and AR (100 µg) was loaded to SDS–PAGE (12%) electrophoresis as per standard protocol [70]. The resolved proteins were electrophoretically wet and transferred onto a nitrocellulose membrane (Advanced Microdevices Pvt. Ltd., Ambala Cantt., India) at 50 V overnight at 4˚C.

The membrane was blocked in TBST (0.05% Tween-20 in Tris-50 mM, 5% (w/v) nonfat milk, pH 8.0) at RT for 2 h with constant shaking, then incubated with specific primary antibodies (rabbit polyclonal anti-StAR primary antibody, a gift from Dr. D.M. Stocco, Texas Tech. University, TX, USA; PG 21 rabbit polyclonal anti-AR primary antibody, a gift from Dr. G.S. Prins, University of Illinois, Chicago, IL) each at dilution of 1:500 for overnight at 4°C. Subsequently, the membrane was washed with TBST three times each for 10 min, followed by incubation with HRP-conjugated goat anti-rabbit secondary antibody (Bangalore Genei, Bangalore, India) at a dilution of 1:2000 for 2 h at RT with continuous shaking.

Immunodetection of proteins was performed with an ECL kit (Thermo Scientific, Rockford, IL, USA) as per the manufacturer's protocol on an X-ray film, and the resulting immunoreactive bands were quantified by Image J Software (NIH, Bethesda, MD, USA). Alpha (α) tubulin protein was used as a loading control; it was detected by a monoclonal mouse TAT-1 primary antibody (a gift from Dr. Keith Gull, University of Oxford, Oxford, OX1, 3 RE) (at a dilution of 1:100 for overnight at 4°C) as per the standard method [71]. The primary antibody was probed with HRP-conjugated goat anti-mouse secondary antibody (Bangalore Genei, Bangalore, India) at a dilution of 1:2000 at RT for 2 h with constant shaking.

Immunostaining of AR in testis

The paraffin-embedded tissue sections were processed for immunostaining of AR in mouse testis as described by Prins *et al.*, and Zhou *et al.* [67-68]. In brief, sections were deparaffinized, rehydrated in graded ethanol series, and washed twice with 0.1M TBS (pH 7.4) for 10 min each. For antigen retrieval, sections were treated with 0.01 M citrate buffer (pH 6.0) for 15 min at 80˚C in an oven; this was followed by three times washing with TBS for 5 min each; endogenous peroxidase activity was blocked by treating the tissue sections with 3% hydrogen peroxide for 15 min at RT. The sections were then pre-incubated with 10% normal goat serum in TBS (1:200) at RT for 1 h to prevent non-specific binding. The sections were incubated with PG-21, a rabbit polyclonal anti-AR primary antibody (a kind gift from Dr. G.S. Prins, University of Illinois, Chicago, IL), at a dilution of 1:50 (diluted with TBS) overnight at RT in a moist chamber. For negative controls, the primary antibody was replaced with TBS.

This was followed by washing three times with TBS for 5 min each. The sections were then incubated successively with biotinylated goat anti-rabbit secondary antibody at 1:200 dilution, followed by streptavidin-HRP conjugate at 1:100 dilution for 1 h each at RT in the moist chamber with extensive TBS washing in between. Following washing, sections were incubated with a chromagen substrate DAB (0.1% 3, 3-diaminobenzidine tetrahydrochloride) (Vector Laboratories, Burlingame, CA) for 5-10 min and then counterstained for 60 sec with Harris hematoxylin. As a final step, the sections were dehydrated gradually with alcohol, cleared with xylene, and cover-slipped with DPX. The negative controls were also processed the same way as others, except the sections were incubated with TBS instead of primary antiserum.

A positive reaction showed the deposition of a reddish product at the site of the antibody-antigen reaction. The images were examined under a Leitz or Leica (Germany) light microscope equipped with a digital camera to identify AR-positive cells. Image J Software (NIH, Bethesda, MD, USA) was used to obtain

the total number of AR-positive cells and mean staining intensity (MSI) by analyzing a definite area around randomly selected at least 5 stage VII-VIII seminiferous tubules per testis from three animals in each group.

Statistical analyses

ANOVA (one-way analysis of variance) followed by Neuman-Keuls' multiple range test was employed to analyze the data. However, body weight and fertility test data were examined using the Student's t-test. Data were expressed as mean ± S.E.M. and considered significant at $p< 0.05$.

RESULTS

Treatment with Terminalia extract (100, 300, and 500 mg/kg BW daily for 35 d) had no impact on body weight and weight of brain, liver, kidney, adrenal gland, and spleen; further, histological features of liver, kidney, and spleen were similar to controls (figures not presented). No significant alterations were noticed in hematological indices (RBC, WBC, Hb, Hct, MCV, MCH, and MCHC), serum levels of ALT and AST, and creatinine in Terminalia-treated mice compared to controls (data not presented). Significant reductions were, however, found in the absolute weights of the testis, epididymis, seminal vesicle, and prostate gland in mice treated with a 500 mg dose of Terminalia compared to controls. Still, the weight of the above reproductive tissues was similar to that of the control group by 42 d of treatment discontinuation (Figure 1 A-B).

Sperm parameters and fertility

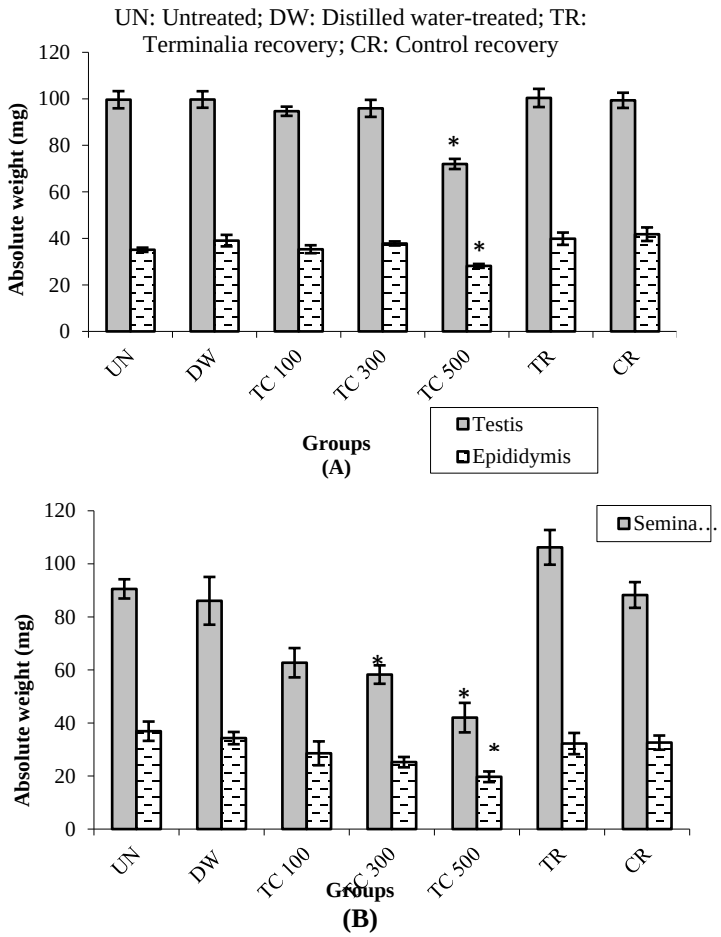
There was a significant decrease in sperm motility, viability, and number in Terminalia-treated mice compared to controls, while an increase in the number of morphologically abnormal spermatozoa was noticed (Figure 1 C); these alterations were severe in those treated with a 500 mg dose (Figure 1 C). When Terminalia (500 mg dose)-treated males were allowed to mate with females at 24 h, 2, 4, and 6 w after discontinuation of the treatment, libido remained unaltered every time (data not presented); the fertility of treated males, however, reduced significantly at 24 h, 2 w, and 4 w after treatment discontinuation (Figure 2 A), since none of the impregnated females showed live implants due to absolute pre-implantation loss (Figure 2 B-C); the post-implantation loss, though, was insignificant in these impregnated females compared to controls (data not presented). By 6 w (42 d) after treatment discontinuation, however, all fertility parameters recovered to control levels (Figure 2 A-C)), though the number of sperm with abnormal morphology remained significantly elevated (Figure 1 C).

Status of oxidative stress in testis

The enzyme activities of SOD and catalase remained unaltered in testes of Terminalia-treated mice compared to controls, though the level of LPO was significantly high in those treated with a 500 mg dose of the extract; By 42 d after treatment discontinuation, the level of LPO in these treated mice was similar to controls (data not presented).

Testicular activities of 3ß- and 17ß-HSDs and serum testosterone level

Significant declines were noticed in the activities of 3ß-HSD and 17ß-HSD in testes and serum testosterone levels in mice treated with 300 and 500 mg doses of Terminalia; though, the 100 mg dose of Terminalia did not affect the 17ß-HSD activity and serum testosterone level (Table 1). By 42 d of treatment discontinuation, the activities of 3ß- and 17ß-HSDs and serum testosterone levels in Terminalia-treated mice recovered to control levels (Table 1).



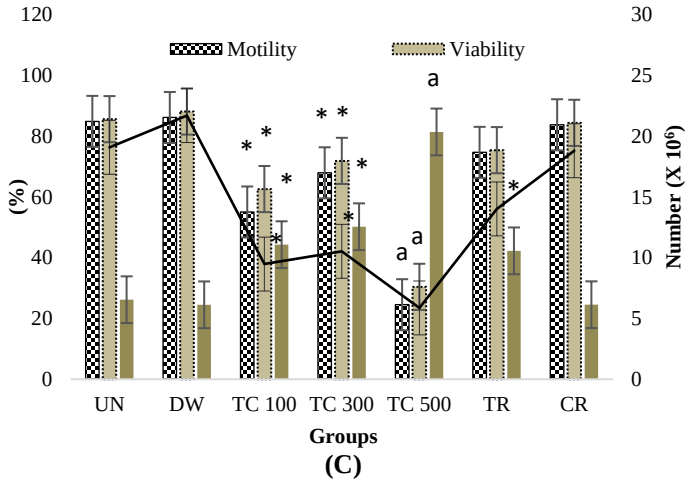
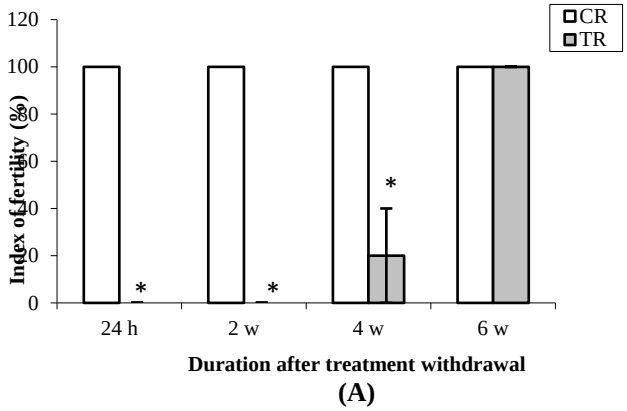
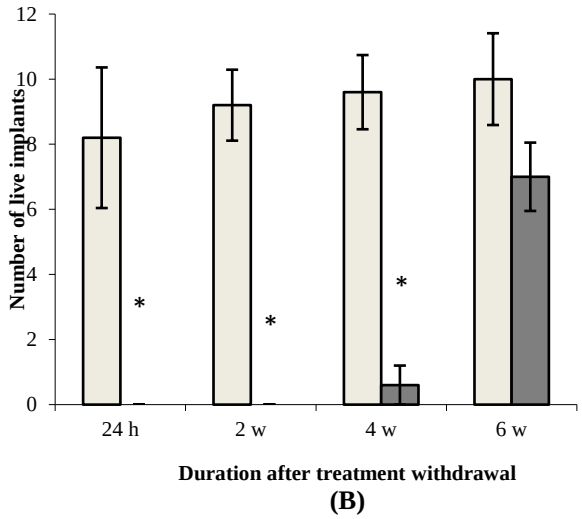


Figure 1: Effect of treatment with *T. chebula* (mg/kg BW for 35 d), and 42 d after discontinuation of the treatment on (A) absolute weights of testis and epididymis; (B) weights of seminal vesicle and prostate gland; (C) sperm parameters. Values are mean ± S.E.M. for five animals; *significantly different from controls; [a] significantly different from controls and those in groups III and IV; [b] significantly different from recovery controls.





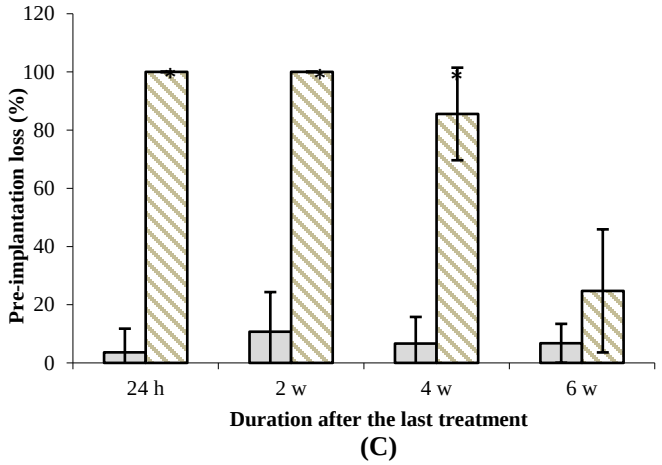


Figure 2: Effect of treatment with *T. chebula* (500 mg/kg BW for 35 d) at intervals after discontinuation of the treatment on (A) fertility of males; (B) pregnancy outcome and (C) pre-implantation loss in impregnated females. Values are mean ± S.E.M. for five animals; *significantly different from controls. Index of fertility = (number of males siring live implants/ number mated) x 100; Pre-implantation loss = (total number of corpora lutea – total number of implantations/ total number of corpora lutea) X 100.

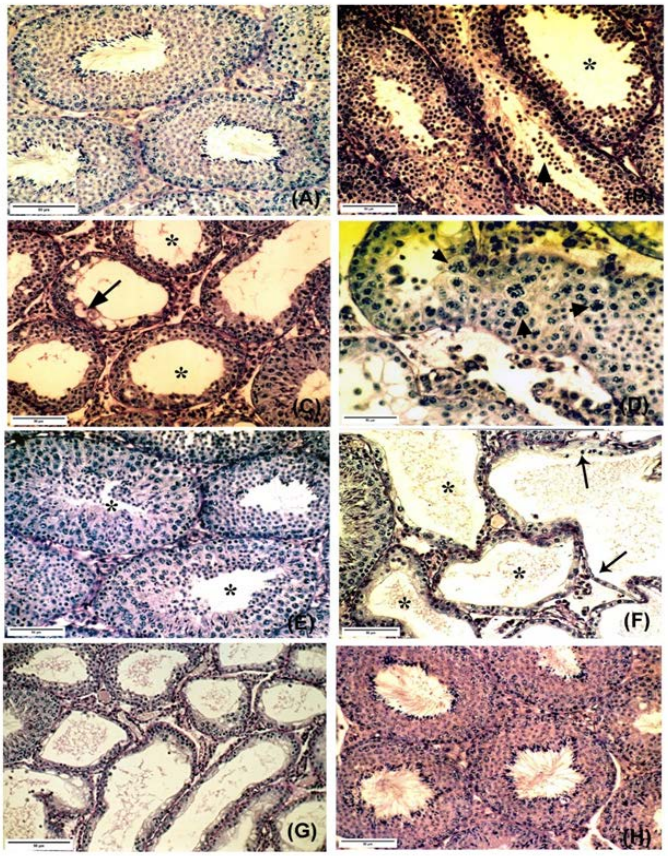


Figure 3: Photomicrographs of PAS-H stained sections of mouse testis. (A) DW-treated control showing normal spermatogenesis

in the seminiferous tubules; (B) After treatment with an aqueous extract (100 mg/kg BW/d for 35 d) of *T. chebula* showing loosening in germinal epithelium (asterisk) in a tubule and detachment of germ cells (arrowhead); (C) After *T. chebula* treatment (300 mg/kg BW/d for 35 d) showing vacuolation (arrow) and reduced height of the germinal epithelium (asterisk); (D) After the same treatment, as in (C), showing tubule containing multinucleate giant cells; (E) After *T. chebula* treatment (500 mg/kg BW/d for 35 d) showing the presence of pycnotic round spermatids (asterisk); (F) After the same treatment, as in (E), showing severe degenerative changes in the tubules. Note the tubules with disorganized germinal epithelium (asterisk), and containing only Sertoli cells and a few germ cells (arrows); (G) After the same treatment, as in (E), showing the presence of several atrophic tubules under low magnification; (H) After *T. chebula* treatment (500 mg/kg BW/d for 35 d and sacrificed 42 d after treatment discontinuation) to show normal appearance of the tubules. Scale bar = 50 µm

Histopathological investigation of testis

The seminiferous tubules in the testes of DW-treated controls showed typical histological features (Figure 3 A). On the other hand, distinct nonuniform degenerations were observed in testes in mice after treatment with Terminalia (100, 300, and 500 mg/kg BW/d) for 35 d, since both normal and affected tubules were present in the same tissue sections. Terminalia treatment induced dose-dependent histological alterations in testes because mice treated with 300 and 500 mg doses frequently contained degenerating and atrophic tubules compared to those treated with 100 mg dose of the plant (Figure 3 F-G). In general, affected tubules showed vacuolation and loosening in the germinal epithelium, detachment of germ cells (Figure 3 B-C), formation of multinucleate giant cells (Figure 3 D), and presence of pycnotic round spermatids (Figure 3 E). In severe cases, as seen in mice treated with a 500 mg dose, the affected tubules contained only Sertoli cells and a few germ cells (Figure 3 F-G). Testes in Terminalia-treated mice showed a significantly elevated percent frequency of affected tubules, reduced tubular diameter, and decreased germinal epithelium height in stage VII seminiferous tubules compared to controls (Table 2). Enumeration of germ cells in stage VII tubules showed a significant reduction in the number of preleptotene, pachytene spermatocytes, and step 7 spermatids in Terminalia-treated mice compared to controls (Table 2). However, the number of type A spermatogonia increased significantly in mice treated with Terminalia at higher doses (300 and 500 mg) (Table 2). By 42 d after treatment discontinuation, the alterations in the seminiferous tubules recovered histologically similar to controls (Figure 3 H). However, the percent frequency of affected tubules remained elevated (Table 2), and the numbers of preleptotene spermatocytes and step 7 spermatids decreased significantly compared to controls (Table 2).

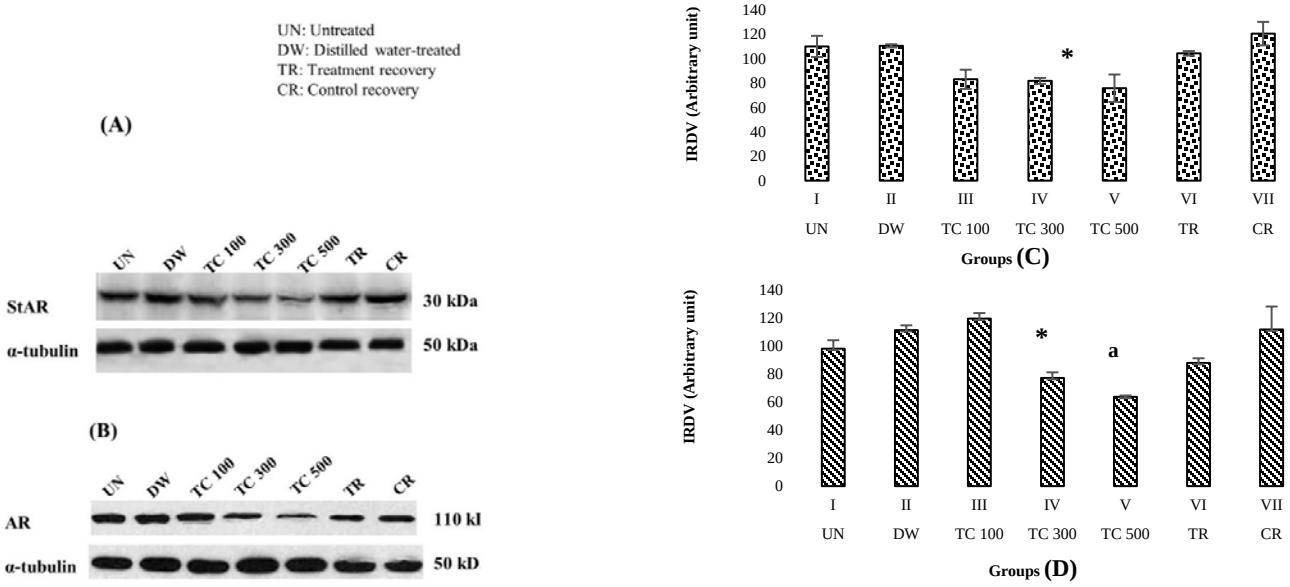


Figure 4: Immunoblot expressions of (A) StAR and (B) AR proteins in mouse testis. (A-B) After *T. chebula* treatments (100, 300, and 500 mg/kg BW/d for 35 d, and sacrificed 24 h after treatment discontinuation). In recovery groups (CR and TR), mice were sacrificed 42 d after treatment discontinuation; (C-D) Densitometric data are presented as mean of integrated relative density value (IRDV) ± S.E.M. of triplicate blots; α-Tubulin was used as a loading control; *significantly ($p<0.05$) different from controls.

Table 1: Effect of treatment with *T. chebula* (mg/kg BW for 35 d) on the testicular steroidogenic enzymes and serum level of testosterone

	Groups/Treatments						
		I	II	III	IV	V	VI#
3β HSD (U/mg tissue/h)	15.42±1.42	14.62±0.33	8.78*±1.56	9.01*±1.85	7.87*±0.60	10.69±1.51	14.93±1.46
17β HSD (U/mg tissue/h)	26.78±2.20	27.17±1.93	20.11±5.18	14.34*±1.97	14.87*±1.61	18.59±3.04	28.64±1.71
Testosterone ng/ml)	1.62±0.08	1.49±0.25	1.14±0.37	0.73*±0.10	0.61*±0.08	1.23±0.11	1.58±0.23

Table 2: Effect of treatment with *T. chebula* (mg/kg BW for 35 d) on the height of germinal epithelium, the diameter of stage VII tubules, number of affected tubules, and corrected counts of germ cells in testis.

	I	II	III	IV	V	VI#	VII #
Height (µm)	65.74±2.75	68.17±2.55	56.68±3.29	53.24±3.29*	38.99±1.78[b]	60.55±1.90	69.49±2.11
Diameter (µm)	193.16±3.43	199.00±2.87	193.32±4.67	178.65±5.58	168.35±3.34*	202.45±5.22	199.20±3.43
Affected tubules (%)	15.00±0.91	13.94±1.49	30.45±3.97*	43.44±5.10*	96.45±1.27[a]	30.11±8.04[c]	15.65±0.52
Spermatogonia A	0.50±0.016	0.43±0.025	0.55±0.029	0.62±0.031*	0.59±0.069*	0.46±0.041	0.45±0.027
Preleptotene spermatocytes	26.94±1.39	22.37±1.05	19.88±1.30*	16.68±0.58*	15.72±1.61*	17.13±1.31[c]	24.59±1.60
Pachytene spermatocytes	28.07±1.30	32.14±0.73	33.44±2.10	19.89±2.10*	17.59±0.79*	22.61±1.20	27.05±1.20
Step 7 spermatids	109.49±5.27	108.54±3.15	86.64±3.85*	61.33±4.35*	54.40±2.39[a]	79.18±7.80[c]	110.17±4.51

I: Untreated (UN); II: Control (DW), III: TC 100; IV: TC 300; V: TC 500, VI: Treatment recovery (TR); VII: DW recovery (CR)

*Values are mean ± SEM for five animals; * significantly different from controls; [b] significantly different from controls and those in group IV; [a] significantly different from controls and those in groups III and IV; [c] significantly different from recovery controls (CR) by ANOVA followed by Newman-Keuls' multiple range test; # Treatments were discontinued after 35 d, and animals were sacrificed 42 d after discontinuation of the treatment.*

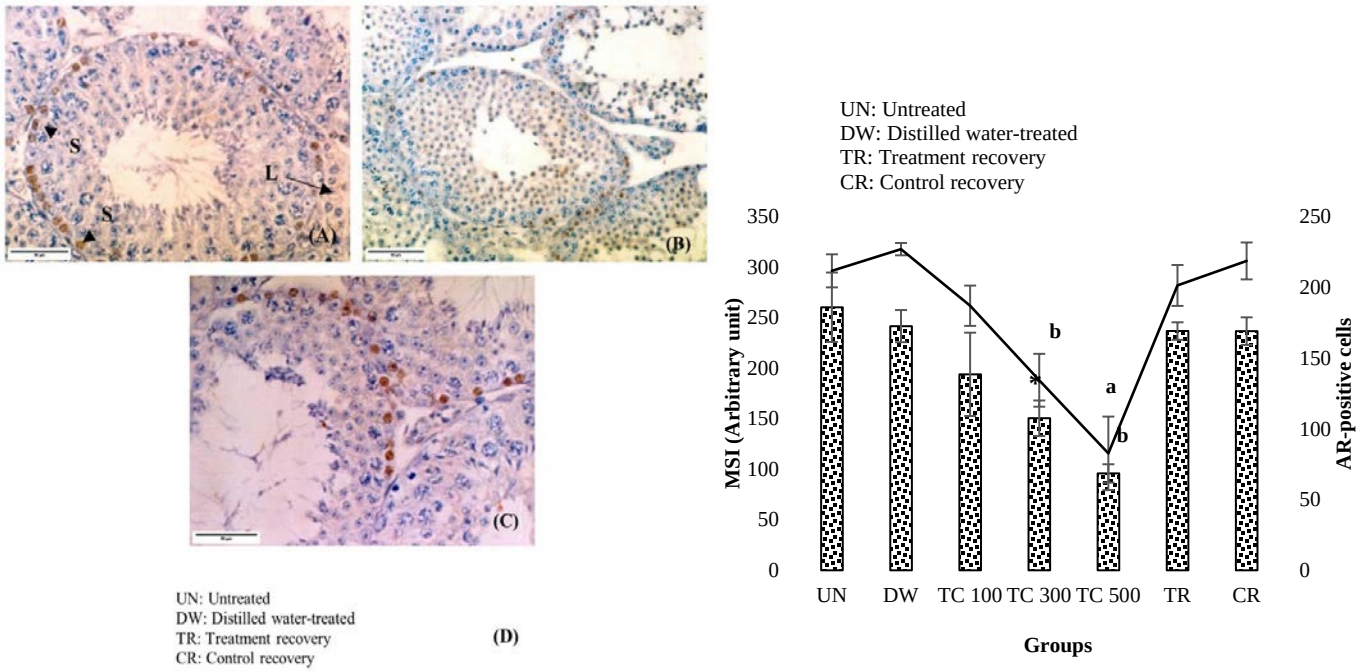


Figure 5: Immunostaining of AR in mouse testis. (A) DW-treated controls to show strong AR immunoreactivity in the nuclei of Sertoli cells (S) and Leydig cells (L); (B) After *T. chebula* treatment (500 mg/kg BW/d for 35 d, and sacrificed 24 h after treatment discontinuation) to show weak immunoreactivity in the above cells; the number of AR-positive cells and MSI index (D) reduced

significantly in the above-treated mice; (C) After *T. chebula* treatment (500 mg/kg BW/d for 35 d and sacrificed 42 d after treatment discontinuation) to show normal AR immunostaining. Immunostaining for AR was analyzed using Image J Software (NIH, Bethesda, MD, USA) to obtain the total number of AR-positive cells and mean staining intensity (MSI) in a definite area around randomly selected 5 stage VII-VIII seminiferous tubules per testis. Values are mean ± S.E.M. of three animals in each group. *significantly different from controls; [a] significantly different from controls and those in groups III-IV; [b] significantly different from controls and those in group III. Scale bar = 50 µm

Immunoblot expressions of testicular StAR and AR proteins

The immunoblot analyses of StAR (Figure 4 A) and AR (Figure 4 B) proteins showed a single immunoreactive band at about 30 and 110 kDa, respectively. Significant reductions were noticed in the testicular expressions of StAR (Figure 4 A & C) and AR (Figure 4 B & D) proteins in mice treated with a 500 mg dose of Terminalia; the 300 mg dose, however, caused a reduction in the expression of AR protein only; further, treatment with 100 mg dose of Terminalia did not affect the expressions of the above proteins compared to controls (Figure 4 A & C; Figure 4 B & D). However, by 42 d of treatment discontinuation, the alterations in the expressions of the StAR and AR proteins recovered to control levels (Figure 4 A & C and Figure 4 B & D).

Immunostaining of AR in testis

In the testis, immunostainable AR was detected in the nuclei of Sertoli and Leydig cells in controls and Terminalia-treated mice (Figure 5 A i-iii). However, a significant decline was noticed in immunostainable AR in testes of Terminalia-treated mice with increasing doses of the extract (Figure 5 A ii; see also Figure 5 B); the number of AR-positive cells and mean staining intensity (MSI) index decreased significantly in mice treated with 300 and 500 mg doses of Terminalia compared to controls and those in group III (see Figure 5 B); the alterations in the above indices, however, were not so marked in mice treated with 100 mg dose of Terminalia compared to controls (Figure 5 B). However, by 42 d of treatment discontinuation, the number of AR-positive cells and MSI index recovered to control levels (Figure 4 A iii; also see Figure 5 B).

DISCUSSION

The results of the present investigation in albino mice indicate that treatment with aqueous bark extract (100, 300, and 500 mg/kg BW) of *T. chebula* for 35 d caused reduction in weight of testis accompanied with nonuniform histologic alterations in the seminiferous tubules, such as vacuolation and loosening in germinal epithelium, detachment of germ cells, and occurrence of pycnotic round spermatids showing marginal condensation of chromatin material, and formation of multinucleate giant cells. It is to be noted here that such a nonuniform response of testis has also been described in mice in our previous studies with Terminalia and after treatment with *Albizia lebbeck*, *Curcuma longa*, *Bacopa monnieri*, *Citrus limon*, and *Coccinia indica*, etc [46-47, 72-75]. It has been suggested that nonuniform injuries in the testis occur because tubules in certain stages of spermatogenesis are sensitive to damage by various treatments [62]. It is known that stages VII-VIII of spermatogenesis are highly testosterone dependent, and any alteration in this hormone leads to a significant decrease in the frequency of the tubules in stages VII-VIII in the testis [76]. The decrease in the percent frequency of tubules VII-VIII after Terminalia treatment has already been demonstrated in our previous studies [46-47]. Treatment with a 500 mg dose of Terminalia caused suppression of spermatogenesis since testes in treated mice showed the highest number of affected tubules, severe reductions in the height of germinal epithelium and diameter of stage VII tubules due to significant premature loss of preleptotene/pachytene and step 7 spermatids compared to controls. The round spermatids appeared to be the target germ cells to the Terminalia treatment since these cells often showed marginal condensation of chromatin, premature detachment from germinal epithelium, and giant cell formation; there was also a marked reduction in the number of step 7 spermatids in stage VII tubules in testes of Terminalia-treated mice as observed in the present study. A significant decline in serum level of testosterone was noticed in Terminalia (300 and 500 mg doses)-treated mice, though 100 mg dose had no such effect. Testosterone is known to support the meiotic division of spermatocytes [77]. Therefore, a reduction in the number of pachytene spermatocytes and step 7 spermatids, as noted in the present study, is likely to be caused due to the decreased level of testosterone. Terminalia treatment adversely affected motility, viability, morphology, and number of spermatozoa in cauda epididymides. The reduction in the sperm number in treated mice is certainly due to the adverse effect of Terminalia on spermatogenesis, since round spermatids were the

most affected germ cells in the testis, as stated above. Further, Terminalia-induced suppression of spermatogenesis was reversible since sperm number returned to the control level following treatment discontinuation. On the other hand, the alterations in motility, viability, and morphology of spermatozoa might result from testosterone-mediated disturbance in the secretory functions of epididymis [78-79]. Reductions in the weights of reproductive organs, viz. epididymis, seminal vesicles, and prostate glands in treated mice, are supposed to result from reduced serum testosterone levels. Terminalia treatment did not affect libido in treated males. However, there was a remarkable suppression of fertility in these males at 24 h, 2 w, and 4 w after treatment discontinuation due to poor sperm parameters and absolute pre-implantation loss in the impregnated females. The reversal of fertility by 6 w (42 d) after treatment discontinuation suggests that the treatment did not affect spermatogonia divisions, as observed in the present study. The absence of any variations in mean body weights or weights of brain, liver, kidney, adrenal, and spleen, serum levels of ALT, AST, and creatinine, and hematological indices (data not presented) advocate that Terminalia did not produce any systemic toxicity in treated mice. This is further supported by the absence of histological alterations in the vital organs (figures not presented).

The results of the present study indicate that Terminalia treatment causes a reduction in serum levels of testosterone in albino mice. A similar observation has been found in mice after treatment with leaf extract of *Citrus limon*, in rats after treatment with ethanolic extract of *Cassia tora*, leaf extract of *Polyscias fruticose*, methanolic extract of *Cassia siamea* [65; 80-82]. It is well documented that the synthesis of testosterone by Leydig cells in the testis depends upon the expression of the StAR protein [83]. In the present study, there was a marked reduction in the expression of StAR protein in mice treated with 500 mg dose of Terminalia; also, the activities of 3β- and 17β-HSD enzymes declined significantly in testes of Terminalia-treated (300 and 500 mg/kg BW) mice. As reported earlier, 3β- and 17β HSD enzymes play a crucial role in testicular androgenesis [84]. Thus, it is expected that the reduced expression of StAR protein and the diminished activities of 3β- and 17β-HSD enzymes in the testis of Terminalia-treated mice caused the inhibition with testicular steroidogenesis, resulting in a decrease in the level of serum testosterone [65,75]. It is well established that testosterone elicits its action through the androgen receptor (AR) in Sertoli cells in seminiferous tubules and Leydig cells in interstitial tissue [85-86]. The results of AR immunostaining in the present study confirm the presence of AR in the nuclei of Sertoli cells and Leydig cells, as reported in similar studies [87,68,85]. AR in Sertoli cells is essential for germ cell nursery, formation of junctional complex, and terminal differentiation of haploid spermatids in testis [88-89]. The lack of functional AR in Leydig cells reduces testicular steroidogenesis, leading to spermatogenesis arrest predominately at the round spermatid stage [90]. Thus, androgens and AR are crucial in spermatogenesis and male fertility, as studied in NOA (non-obstructive azoospermia) groups [86]. Treatment with Terminalia (300 and 500 mg/kg BW) caused a marked reduction in the expression of AR protein in albino mice; this is further supported by a significant decline in the number of AR-positive cells and MSI index in testes of above-treated mice compared to controls. Terminalia-induced decrease in the testicular expression of StAR and AR proteins, as observed in the present study, have also been reported after treatment with ethanolic extract of young papaya seed (*Carica papaya* Linn) in albino mice and VPA-treated rats [91-93]. Therefore, lowered activities of 3β- and 17β HSD enzymes and downregulation of StAR and AR proteins might be the causes of reduced steroidogenesis and lowered serum testosterone levels in Terminalia-treated mice, as reported in several studies [94]. However, the exact mechanism of Terminalia's action on downregulating StAR and AR proteins is still unknown. There was no significant alteration in the activities of antioxidant enzymes SOD and catalase in testes of Terminalia-treated mice, which suggests that the treatment did not induce oxidative stress in testis; the increased LPO level in testes of mice treated with 500 mg dose of Terminalia, however, might be the result of severe degenerative alterations in seminiferous tubules.

CONCLUSION

The results of the present study suggest that Terminalia (*T. chebula*) treatment causes a reduction in the expressions of StAR and AR proteins and the activities of 3β- and 17β-HSD enzymes, leading to decreased testicular steroidogenesis and subsequently lowered serum testosterone levels in albino mice. Terminalia-induced alteration in the above steroidogenic markers was, however, reversible following discontinuation of the treatment. Thus, *T. chebula* reversibly affects spermatogenesis and fertility in albino mice due to altered serum testosterone without signs of toxicity.

Significance of work and its limitations

The findings of the present work in albino mice advocate the contraceptive potential of Terminalia. The study also demonstrates the mechanism of action of antifertility effects of *T. chebula* in males. Only a few studies were conducted in laboratory animals, such as mice and rats, to explain the mechanism of inhibitory action of plant extracts on spermatogenesis [66, 76]. Further, no study in albino has reported the effect of Terminalia treatment on the testicular expression of AR protein. However, more studies are needed to be carried out in other model animals to test the contraceptive efficacy of Terminalia. There are many drawbacks associated with plant-based products for fertility control. These include pronounced variations in the antifertility activity of the same plant in different animal models; the time and place of collection, appropriate identification, extraction method, and drug treatment strategy must be discussed while inferring the results. It should be noted that many plant-based drugs are extracts of whole plants and synergistic interactions between different active components of the plants are crucial for their efficiencies; in several cases, it has been established that a crude extract has exhibited better contraceptive efficacy than an isolated active compound.

ETHICAL APPROVAL

The study received ethical approval from the Mahatma Gandhi Kashi Vidyapith, Varanasi (India), Local Ethics Committee.

ACKNOWLEDGEMENTS

The authors thank Professor Shio Kumar Singh, Department of Zoology, Banaras Hindu University, Varanasi (India), for his valuable guidance and suggestions and for providing laboratory facilities.

FINANCIAL ASSISTANCE

NIL

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Prakash Chandra Gupta designed the research study, performed the experiments, analyzed the data, and wrote the manuscript. Laxmi Yadav critically revised the first draft and contributed to the revision and final draft.

REFERENCES

[1] Chaudhury R. Plant contraceptives: translating folklore into scientific application. Advances in Maternal and Child Health,. (Jelliffe DB and Jelliffe, EEP ed.), Oxford University Press, Oxford, pp.58–74 (1985)

[2] Sharma RS, Rajalakshmi M, Jeyaraj DA. Current status of fertility control methods in India. *J Biosci*, **26**, 391–405 (2001)

[3] Kaur R, Sharma A, Kumar R, Kharb R. Rising Trends towards Herbal Contraceptives. *J Nat Prod Plant Resour*, **1(4)**, 5-12 (2011)

[4] Verma S, Yadav A. Rising trends towards the development of oral herbal male contraceptive: an insight review. *Futur J Pharm Sci*, **7(1)**, 1-15, (2021)

[5] Dhanapal R, Ratna JV, Sarathchandran I, Gupta M. Reversible antispermatogenic and antisteroidogenic activities of *Feronia limonia* fruit pulp in adult male rats. *Asian Pac J Trop Biomed*, **2(2)**, S1024-S1030 (2012)

[6] Borokini FB, Oladipo GO, Komolafe OY, Ajongbolo KF, Oladipo MC. *Abelmoschus esculentus* Moench L. leaf inhibited lipid peroxidation and deproteination, and caused dose-dependent spermatogenesis and impaired hormonal synchronism in Wistar rats. *Appl Food Res*, **2(2)**, 100179 (2022)

[7] Verma PK, Sharma A, Joshi SC, Gupta RS, Dixit VP. Effect of isolated fractions of *Barleria prionitis* root methanolic extract on reproductive function of male rats: Preliminary study. *Fitoterapia*, **76(5)**, 428-432 (2005)

[8] Zhang X, Han F, Gao P, Yu D, Liu S. Bioassay-guided fractionation of antifertility components of castorbean (*Ricinus communis* L.) seed extracts. *Nat Prod Res*, **21(11)**, 982-989 (2007)

[9] Ashok P, Meenakshi B. Contraceptive effect of *Curcuma longa* (L.) in male albino rat. *Asian J Androl*, **6(1)**, 71-74 (2004)

[10] Chauhan A, Agarwal M, Kushwaha S, *et al.* Antifertility studies of *Aegle marmelos* Corr., an Indian medicinal plant on male albino rats, Egypt *J Biol*, **10(1)**, 28-35 (2008)

[11] Gupta RS, Kachhawa JBS, Chaudhary R. Antispermatogenic, antiandrogenic activities of *Albizia lebbeck* (L.) Benth bark extract in male albino rats. *Phytomedicine*, **13(4)**, 277-283 (2006)

[12] Hyacinth AA, Nwocha UC. Antifertility activity of aqueous ethanolic extract of *Hymenocardia acida* stem bark in female rats. *Iran J Reprod Med*, **9(3)**, 217-222 (2011)

[13] Lucinda LMF, Rocha CB, Reboredo MM, Faria VC, Sá R. Assessment of sperm production and reproductive organs of Wistar rats to long-term exposure of *Caesalpinia ferrea*. *An Acad Bras Cienc*, **82**, 907-914 (2010)

[14] Vasudeva N, Rai G, Sharma SK. Anti-spermatogenic Activity of *Butea monosperma* (Lam.) Kuntze Root. *Asian J Biol Sci*, **4(8)**, 591-600 (2011)

[15] Agarwal M, Sharma P, Kushwaha S. Antifertility efficacy of 50% ethanolic extract of *Calendula officinalis* in male rats. *Int J Pharm Pharm Sci*., **3(suppl. 5)**, 192-196 (2011)

[16] Dhanapal R, Ratna JV, Sarathchandran I, Gupta M. Reversible antispermatogenic and antisteroidogenic activities of *Feronia limonia* fruit pulp in adult male rats. *Asian Pac J Trop Biomed*, **2(2)**, S1024-S1030 (2012)

[17] Jain S, Jain DK, Balekar N, Jain A. Antifertility effect of chronically administered *Tabernaemontana divaricata* leaf extract on female albino mice. *Asian Pacific J Trop Dis*, **2(suppl. 1)**, 547-551 (2012)

[18] Sharma A, Sharma P, Chaturvedi M, Joshi SC. Contraceptive Efficacy of *Citrullus colocynthis* Methanolic Extract in Male Rats. *Am J Phytomedicine Clin Ther*, **2**, 229-241 (2014)

[19] Saxena P, Gupta R, Gupta RS. Contraceptive studies of isolated fractions of *Cuminum cyminum* in male albino rats. *Nat Prod Res*, **29(24)**, 2328-2331 (2015)

[20] Bhakta S, Das SK. A new approach of herbal contraception in male Swiss albino mice by *Hibiscus rosasinensis*: An in vivo study. *Int J Nat Soc Sci*, **3(3)**, 24-29 (2016)

[21] Naik BS, Dangi NB, Sapkota HP, Wagle N, Nagarjuna S, Sankaranand R. Phytochemical screening and evaluation of anti-fertility activity of *Dactyloctenium aegyptium* in male albino rats. *Asian Pacific J Reprod*, **5(1)**, 51-57 (2016)

[22] Kamble A, Reddy C, Patili S. Testicular Activity of Mice Treated With MeOH Extract of *Achyranthes aspera* Leaves. *J Adv Med Sci Appl Technol*, **3(2)**, 93 (2017)

[23] Kagbo HD, Obinna VC. Antifertility Effect of *Costus Lucanuscianuss* tem Extract in Male Albino Rats. *Int J Sci Technoledge*, **5(12)**, 119-123 (2017)

[24] Ogundola AF, Akhigbe RE, Saka WA, *et al.* Contraceptive potential of *Andrographis paniculata* is via androgen suppression and not induction of oxidative stress in male Wistar rats. *Tissue Cell*, **73**, 101632 (2021)

[25] Adeyemi AA, Haastrup AM, Alake OO, Aramide IL, Oguntola O. Semen quality and testosterone concentration in male rabbits fed *Carica papaya* leaf meal. *Nigerian J Animal Prod*, 719-722 (2024)

[26] Pokharkar RD, Saraswat RK, Kotkar S. Survey of Plants Having Antifertility Activity From Western Ghat Area of Maharashtra State. *J Herb Med Toxicol*, **4(2)**, 71-75 (2010)

[27] Oyewole T, Onumaegbu C, Makinde OM, Fapohunda SO. Bio-prospecting for fertility in humans: An update on herbal therapy. *Asian J Pharm Pharmacol*, **7(2)**, 92-99 (2021)

[28] Gupta PC. Biological and pharmacological properties of *Terminalia chebula* Retz. (haritaki)- an overview. *Int J Pharm Pharm Sci*, **4(suppl. 3)**, 62-68 (2012)

[29] Sato Y, Oketani H, Singyouchi K, *et al.* Extraction and purification of effective antimicrobial constituents of *Terminalia chebula* Retz. against methicillin-resistant Staphylococcus aureus. *Biol Pharm Bull*, **20(4)**, 401-404 (1997)

[30] Kannan P, Ramadevi SR, Hopper W. Antibacterial activity of *Terminalia chebula* fruit extract. *African J Microbiol Res*, **3(4)**, 180-184 (2009)

[31] Mehmood Z, Ahmad I, Mohammad F, Ahmad S. Indian medicinal plants: A potential source for anticandidal drugs. *Pharm Biol*, **37(3)**, 237-242 (1999)

[32] Vonshak A, Barazani O, Sathiyamoorthy P, Shalev R, Vardy D, Golan-Goldhirsh A. Screening South Indian Medicinal Plants for Antifungal Activity Against Cutaneous Pathogens. *Phyther Res*, **17(9)**, 1123-1125 (2003)

[33] Kurokawa M, Nagasaka K, Hirabayashi T, *et al.* Efficacy of traditional herbal medicines in combination with acyclovir against herpes simplex virus type 1 infection in vitro and in vivo. *Antiviral Res*, **27(1-2)**, 19-37 (1995)

[34] Badmaev V, Nowakowski M. Protection of epithelial cells against influenza A virus by a plant-derived biological response modifier Ledretan-96. *Phyther Res*, **14(4)**, 245-249 (2002)

[35] Kaur S, Grover IS, Singh M, Kaur S. Antimutagenicity of hydrolyzable tannins from *Terminalia chebula* in *Salmonella typhimurium*. *Mutat Res Toxicol Environ Mutagen*, **419(1-3)**, 169-179 (1998)

[36] Chekdaengphanao P, Jaiseri D, Sriraj P, *et al.* Anticancer activity of *Terminalia chebula*, *Terminalia bellirica*, and *Phyllanthus emblica* extracts on cholangiocarcinoma cell proliferation and induction of apoptosis. *J Herb Med*, **35**, 100582 (2022)

[37] Lee HS, Won NH, Kim KH, Lee H, Jun W, Lee KW. Antioxidant effects of aqueous extract of *Terminalia*

chebula in vivo and in vitro. *Biol Pharm Bull*, **28(9)**, 1639-1644 (2005)

[38] Sabu MC, Kuttan R. Anti-diabetic activity of medicinal plants and its relationship with their antioxidant property. *J Ethnopharmacol*, **81(2)**, 155-160 (2002)

[39] Lin K, Zhou M, Leng C, *et al.* Neuroprotective Effect of Polyphenol Extracts from *Terminalia chebula* Retz. against Cerebral Ischemia-Reperfusion Injury. *Molecules*, **27(19)**, 6449 (2022)

[40] Tasduq SA, Singh K, Satti NK, Gupta DK, Suri KA, Johri RK. *Terminalia chebula* (fruit) prevents liver toxicity caused by sub-chronic administration of rifampicin, isoniazid and pyrazinamide in combination. *Hum Exp Toxicol*, **25(3)**, 111-118 (2006)

[41] Suchalatha S, Devi CS. Protective effect of *Terminalia chebula* against experimental myocardial injury induced by isoproterenol. *Indian J Exp Biol*, **42(2)**, 174-178 (2004)

[42] Hamada SI, Kataoka T, Woo JT, *et al.* Immunosuppressive effects of gallic acid and chebulagic acid on CTL- mediated cytotoxicity. *Biol Pharm Bull*, **20(9)**, 1017-1019 (1997)

[43] Gandhi NM, Nair CKK. Radiation protection by *Terminalia chebula*: Some mechanistic aspects. *Mol Cell Biochem*, **277(1-2)**, 43-48 (2005)

[44] Aher V, Wahi AK. Immunomodulatory activity of alcohol extract of *Terminalia chebula* retz combretaceae. *Trop J Pharm Res*, **10(5)**, 567-575 (2011)

[45] Srivastav A, Chandra A, Singh M, *et al.* Inhibition of hyaluronidase activity of human and rat spermatozoa in vitro and antispermatogenic activity in rats in vivo by *Terminalia chebula*, a flavonoid rich plant. *Reprod Toxicol*, **29(2)**, 214-224 (2010).

[46] Gupta PC. Reversible Contraceptive Potential of Harad in Male Albino Mice. *Int J Pharm Pharm Sci*, **9(1)**, 288-296 (2017)

[47] Gupta PC, Yadav L. Evaluation of antispermatogenic and antifertility properties of *Terminalia chebula* (Retz.) in albino mice. Int J Reprod Contracep Obstet Gynecol, **13(7)**, 1774-1784 (2024)

[48] World Health Organization. WHO Chemical Guidelines, CG-03, 1001A/Ip. *Geneva WHO*, (1986)

[49] CPCSEA. Guidelines on the Regulation of Scientific Experiments on Animals. Government of India, New Delhi (2007)

[50] Gupta PC. Evaluation of in vitro Spermicidal Potential of *Mimusops elengi* Linn. (Bakul) in Wild Mice. *Indian J Sci*, **11(27)**, 07-14 (2014)

[51] World Health Organisation. Who Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction. Vol. 19, Cambridge university press, (1996)

[52] World Health Organisation. Examination and processing of human semen. Vol. 443, Geneva, *World Heal Organ*, (2010)

[53] Wyrobek AJ, Bruce WR. Chemical induction of sperm abnormalities in mice. *Proc Natl Acad Sci U S A*, **72(11)**, 4425-4429 (1975)

[54] Zaneveld L, Polakovski K. Collection and physical examination of the ejaculate. *Tech Hum Androl*, 147 (1977)

[55] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem*, **95(2)**, 351-358 (1979)

[56] Das K, Samanta L, Chainy GBN. *A Modified Spectrophotometric Assay of Superoxide Dismutase Using Nitrite Formation by Superoxide Radicals*, 37 (2000)

[57] Aebi H. Catalase. Methods of enzymatic analysis. Elsevier, pp.673-84 (1974)

[58] Talalay P. [69] Hydroxysteroid dehydrogenases. Hydroxysteroid + DPN + (TPN+) ⇄ Ketosteroid DPNH (TPNH) + H+. *Methods Enzymol*, **5(C)**, 512-526 (1962)

[59] Jarabak J, Adams JA, Williams-Ashman HG, Talalay P. Purification of a 17β-hydroxysteroid dehydrogenase of human placenta and studies on its transhydrogenase function. *J Biol Chem*, **237**, 345–57 (1962)

[60] Ghai C. *A Textbook of Practical Physiology*. JP Medical Ltd, (2013)

[61] Reitmann S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *Am J Clin Pathol*., 28(1), 56-63 (1957).

[62] Russell LD, Ettlin RA, Hikim APS, Clegg ED. Histological and Histopathological Evaluation of the Testis. *Int J Androl*, **16(1)**, 83-83 (1993)

[63] Abercrombie M. Estimation of nuclear population from microtome sections. *Anat Rec*, **94(2)**, 239-247 (1946)

[64] Russell LD. Mammalian spermatogenesis. *Histol Histopathol Eval testis*. (1990)

[65] Singh N, Singh SK. *Citrus limon* extract: Possible inhibitory mechanisms affecting testicular functions and fertility in male mice. *Syst Biol Reprod Med*, **62(1)**, 39-48 (2016)

[66] Salewski E. Färbemethode zum makroskopischen Nachweis von Implantationsstellen am Uterus der Ratte. *Naunyn Schmiedebergs Arch Exp Pathol Pharmakol*, **247(4)**, 367 (1964)

[67] Prins GS, Birch L, Greene GL. Androgen receptor localization in different cell types of the adult rat prostate. *Endocrinology*, **129(6)**, 3187-3199 (1991)

[68] Zhou Q, Nie R, Prins GS, Saunders PTK, Katzenellenbogen BS, Hess RA. Localization of androgen and estrogen receptors in adult male mouse reproductive tract. *J Androl*, **23(6)**, 870-881 (2002)

[69] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*, **193(1)**, 265-275 (1951)

[70] Laemmli UK. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*, **227**, 680-685 (1970)

[71] Woods A, Sherwin T, Sasse R, MacRae TH, Baines AJ, Gull K. Definition of individual components within the cytoskeleton of *Trypanosoma brucei* by a library of monoclonal antibodies. *J Cell Sci*, **93(3)**, 491-500 (1989)

[72] Gupta RS, Kachhawa JBS, Chaudhary R. Antispermatogenic, antiandrogenic activities of *Albizia lebbeck* (L.) Benth bark extract in male albino rats. *Phytomedicine*, **13(4)**, 277-283 (2006)

[73] Mishra RK, Singh SK. Reversible antifertility effect of aqueous rhizome extract of *Curcuma longa* L. in male laboratory mice. *Contraception*, **79(6)**, 479-487 (2009)

[74] Singh A, Singh SK. Evaluation of antifertility potential of Brahmi in male mouse. *Contraception*, **79(1)**, 71-79 (2009)

[75] Verma HP, Singh SK. Antifertility efficacy of *Coccinia indica* in male mice and its possible mechanisms of action on spermatogenesis. *Gen Comp Endocrinol*, **241**, 89-99 (2017)

[76] Hess RA, De Franca LR. Spermatogenesis and cycle of the seminiferous epithelium. Adv Exp Med Biol, **636**, 1-15, (2008)

[77] McLachlan RI, O'Donnell L, Meachem SJ, *et al.* Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys, and man. *Recent Prog Horm Res*, **57**, 149-179 (2002)

[78] Khole V. Epididymis as a target for contraception. *Indian J Exp Biol*, **41(7)**, 764-772 (2003)

[79] Cornwall GA. Epididymis: Sperm maturation and motility. Encyclopedia of Reproduction. pp.292–7 (2018)

[80] Khan S, Mali PC. Reversible Antifertility Effect of *Cassia tora* Linn in Male Rats. *Int J Life-Sciences Sci Res*, **3(5)**, 1415-1423 (2017)

[81] Boye A, OseiOwusu A, Koffuor G, Barku V, Asiamah E, Asante E. Assessment of *Polyscias fruticosa* (L.) Harm (Araliaceae) leaf extract on male fertility in male Wistar rats. *J Intercult Ethnopharmacol*, **7(1)**, 1 (2018)

[82] Dewal S, Lakhne R, Gupta RS. Antifertility activity of *Cassia siamea* (stem bark) in male albino rats and phytochemical analysis by GC-MS technique. *J Pharmacogn Phytochem*, **7(3)**, 3050-3053 (2018)

[83] Clark BJ, Stocco DM. StAR - A tissue specific acute mediator of steroidogenesis. *Trends Endocrinol Metab*, **7(7)**, 227-233 (1996)

[84] Ge RS, Li X, Wang Y. Leydig Cell and Spermatogenesis. *Adv Exp Med Biol*, **1288**, 111-129 (2021)

[85] Hill CM, Anway MD, Zirkin BR, Brown TR. Intratesticular androgen levels, androgen receptor localization, and androgen receptor expression in adult rat sertoli cells. *Biol Reprod*, **71(4)**, 1348-1358 (2004)

[86] Kuyucu Y, Coşkun G, Şaker D, *et al.* Immunohistochemical examination of androgen receptor and estrogen receptor alpha expressions in obstructive and non-obstructive azoospermia. *Syst Biol Reprod Med*, **67(6)**, 463-470 (2021)

[87] Zhu LJ, Hardy MP, Inigo I V., Huhtaniemi I, Bardin CW, Moo-Young AJ. Effects of androgen on androgen receptor expression in rat testicular and epididymal cells: A quantitative immunohistochemical study. *Biol Reprod*, **63(2)**, 368-376 (2000)

[88] Wang RS, Yeh S, Chen LM, *et al.* Androgen receptor in Sertoli cell is essential for germ cell nursery and junctional complex formation in mouse testes. *Endocrinology*, **147(12)**, 5624-5633 (2006)

[89] Holdcraft RW, Braun RE. Androgen receptor function is required in Sertoli cells for the terminal differentiation of haploid spermatids. *Development*, **131(2)**, 459-467 (2004)

[90] Wang RS, Yeh S, Tzeng CR, Chang C. Androgen receptor roles in spermatogenesis and fertility: Lessons from testicular cell-specific androgen receptor knockout mice. *Endocr Rev*, **30(2)**, 119-132 (2009)

[91] Satriyasa BK, Indrayani AW, Arijana IGKN, Ruspawan DM, Ryalino C. Ethanolic extracts of young papaya seed (*Carica papaya*, linn) decrease spermatocytes, leydig cells, and androgen receptor of leydig cells of adult mice (*Mus musculus*). *J Glob Pharma Technol*, **12(1)**, 167-171 (2020)

[92] Ruspawan IDM, Bakta IM, Karmaya INM, Satriyasa BK, Arijana IGKN. Ethanol extract of young papaya seeds (*Carica Papaya*, L) lower the level of testosterone, spermatozoa count and expression of androgen receptors in sertoli cells of adult mice (*Mus Musculus*). *Biomed Pharmacol J*, **14(1)**, 207-213 (2021)

[93] Sitthichai I, Wannisa S, Apichakarn S, Bungorn S. Protective Effect of *Momordica cochinchinensis* (L.) Spreng Aril Extract on Essential Testicular Markers in Rats Induced with Valproic Acid. *Int J Morphol*, **35(3)**, 992-999 (2017)

[94] Dhanabalan S, Mathur PP. Low dose of 2,3,7,8 tetrachlorodibenzo-p-dioxin induces testicular oxidative stress in adult rats under the influence of corticosterone. *Exp Toxicol Pathol*, **61(5)**, 415-423 (2009)