ISSN (Online) 2249-6084 (Print) 2250-1029

International Journal of Pharmaceutical and Phytopharmacological Research (eIJPPR)
[Impact Factor – 0.852]

Journal Homepage: www.eijppr.com

Research Article

Safety Evaluation of *Tribulus Terrestris* on the Male Reproductive Health of Laboratory Mouse

Mrinalini Kumari, Praveen Kumar and Poonam Singh*
Department of Zoology, MMV, Banaras Hindu University, Varanasi-221005, India.

Article info

Article History:
Received 11 November 2014
Accepted 29 January 2015

Keywords:
Epididymis, Infertility, Testis, *Tribulus terrestris* (TT)

Abstract

Tribulus terrestris (TT) has emerged as an instant plant for the treatment of sexual dysfunctions and fertility related disorders in the males. The present study was aimed to assess the safety efficacy of the fruit extract of TT on the male reproduction. Animals of Group I served as control while that of II and III were administered with 100mg/kgBW/day and 200mg/kgBW/day of the fruit extract of TT, respectively, for 28 days. Testicular histology, sperm parameters, serum clinical biochemistry (SGOT, SGPT and creatinine) and tissue biochemistry (fructose in the seminal vesicle, sialic acid in the epididymis, antioxidant enzymes activity, LPO, LDH and ALP in the testis) were carried out to establish the safety of the fruit extract. Safety of the extract was evidenced by the unaltered body weight and serum clinical biochemistry. Administration of the fruit extract of TT neither interfered with the weights of the reproductive organs nor altered the sperm indices in the cauda epididymidis as well as the spermatogenic activity in the testis. The unaltered androgen - dependent biochemical markers i.e. sialic acid in the epididymis and fructose in the seminal vesicle indicated the normal status of the testosterone level. Unaltered activities of testicular antioxidant enzymes and the level of LPO suggest that the fruit extract does not cause oxidative stress. Further the unaltered activities of LDH and ALP in the testis represent normal physiological activity of the organ that could be correlated with the uninterrupted spermatogenic activity. It can, therefore, be concluded that the fruit extract of this herb could effectively be used as a natural remedy in treatment of male reproductive disorders without causing any side effects.

1. INTRODUCTION

Since ancient times, plants are being used globally across varied cultures throughout the known civilizations as a valuable and safe natural source of medicine and as agents of therapeutic, industrial and environmental utilities. Medicinal plants are the part of human society from the early ancient civilization, used to combat diseases because of the presence of certain active components of the therapeutic values [1]. The Indian and Chinese systems of traditional medicinal plants are well established as curatives for numerous ailments, with written records dating back to thousands of years. The World Health Organization (WHO) reports suggests, 70%–80% of the world population relying on the plants for primary healthcare [2, 3].

Our country, India, is a varietal emporium of medicinal plants and alone possesses almost 8.0% of the estimated biodiversity of the world with around 0.126% million species [4]. The use of medicinal plants, possessing pharmacologically active components, is extensively increasing in the world due to their natural property and minimum or no side effects [5]. Therefore, in the recent years, the modulation of the diseased states by using medicinal plant products as possible therapeutic measures has emerged as a subject of active scientific investigations and much attention is being focused on the use of medicinal herbs to prevent and control the diseases [6].

Plants have a long folklore of use in aiding fertility. In the past few years, some of the traditional herbs have emerged as an 'instant' treatment for sexual and erectile dysfunctions [7]. Findings of Riaz and coworkers [8] have justified the traditional use of herbal combinations of *Tribulus terrestris*, *Withania somnifera, Mucuna pruriens and Argyreia speciosa* in improving the sexual dysfunction and other fertility disorders. These authors have, further, put forth the scope of combination of the herbal extracts in preventing the infertility states. Later, Adaay and Mattar [9] have reported the beneficial effects of herbal combinations of *Tribulus terrestris, Phoenix dactylifera* and *Nasturtium officinale* on semen quality, sex hormones and reproductive performance. Among the combination of these herbs, *Tribulus terrestris*, being used as a folk medicine against various diseases [10], has emerged as a new source of antioxidant for infertility therapy [11]. This plant has been used in Ayurveda and Chinese medicine for a variety of ailments [12] (Qin and Yu, 1998). Phytochemical analysis of the whole plant of TT shows the existence of major chemical constituents such as flavonoids, steroidal saponins, alkaloids and lignanamides [13, 14]. The fruit of TT also contains a number of different chemical substances including saponins, glycosides, alkaloids, resins, tannins, sugar, sterols and essential oil [15, 16]. Among the saponins isolated from the fruit extract of TT, protodioscin is the most popular active constituent present in the extract [17]. The existence of flavonoid compound in the ethanolic fruit extract of TT has also been well documented [14, 18]. Reports suggest that the plant extract of TT bears several pharmacological properties such as anabolic, pain-relieving, cardioprotective, antimicrobial, antihelminthic, antiurolithiatic, diuretic, central nervous system stimulatory, gastrointestinal protective, anticancereous, anticytotoxic, hepatoprotective, anti-inflammatory, antidiabetic, antilipidemic, antihypertensive, aphrodisiac and antioxidative properties [19-22]. The latter two properties raise the scope of the plant extract of TT in infertility therapy.

Therefore, in order to validate the use of *Tribulus terrestris* in case of male infertility, the aim of the present study is to explore its safety and efficacy taking laboratory mouse as an experimental model.

Corresponding Author:
Dr. Poonam Singh,
Associate Professor, Department of Zoology,
MMV, Banaras Hindu University, Varanasi-221005, India.
Tel. No.: +91-9450181646
Email: poonom@gmail.com

2. MATERIALS AND METHODS

2.1 Animal selection

Thirty Swiss strain adult (12 weeks) male mice weighing about 25-30g were used for the present investigation. The animals were housed under standard laboratory conditions and maintained on pelleted diet and water *ad libitum.* Approval from the Animal Ethical Committee, Banaras Hindu University, Varanasi, India was obtained for the animal study plan (No. Dean/11-12/CAEC/263).

2.2 Experimental design, drug and dosage

After recording the initial body weights all the animals were divided into three groups of ten each. Mice of Group I served as vehicle-treated control while that of Groups II and III were administered with TT at the doses of 100mg/kgBW/day and 200mg/kgBW/day, respectively, for 28 consecutive days.

The fruit of TT was purchased from the local market of Varanasi and got identified from the Department of Botany, Banaras Hindu University (Voucher No. Zygo-2013-1). The extract of the fruit was prepared by adopting the method of Hussain and coworkers [23] and dissolved in distilled water for administration. The extract of both the doses were administered orally through gavage.

2.3 Animal sacrifice and collection of the reproductive organs

After recording the final body weights, the animals were sacrificed by cervical dislocation. Blood was collected by cardiac puncture to measure the level of serum testosterone. The testis, epididymis, seminal vesicle, liver and kidney were dissected out and processed for the following studies:

2.4 Weight of the reproductive organs

The testis, epididymis and seminal vesicle were blotted, free of blood and weighed to calculate the gonadosomatic index by using the following formula:

Gonadosomatic Index (GSI) = (Gonad weight/total body weight) ×100

2.5 Testicular histology

Bouin's fixed testis was dehydrated and embedded in paraffin. Sections of 5µm thickness were taken from the mid portion of each testis, stained with Periodic Acid Schiff reagent followed by counterstaining with Ehlrich's Haematoxylin.

2.6 Sperm assessment

The sperm motility, viability and count were assessed according to the WHO laboratory manual [24]. Evaluation of sperm abnormality was based on the criteria of Wyrobek and Bruce [25] and Zaneveld and Polakoski [26].

2.7 Serum testosterone assay

Serum testosterone was measured by ELISA, as described in the instructions provided in the kit (LDN, Nordhorn).

2.8 Biochemical estimations

Concentrations of epididymal sialic acid and seminal vesicular fructose were estimated by the methods of Aminoff [27] and Lindner and Mann [28], respectively.

2.9 Antioxidant enzyme estimations

The testis from each mouse was dissected out carefully and washed with ice-cold physiological saline solution. The tissue was weighed and 10% homogenate was prepared in ice-cold phosphate buffer (0.05M, pH 7.0) by the method of Vaithinathan and coworkers [29]. The supernatant was used for the enzyme assays after estimating the protein content by the method of Lowry and coworkers [30] using bovine serum albumin as a standard. All the readings were measured spectrophotometrically at the specified O.D.

Superoxide dismutase

Superoxide dismutase (EC 1.15.1.1) was assayed by the method of Marklund and Marklund [31]. The enzyme activity was expressed as units per milligram protein.

Catalase

Catalase (EC 1.11.1.6) was assayed by the method of Claiborne [32]. The enzyme activity was expressed as micromoles H_2O_2 consumed per min per milligram protein.

Glutathione peroxidase

Glutathione peroxidase was assayed by the method of Flohe and Gunzler [33] and activity was expressed in units per milligram of protein.

Lipid peroxidation estimation:

The concentration of malonaldehyde (MDA) was measured in the tissue supernatant using MDA concentration as a surrogate measure [34]. The values were expressed as nanomoles MDA produced per milligram protein.

2.10 Testicular functional markers

Lactate Dehydrogenase

The activity of lactate dehydrogenase (LDH) was estimated in the tissue supernatant using LDH (P-L) kit (Mod. IFCC method).

Alkaline phosphatase

The activity of alkaline phosphatase (ALP) was estimated in the tissue supernatant using COGENT diagnostic kit.

2.11 Liver and Kidney Function tests

The activities of SGOT and SGPT were determined in the serum using AVECON kit. The creatinine level in the serum was determined using reagent kit of CHEMPAK (Reckon Diagnostic Pvt. Ltd.).

2.12 Statistical analysis

All the data were analyzed statistically by one way ANOVA followed by Newman-Keul's test. Values were considered significant at $p < 0.05$.

3. RESULTS

3.1 Body Weight

No significant differences were observed in the initial and final body weights when the treated groups were compared with the controls (Fig. 1A).

3.2 Weight of the reproductive organs

The weights of the testis, epididymis and seminal vesicle of the mice administered with low and high doses of the fruit extract of TT exhibited no significant alterations as compared with the controls (Fig. 1B).

3.3 Histological study of the testis

The testis of the mice of the vehicle-treated control exhibited normal histological features (Fig. 2A). The tubular compartment of the testis was comprised of the seminiferous tubules exhibiting full swing of spermatogenesis with successive stages of transformation from spermatogonia to spermatozoa. In the seminiferous tubules, the seminiferous epithelium was formed by two types of cells: the non-germinative Sertoli cells and the spermatogenic germ cells. The germ cells in the seminiferous tubules of the controls were well organized with constant proliferation and maturation with the formation of spermatozoa. The Sertoli cells were well developed. In the intertubular spaces the connective tissue contained blood and lymph vessels, fibroblasts, elastic fibers and well-structured Leydig cells. Oral administrations of 100mg/kgBW/day and 200mg/kgBW/day of the fruit extract of TT did not interfere in the spermatogenic activity and exhibited normal histological features similar to that of the controls (Fig. 2B-2C). Leydig cells also presented normal histology.

3.4 Sperm Assessment

The percentage of sperm motility, viability, morphological abnormality and the sperm count remained unaltered in the mice administered with low and high doses of TT (Fig. 3A-3B).

3.5 Serum testosterone level

Administration of TT at any dose (100mg/kgBW/day or 200mg/kgBW/day) did not alter the level of serum testosterone, thus remained comparable to the control. (Fig. 4).

3.6 Biochemical estimations

The concentrations of sialic acid in the epididymis and fructose in the seminal vesicle also remained unaltered in the mice administered with the low and high doses of the fruit extract of TT as compared with the controls (Fig. 5A-5B).

3.7 Antioxidant enzyme activities

No significant alterations were observed in the activities of testicular SOD, CAT and GPx in the mice administered with the low and high dose of the fruit extract of TT as compared with the controls (Fig. 6A-6C).

3.8 Level of LPO

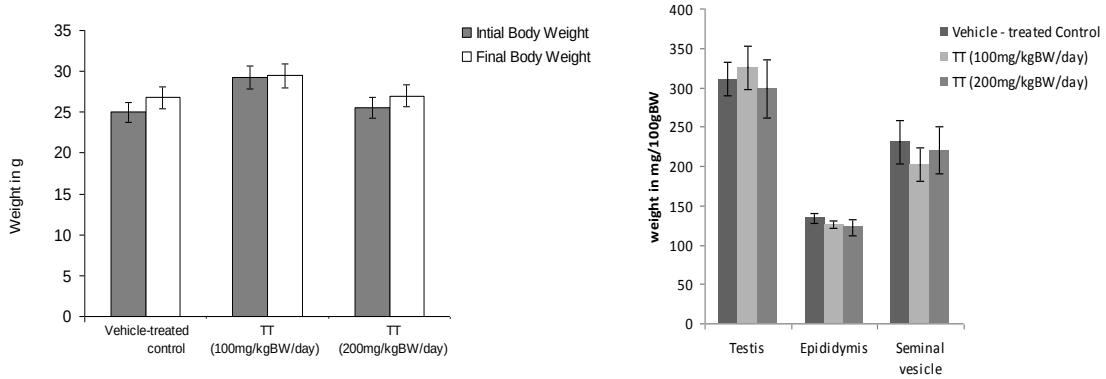
The level of testicular LPO also remained unaltered in the mice administered with the low and high dose of the fruit extract of TT as compared with the control (Fig. 6D).

3.9 Testicular functional markers

The activities of LDH and ALP in the testis exhibited no significant alterations in the mice administered with the low and high dose of the fruit extract of TT as compared with the control (Fig. 7A-7B).

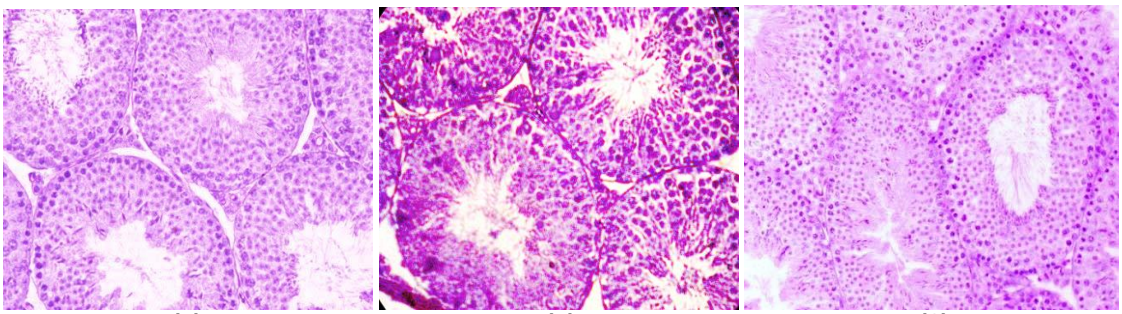
3.10 Liver and Kidney function tests

The activities of SGOT and SGPT and the level of creatinine in the serum remained unaltered in the mice administered with any dose of the fruit extract of TT, as compared with the control (Fig. 8A-8C).



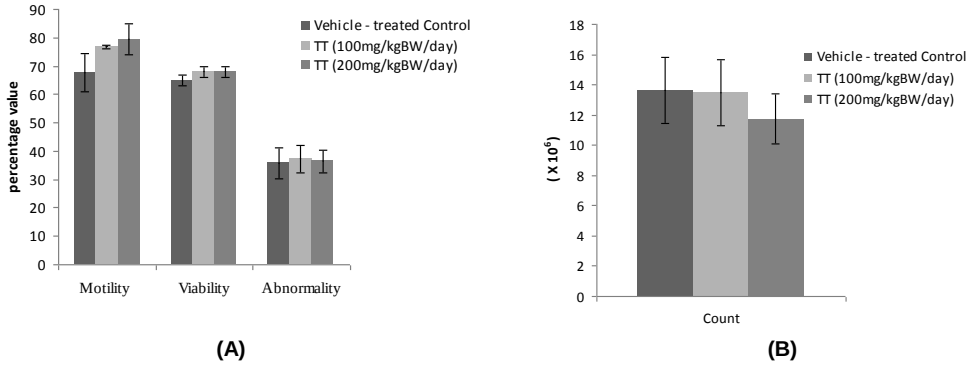
(A) **(B)**

Fig. 1: Effect of oral administration of the fruit extract of TT (100mg/kgBW/day and 200mg/kgBW/day) on the **(A)** body weight **(B)** weights of the reproductive organs (values are mean ± SEM of five animals)



(A) **(B)** **(C)**

Fig. 2 T.S. of the testis of **(A)** control mouse showing normal histological features, **(B)** the mouse administered orally with 100mg/kgBW/day of the fruit extract of TT showing histological features similar to the control and **(C)** the mouse administered orally with 200mg/kgBW/day of the fruit extract of TT showing histological features similar to the control.



(A) **(B)**

Fig. 3: Effect of oral administration of the fruit extract of TT (100mg/kgBW/day and 200mg/kgBW/day) on the **(A)** percentage of sperm motility, viability and abnormality, and **(B)** sperm count in the cauda epididymidis (values are mean ± SEM of five animals)

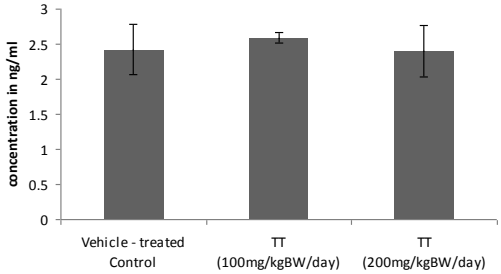
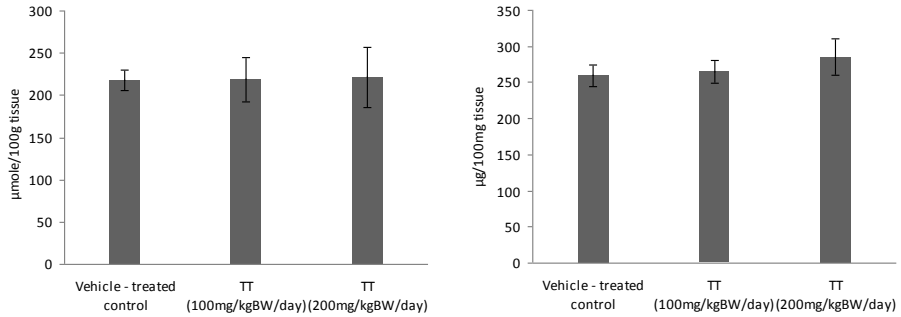
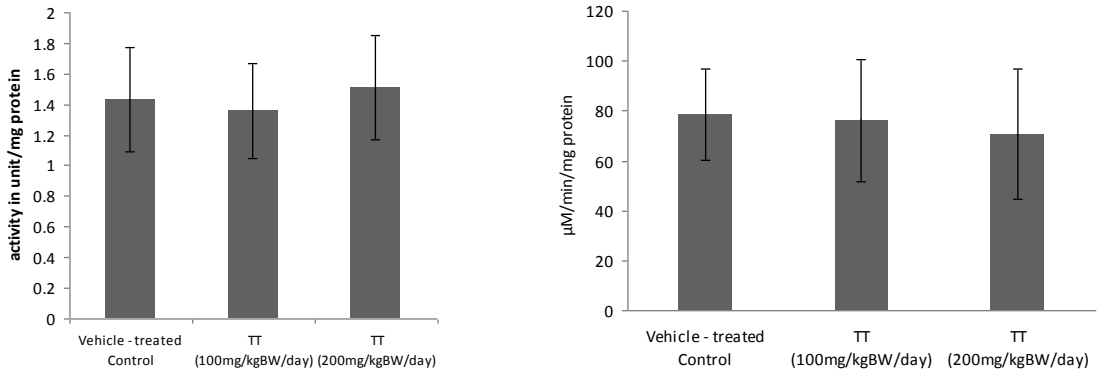


Fig. 4: Effect of oral administration of the fruit extract of TT (100mg/kgBW/day and 200mg/kgBW/day) on the serum testosterone level (values are mean ± SEM of five animals)



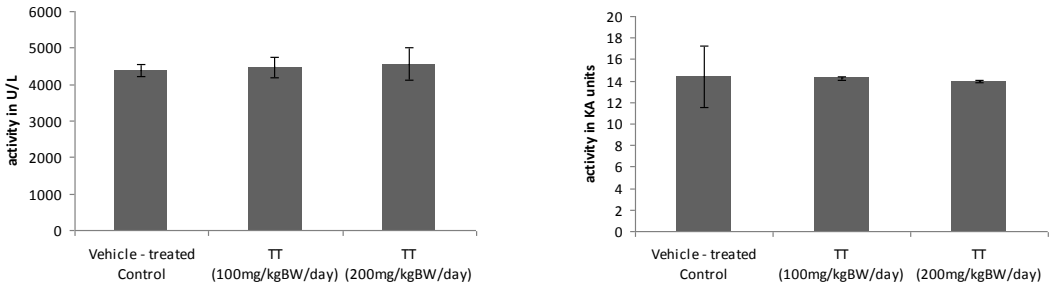
(A) **(B)**

Fig. 5: Effect of oral administration of the fruit extract of TT (100mg/kgBW/day and 200mg/kgBW/day) on the concentration of **(A)** sialic acid in the epididymis and **(B)** fructose in the seminal vesicle (values are mean ± SEM of five animals)



(C) **(D)**

Fig. 6: Effect of oral administration of the fruit extract of TT (100mg/kgBW/day and 200mg/kgBW/day) on the **(A)** activity of SOD, **(B)** activity of catalase, **(C)** activity of GPx, and **(D)** level of LPO in the testis (values are mean ± SEM of five animals)



(A) **(B)**

Fig. 7: Effect of oral administration of the fruit extract of TT (100mg/kgBW/day and 200mg/kgBW/day) on the activity of **(A)** LDH and **(B)** ALP in the testis (values are mean ± SEM of five animals)

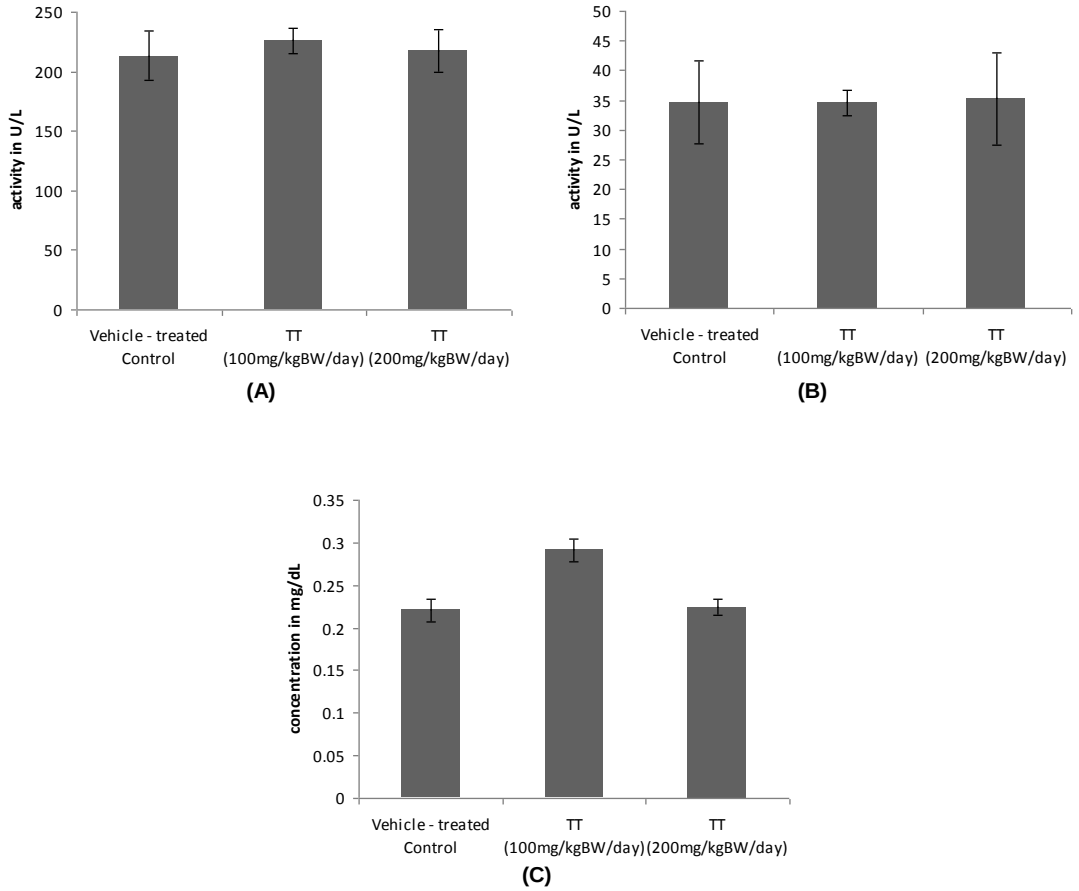


Fig. 8: Effect of oral administration of the fruit extract of TT (100mg/kgBW/day and 200mg/kgBW/day) on the activity of **(A)** SGOT **(B)** SGPT **(C)** Creatinine in the serum (values are mean ± SEM of five animals)

4. DISCUSSION

The present study focuses the safety and efficacy of the fruit extract of TT taking reproductive health of the males into consideration.

No significant alteration in the body weight of the TT-treated mice suggest that the fruit extract of TT is not interfering in the metabolic activity. This is in contrast to the finding of Singh and coworkers [35] who have reported increased body weight following administration of 6mg/kgBW/day of the fruit extract of TT in rats for 30 consecutive days, thus, indicating its anabolic property. The confliction in the present result and that of Singh and coworkers [35] may be due to variations in the response of different species towards the body weight against herbal extracts.

The weight of the male reproductive organs provides a useful fertility/reproductive risk assessment in the experimental studies. It is expected that there should be no difference in the organs weight between animals of similar body weight unless some specific organs are affected by the treatment [36]. Testicular size is the best primary assessment for spermatogenesis because the tubules and germinal elements account for approximately 98% of the testicular weight [37]. Its weight is a general indicator of overall testicular health, reflecting changes in the seminiferous tubule fluid retention or the germ cell loss [38]. The weights of the testis as well as the epididymis are associated with the spermatozoa content [39]. In the present study, the unaltered weight of the testis in the TT-treated mice is indicating the overall normal testicular health and effectively reflecting the germ cell differentiation and spermatogenesis. No difference in the weights of the epididymis in the group administered with low and high dose of TT could be correlated with the unaltered weights of the testis and is an indicator of the normal spermatozoal content. Since, the weight of the seminal vesicle is an indicator of the testosterone level [40], therefore, its unaltered weight in the TT-treated mice indicates the normal androgenic activity.

An ICH guideline (Step 4, 29 November, 1995, amended in November, 2000) recommends histopathological examination of the testis as the most sensitive method for the detection of the effects of drugs on spermatogenesis and fertility. The testis consists of two important functional components: the interstitial cells of Leydig and the seminiferous tubules. The seminiferous tubules give rise to spermatozoa through a process known as spermatogenesis. The regulation of spermatogenesis is quite complex and requires testosterone from the Leydig cells present in the intertubular spaces and a supportive role of the Sertoli cells within the seminiferous tubule. In the present study, the well organized, full population of the germ cells and unaltered structure of the Sertoli cells in the seminiferous tubules as well as the normal appearance of Leydig cells in the intertubular space reflects the uninterrupted regulation of spermatogenesis in the testis of the TT-treated mice.

An ICH guideline (Step 4, 29 November, 1995, amended in November, 2000) recommends sperm analysis including the sperm morphology as an optional procedure for the confirmation or better characterization of the observed toxicity, such as pathological changes or decreased fertility. Sperm count is one of the most sensitive tests for spermatogenesis and highly correlated with the fertility [41] whereas sperm motility is a chief parameter of post-testicular function [42]. Sperm vitality is one of the basic elements of semen analysis and is especially important in samples where many spermatozoa are immotile, to distinguish between immotile dead sperms and immotile live sperms [43]. Therefore, the unaltered sperm motility, viability and count reflect the normal testicular function and male fertility in the mice administered with the fruit extract of TT.

The structural and functional integrity of the accessory sex glands in the males are androgen-dependent [44]. According to Gonzales [44] measurement of seminal fructose, used as a marker of seminal vesicular function, is essential for spermatozoal metabolism and motility as an energy source. The synthesis and/or secretion of sialic acid in the epididymis is also under androgenic control [45]. Therefore, no alterations in the levels of seminal vesicular fructose and epididymal sialic acid following administration of TT reflect the optimum serum testosterone level.

SOD, a superoxide free radical scavenging enzyme, is considered as the first line of defense against the deleterious effect of oxygen radicals in the cells which scavenges reactive oxygen radical species by catalyzing the dismutation of O^{2-} radical to H_2O_2 and O_2. Thus, the presence of SOD in various compartments of our body

enables it to dismutate O^2- radicals immediately and protects the cells from oxidative damage. CAT is as an antioxidant enzyme which removes H_2O_2, generated by SOD, to extremely reactive molecule such as OH [46], thereby converting it into H_2O and O_2. GPx, present in the cytoplasm, is another antioxidant enzyme that catalyses the reduction of variety of hydrogen peroxide (ROOH and H_2O_2) using glutathione (GSH) as a substrate, thereby protecting the mammalian cells against oxidative stress [47]. In the present study, absence significant alterations in the activities of SOD, CAT and GPx following administration of the fruit extract of TT, suggests the maintained balance between ROS generation and elimination, thus, indicating healthy condition of the testis.

The level of LPO products has been widely used as an index of oxidative stress [48]. Hence, its unaltered level in the testis, following administration of the fruit extract of TT, further supports the balance between ROS generation and elimination indicating that the metabolism and functions of the testis are not deregulated.

According to Pant and Srivastava [49] the LDH activity has a direct effect on testicular functions such as sperm count and sperm production, as well as sperm morphology. Alkaline phosphatase is also widely distributed in the testis and plays an important role in the physiology of sperm [50]. No significant alterations in the activities of testicular LDH and ALP, following administration of the fruit extract of TT, indicate that the extract is not exerting any direct effect on the testis and thus maintains its normal physiology.

The SGOT and SGPT are the reliable determinants of liver parenchymal injury [51]. The measurement of serum creatinine is used as an index of renal function in the clinical practice [52]. The present study reveals that administration of both the doses of TT did not affect the levels of SGOT, SGPT and creatinine hence, indicating its safe use.

5. CONCLUSION

The fruit extract of TT could effectively be used as a natural supplement for male reproductive disorders. The extract up to the dose of 200mg/kgBW/day could be safely used as supplement or in treatment of various diseases for a month without any side-effect on the male reproduction.

6. ACKNOWLEDGEMENTS

The authors are thankful to Banaras Hindu University, Varanasi, for providing financial assistance through CRET, University Grants Commission, New Delhi. The first and second authors are thankful to University Grant Commission, New Delhi, for providing financial assistance to carry out the present work.

REFERENCES

1. Britto AJD, Gracelin DHS. Comparative phytochemical screening of flowers of a few medicinal plants, *Int. J. Appl. Biol. Pharm. Technol.*, 2011, 2: 19-22.
2. World Health Organization. Summary of WHO guidelines for the assessment of herbal medicines, *Herbal Gram.*, 1993, 28: 13-14.
3. McKay DL, Blumberg JB. A review of the bioactivity of South African herbal teas: Roobos (*Aspalathus linearis*) and Honey comb (Cyclopia intermedia), *Phytother. Res.,* 2007*,* 21: 1-16.
4. Jain B, Kumane CJ, Bhattacharya SS. Medicinal flora of Madhya Pradesh and Chattisgarh: A Review, *Indian J. Trad. Knowl.*, 2006, 5: 237-242.
5. Gurib-Fakim A. Medicinal plants: traditions of yesterday and drugs of tomorrow, *Mol. Aspects Med*., 2006, 27: 1-93.
6. Devasagayam TPA. Introduction to serial reviews: Recent advances in Indian herbal drug research, *J. Clin. Biochem. Nutr.,* 2007, 40: 73.
7. Adimoelja A. Phytochemicals and the breakthrough of traditional herbs in the management of sexual dysfunctions, *Int. J. Androl.*, 2002, 23: 82-84.
8. Riaz A, Khan RA, Ahmed S, Afroz S. Assessment of acute toxicity and reproductive capability of a herbal combination, *Pak. J. Pharm. Sci.*, 2010, 23: 291-294.
9. Adaay MH, Mattar AG. Effect of aqueous and ethanolic extracts of *Tribulus terrestris, Phoenix dactylifera* and *Nasturtium officinale mixture* on some reproductive parameters in male mice, *J. Baghdad Sci.,* 2012, 9: 640-650.
10. Adaikan PG, Gauthaman K, Prasad RN. History of herbal medicines with insight on the pharmacological properties of *Tribulus terrestris*, *The Aging Male*, 2001, 4: 163-169.
11. Zheleva-Dimitrova D, Obreshkova D and Nedialkov P. Antioxidant activity of Tribulus terrestris- A natural product in infertility therapy, *Int. J. Pharm. Pharmaceut. Sci.,* 2012, 4: 508-511.
12. Qin S, Yu B. Investigation on resources and commodities of Chinese medicinal herbs of Jili, *Zhongyao Zazhi*, 1998, 23: 709-711.
13. El-Tantawy WH, Hassanin LA. Hypoglycemic and hypolipidemic effects of alcoholic extract of *Tribulus alatus* in streptozotocin-induced diabetic rats: A comparative study with *T. terrestris* (Caltrop), *Indian J. Exp. Biol.*, 2007, 45: 785-790.
14. Gomathi S, Shanmugapriya A, Bharathi V, Gayathri G, Karpagam T. Antimicrobial activities and phytochemical studies of aqueous and ethanolic fruit extracts of *Tribulus terrestris*, *IJPI'S J. Pharma. Herbal Formul.*, 2012, 2: 47-51.
15. Raja M, Venkataraman R. Pharmacognostical studies on *Tribulus terrestris* and *Tribulus alatus. Der Pharmacia Sinica*, 2011, 2: 136-139.
16. Kumar A, Bhardwaj A. Comparative, qualitative and quantitative chemotypic characterization among North India *Tribulus terrestris*, *Int. Res. J. Pharm*, 2012, 3: 212-218.
17. Koumanov F, Bozadjieva E, Andreeva M, Platonva E, Ankov V. Clinical trial of *Tribestan*, *Expt. Med.,* 1982, 1: 2-4.
18. Mitra N, Mohammad-Mehndi D, Mohammad-Reza Z. *Tribulus terrestris* (Zygophyllaceae) flavonoid compounds, *Int. J. Mod. Bot.,* 2012, 2: 35-39.
19. Mohd J, Ansari JA, Ali A, Ahmed J, Ali M, Tamboli E. Pharmacological scientific evidence for the promise of *Tribulus terrestris*, *Int. Res. J. Phar,* 2012, 3: 403-406.
20. Bhandari B, Deepti C, Kohli SK. Pharmacological effects of *Tribulus terrestris*. A Review. *Int. J. Contem. Med*. 2013, 1.
21. Verma P, Galib, Patgiri BJ, Prajapato PK. *Tribulus terrestris:* A phytopharmacological review, *J. Ayur. Hol. Med.*, 2013, 1: 37-43.
22. Hashim S, Bhakt T, Marwat KB, Jan A. Medicinal properties, phytochemistry and pharmacology of *Tribulus terrestris* (Zygophyllaceae), *Pak. J. Bot.*, 2014, 46: 399-404.
23. Hussain AA, Muhammad AA, Ibrahim HH, Abbas A. Study of the biological activities of *Tribulus terrestris* extracts, *World Acad. Sci. Engin. Technol.*, 2009, 57: 433-435.
24. World Health Organization. WHO laboratory manual for the examination of human semen and semen-cervical mucus interaction. New York: Cambridge University Press, Cambridge, 2010.
25. Wyrobek AJ, Bruce WR. Chemical induction of sperm abnormalities in mice, *Pro. Natl. Acad. Sci.,* USA, 1975, 72: 4425-4429.
26. Zaneveld L, Polakoski KL. Collection and physical examination of the ejaculate. In: Hafez ESE, ed. Techniques of Human Andrology, New York: Elseveir/North Holland Press, 1977, 147-172.
27. Aminoff D. Methods for the quantitative estimation of N-acetyl neuramic acid and their application to hydrolysates of sialomucoids, *Biochem. J.*, 1961, 81: 384-392.
28. Lindner HR, Mann T. Relationship between the content of androgenic steroids in the testes and the secretory activity of the seminal vesicles in the bull, *J. Endocrinol.*, 1960, 21: 341-360.
29. Vaithinathan S, Saradha B, Mathur PP. Methoxychlor-induced alteration in the levels of HSP70 and clusterin is accompanied with oxidative stress in adult rat testis, *J. Biochem. Molecular Toxicol.*, 2008, 23: 29-35.
30. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, 1951, 193: 265-275.
31. Marklund S, Marklund G. Involvement of superoxide anion radical in antioxidation of pyrogallol and a constituent assay for superoxide dismutase, *Eur. J. Biochem.,* 1974, 47: 469-474.

32. Claiborne A. Catalase activity, In: Greenwald R (ed) CRC Handbook of methods for oxygen radical research, CRC Press, Boca Raton, Fla., 1985, 283-284.
33. Flohe L, Gunzler WA. Assay of glutathione peroxidase, In: Methods Enzymol., (ed. Packer L.), Academic Press, New York, 1984, 105: 114-121.
34. Okhawa H, Ohishi N, Yagi K. Assay of lipid peroxide in animal tissue by Thiobarbituric Acid Reaction, *Anal. Biochem.*, 1979, 95: 351-358.
35. Singh PK, Singh AP, Gupta AK, Chaudhary S. Beneficial effects of aqueous fruit extract of *Tribulus terrestris* on testicular and serum biochemistry of albino rats, *J. Ecophysiol. Occup. Hlth.,* 2009, 9: 217-223.
36. Bailey SA, Zidell RH, Perry RW, Relationship between organ weight and body/brain weight in the rat: What is the best analytical endpoint? *Toxicol. Pathol*., 32, 448-466, 2004.
37. Sherines RJ, Howards SS. Male fertility, In: Harrison JH, Gittes RF, Perimutter AD, Stamey TA, Walsh PC, editors, Campbell's Urology, Philadelphia: Saunders WB. Co. 1987, 715.
38. Creasy DM. Histopathology of the male reproductive system II: Interpretation, Current protocols in toxicology (Maines, MD, eds.), John Wiley and Sons, Inc., Hoboken NJ. 2002, 2: 16.4.1-16.4.14.
39. Robaire B, Zirkin BR. Hypophysectomy and simultaneous testosterone replacement: Effects on male reproductive tract and epididymal Δ4-5 α reductase and 3β-hydroxysteroid dehydrogenase, *Endocrinol.*, 1981, 109: 1225-1233.
40. Elbetieha A, Al-Thani AS, Al-Thani RK, Darmani H, Owais W. Effects of chronic exposure of cobalt chloride on the fertility and testes in mice, *J. Appl. Biol. Sci.*, 2008, 2: 1-6.
41. El-Kashoury AA, Salama AF, Selim AI, Mohamed RA. Animal model study of reproductive toxicity of the chronic exposure of the dicofol, *Life Sci. J.,* 2009, 6: 1-18.
42. Cosentino MJ, Cockett AT. Structure and function of the epididymis, *Urol. Res.,* 1986, 14: 229-240.
43. Bjorndahl L, Soderlund I, Kvist U. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessement, *Hum. Reprod.*, 2003, 18: 813-816.
44. Gonzales GF, Functions of seminal vesicle and their role on male fertility, *Asian J. Androl.*, 2001, 3: 251-258.
45. Rajalakshmi M. Physiology of the epididymis and spermatozoa, *J. Biosci.*, 1985, 7: 191-195.
46. Storey BT. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa, *Mol. Hum. Reprod.,* 2013, 3: 203-213.
47. Nakazawa H, Genka C, Fujishima M. Pathological aspects of active oxygen/ free radicals, *Jpn. J. Petrol.,* 1996, 46: 15-32.
48. Halliwell B, Gutteridge JMC, Cross CE. Free radicals, antioxidants, and human disease: where are we now? *J. Lab. Clin. Med.,* 1992, 119: 598-620.
49. Pant N, Srivastava SP. Testicular and spermatotoxic effects of quinalphos in rats, *J. Appl. Toxicol,* 2003, 23: 271-274.
50. Breton S, Smith PTS, Lui B, Brown D. Acidification of the male reproductive tract by a proton pumping (H+)- ATPase, *Nat. Med.,* 1996, 2: 470-472.
51. Moss DW, Henderson AR, Kachmar JF. In: Fundamentals of clinical chemistry. 3rd edition (Ed: Tietz NW), WB Saurders, Philadelphia, 1987, 346-42.
52. Perrone RD, Madias NE, Levey AS. Serum creatinine as an index of renal function: new insights into old concepts, *Clin. Chem.*, 1992, 38: 1933-1953.