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ANTI-SPERMATOGENIC EFFECT OF THEVETIA PERUVIANA LEAVES IN ALBINO RAT

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ABSTRACT: This study was conducted to explore the anti-spermatogenic efficacy of hydro-methanol (2:3) extract of *Thevetia peruviana* leaves (TPHmLE) on rat. Rats of treatment group were orally treated with TPHmLE at the dose of 40 mg/0.25 ml DW/100 g of body weight over a period of 28 days and control group received only vehicle. The anti-fertility activity of TPHmLE was observed by significant reduction ($P<0.05$) of the organo-somatic indices, epididymal sperm count, motility, viability and hypo-osmotic swelling (HOS) test. The gonadotropins (LH, FSH), testosterone, interstitial Leydig's cell count as well as 17β, hydroxyl steroid-dehydrogenase (17β-HSD) and Δ^5, 3β - hydroxysteroid dehydrogenase (Δ^5, 3β-HSD) activities in testis were decreased significantly in respect to control after the treatment of TPHmLE. Testicular superoxide dismutase (SOD) and peroxidase (POD) activities were significantly decreased in treated group but the lipid peroxidation status was significantly elevated indicated by high thiobarbituric acid reactive substance (TBARS), conjugated diene (CD) levels. From histological point of view, significant damage in germ cells of seminiferous tubule was observed by Feulgen, PAS, heamatoxylin-eosin and Sudan black stain. Activities of testicular acid phosphatase (ACP), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) that are the important metabolic toxicity indicators were not altered significantly in TPHmLE-treated group in respect to control. These results suggested that the *Thevetia peruviana* (TPHmLE) possess anti-spermatogenic activity by suppressing the gonadotrophins and testosterone and or by generation of oxidative free radicals that ultimately damage the germ cells and alter the spermatogenesis without any metabolic toxicity.

INTRODUCTION: Family planning has very crucialrole to minimize the population growth rate of the world as well as to achieve the sustainable development goal [1, 2]. Several studies showed that safe and proper family planning services in the country significantly decrease maternal and childhood mortality [3]. Different birth control techniques were used by females these are the barrier, surgical, withdrawal, retrograde ejaculation method, and medication [4].

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Most of them lead to life-threatening problems such as cerebral stroke, hypertension, cardiac stroke, tumor, abdominal pain, diabetes, nausea, and irregularities of menstrual changes [5]. Even after decades of research, no male contraceptive without side effects is not available.

Some hormonal contraceptives are administrated orally or in an injectable form that acts as Ca^{2+} channel blockers by hampering the lipid metabolic activity of the sperm and preventing fertilization [6, 7]. Excessive use of Non-oxynol-9 (N-9), a widely available spermicidal agent has a chance of increasing inflammation, ulceration and also increases the risk of HIV-1 infection [8]. Natural products are safer compared to synthetic components for humans and the environment [9].

Globally, about 80% of the total population use contraceptives which came from traditional folkloric medicinal plants or their extracts as these are easily available and low-cost [10]. Gossypol used as a male contraceptive derived from cotton seed inhibits the spermatogenesis process [6]. The major side effect of gossypol is the change in electrolyte status [11, 12], particularly causes hypokalaemia which enhances cardiac arrhythmias and hypokalaemic paralysis [13]. It is necessary to discover a safe, effective reliable, and reversible male contraceptive without changing libido. *Thevetia peruviana* frequently known as yellow oleander belongs to the *Apocynaceae* family and evergreen Indian tropical tree. *T. peruviana* is widely used as a folk medicine. Phytoconstituents derived from the bark of this tree have anti-spermatogenic activity in experimental animal models [14, 15]. LD_{50} of this leaf extract is 3.083 g/kg [16].

In-vitro spermicidal effect of *T. peruviana* leaves in human spermatozoa has already been proved by us [17, 18]. There is no available information till now regarding *in-vivo* male contraceptive effects of *T. peruviana* leaves in rats. Now this study was conducted to search out whether this plant extract has any antiandrogenic effect that suppresses the spermatogenesis process or shows anti-spermatogenic efficacy by the generation of oxidative stress.

MATERIAL AND METHODS:

Preparation of Plant Extract: *T. peruviana* leaves were collected from the local area of Midnapore, India and authenticated (specimen no. VU/CM/104) by Prof. Prakash Karmakar, the taxonomist of Botany Department, Vidyasagar University, West Bengal, India. Leaves were dried in the shed separately at normal ambient temperature for a week, then incubated at 37^{o}C for 24 hr to crush it. 50 g of each crushed material was suspended in 1000 ml of hydro-methanol (2:3) for 36 hr. The slurry was stirred intermittently and left overnight. The mixture was then filtered and dried by a rotary evaporator. The remainder was collected for treatment with deionizing water at a fixed dose [17].

Chemical Constituents: ELISA kits of Testosterone, LH, and FSH kits were purchased from Wuhan Fine Biological Technology Co. Ltd. SGPT and SGOT kits were purchased from Span Diagnostic Limited, Surat, India.

Animals and Treatment Schedule: Adult male fertile rats (weighting, 120 ± 10 g), were selected. These rats were kept for 7 days prior to experimentation in cages under standard circumstances (12 hr light/12 hr dark at 25 ± 2^{o}C) for acclimatization. They were provided a standard chew diet and water *ad-libitum* with proper care throughout the experiment phase. This study was approved by the institutional ethical committee (Registration No. VU/IAEC/CPCSEA/4/6/2022, DOR-26/04/2022). Rats were distributed into two groups each group contained six rats as follows.

Group I: (Control): Rats were orally treated with 0.25 ml distilled water/100 g of body weight /day for 28 days.

Group II: (Treated): Rats were orally treated with hydro-methanol (2:3) leaves extract of *T. peruviana* (TPHmLE) at the dose of 40mg/0.25 ml distilled water/100 g of body weight for 28 days.

After 28 days of treatment, body weights were recorded and sacrificed by the euthanasia technique. Blood was taken in a clot vial and separated serum was stored at -20°C for further biochemical, enzymatic and hormonal assay. Testes, epididymis, prostate, and seminal vesicle were isolated and their weight was documented. From all rats, the left testis was stored (-20°C) for enzymatic analysis and the right one was kept in Bouin's fluid for histological studies.

Epididymal Sperm Count, Motility, Viability and Hypo-osmotic Swelling (HOS) Test: Spermatozoa were collected from the caudal epididymis of each rat and counted by Neubauer chamber under the microscope (400X) as per standard procedure [19]. Spermatozoan motility was observed from one drop (10 µl) of caudal suspension and result was expressed as percentage [20]. Sperm viability was assessed by the eosin-nigrosine staining method [21]. The HOS test was performed for recognition of membrane intactness. Prepared HOS solution was mixed with epididymal semen sample (10:1) and incubated for 30 min at 37^{o}C. Single drop (10 µl) of mixture placed on slide and covered by cover slip [22].

Coiled-tail sperm were counted under microscope (400X) and expressed as a percentage.

Estimation of Testicular 17β-hydroxyl Steroid-Dehydrogenase (17β-HSD) and Δ^5, 3β Hydroxysteroid Dehydrogenase (Δ^5, 3β-HSD) Activities: Testicular 17β-HSD activity was estimated by conventional method [23] at 340 nm against a blank in UV spectrophotometer (Thermo Fisher Scientific, Shanghai, China). The standard method of Talalay [24] was used for the assessment of testicular Δ^5, 3β-HSD activity in a UV spectrophotometer cuvette at 340 nm against blank.

Estimation of Serum LH, FSH and Testosterone Levels: Serum testosterone, LH and FSH hormones were analysed by using ELISA kit [25]. Tests were performed according to the instruction of manufacturer and finally measured by ELISA plate analyser (Robonik, India Pvt. Ltd., India).

Estimation of Testicular Superoxide Dismutase (SOD) and Peroxidase (POD) Activities: The SOD activity from testicular tissue homogenate were performed by standard protocol [26]. 50 mM Tris buffer (pH 8.4) and 10 Mm pyrogallol was mixed with the supernatant. Absorbance of sample was taken against Tris buffer as a blank at 420 nm using UV spectrophotometer. Activity of POD was measured by standard method [27] from homogenised testicular supernatant at 420 nm.

Estimation of Testicular Thiobarbituric acid Reactive Substance (TBARS) and Conjugated Diene (CD): Testicular TBARS was estimated by conventional protocol [28] by using thiobarbituric acid-trichloro acetic acid (TBA-TCA) mixture in spectrophotometer cuvette and OD was noted at 535 nm. CD was quantified by the established biochemical method [29] from homogenized testis sample. Amount of formed hydro-peroxide was estimated by UV spectrophotometer at 233 nm and expressed in nM/mg of tissue

Estimation of GOT and GPT in Serum and Acid Phosphatase (ACP) Activity in Testis: SGOT and SGPT activities were quantified by standard method [30] in semi-auto analyser (Robonik, Prietest TOUCH, India PVT. LTD., India). The ACP activity in testicular homogenate was determined by standard protocol using p-nitrophenyl phosphate (p-NPP) as substrate [31].

Histomorphological and Histochemical Studies: Paraffin-embedded testicular blocks were sectioned by semi-automated microtome (Leica RM2245, Germany) at 5 μm thickness from each rat of both groups. The following staining method were performed:

Haematoxylin-eosin (HE) Stain: Deparaffinized testicular sections were stained with haematoxylin-eosin by standard method [32]. All sections were observed under the microscope (400X) using a computerized image analyser (Olympus CX21i LED- Magcam DC5). Mean seminiferous tubule diameter (MSTD), epithelial height (EH) and basement membrane thickness (BMT) was measured in micrometres and Leydig`s cells were counted in the interstitial space of seminiferous tubule (ST) from ten successive fields of HE stained testicular section. The grade of testicular dystrophy and spermatogenesis was evaluated from histopathological view by applying Johnsen's mean testicular biopsy score of (JMTBS) criteria [33]. A score is 1 to 10 was given to each ST according to the appearance or disappearance of germinal cells.

JMTBS 1: Completely absent of germinal epithelial cells in STand tubular sclerosis.

JMTBS 2: No germ cells, only Sertoli cells.

JMTBS 3: Spermatogonia only.

JMTBS 4: No spermatids but few spermatocytes.

JMTBS 5: Absence of spermatids with numerous spermatocytes.

JMTBS 6: No late spermatids only limited early spermatids, arrest of spermatogenesis at the spermatid stage and disturbance in spermatid differentiation.

JMTBS 7: No late spermatids and many early spermatids.

JMTBS 8: Limited late spermatids.

JMTBS 9: Numerous late spermatids and unsystematic tubular epithelium.

JMTBS 10: Complete spermatogenesis.

Testicular Feulgen's Stain: Testis sections of both groups were stained with Feulgen stain for

qualitative analysis as well as assessment of the DNA contained in the testicular cell. The sections were taken in working hydrochloric acid solution at 60°C for 10 min and then Schiff reagent for 45 min. The slide was washed and 0.2% light green solution was applied for 1 min as a counter stain [34].

Testicular Periodic Acid Schiff (PAS) Stain: The testicular sections were exposed to the periodic acid solution (0.5%), Schiff reagent and counter-stained by haematoxylin and then observed under microscope (400X) [35].

Testicular Sudan Black Stain: Deparaffinized testicular sections were exposed with Sudan black solution followed by counter-stained, maturation, mounting, and observation under microscope [36].

Statistical Analysis: The results were reported as mean ± SEM. The comparisons between the mean values of different groups were assessed by two-tail t-test [37].

The significance level was established at p <0.05, and the statistical package used was SPSS 16.0 software which was run on Microsoft Windows 10 operating system.

RESULTS:

Body Weight and Organo-somatic Indices: Body weight and organo-somatic indices of testis, epididymis, seminal vesicles and prostate were decreased significantly in respect to control after the treatment of TPHmLE for 28 days **Table 1**.

TABLE 1: EFFECT OF TPHmLE ON BODY WEIGHT AND ORGANO-SOMATIC INDICES (g%)

Group	Body weight (g)		Organo-somatic index (g%)			
	Initial	Final	Testis	Epididymis	Prostate	Seminal vesicle
Control	122.58 ± 0.92	147.55 ± 1.84	1.51 ± 0.01	0.54 ± 0.01	0.16 ± 0.01	0.64 ± 0.01
Treated	121.07 ± 0.72	130.10 ± 0.90*	1.24 ± 0.01*	0.42 ± 0.01*	0.12 ± 0.01*	0.39 ± 0.01*

Data represented as Mean ± SEM, n = 6. Two-tail t-test was performed, and the asterisk (*) in each vertical column indicated significant different (P < 0.05).

Epididymal Sperm Count, Motility, Viability and HOS Test: Epididymal sperm count, percentage of motile spermatozoa, percentage of viable spermatozoa and HOS positive (coiled tail) spermatozoa were decreased significantly in TPHmLE treated group when compared with the control group **Table 2, Fig. 1**.

TABLE 2: CHANGES IN EPIDIDYMAL SPERM COUNT, MOTILITY, VIABILITY AND HYPO-OSMOTIC SWELLING (HOS) TEST

Group	Sperm count (million/ml of epididymal fluid)	Sperm motility (%)	Sperm viability (%)	Hypo-osmotic swelling test (%)
Control	23.16 ± 0.87	78.33 ± 4.48	84.50 ± 3.07	83.16 ± 2.99
Treated	7.20 ± 0.36*	28.66 ± 1.15*	31.17 ± 1.51*	29.83 ± 1.53*

Data represented as Mean ± SEM, n = 6. Two-tail t-test was performed, and the asterisk (*) in each vertical column indicates significant difference (P < 0.05).

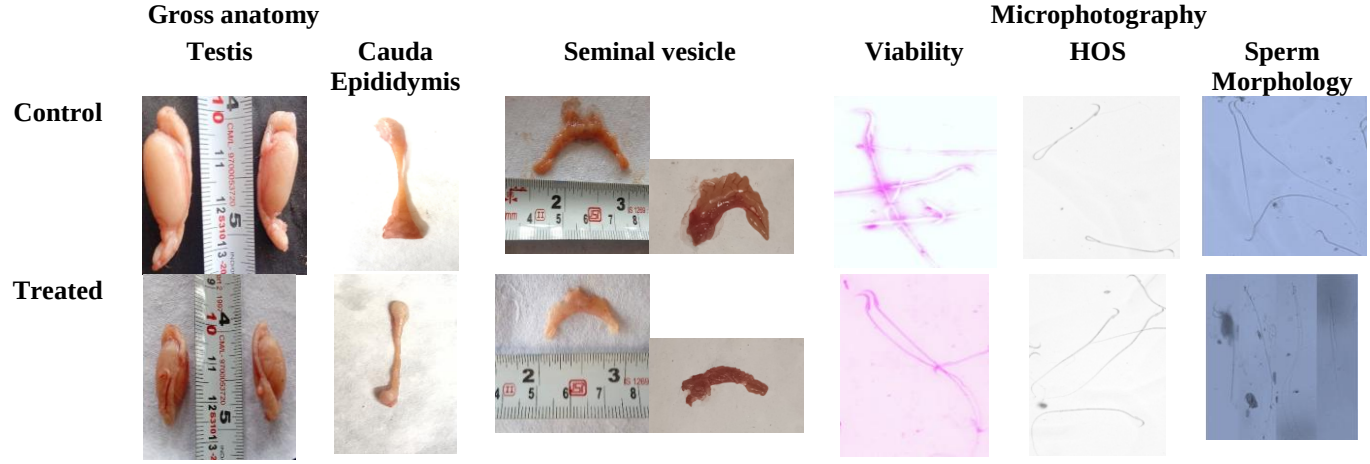


FIG. 1: GROSS ANATOMICAL OBSERVATION OF TESTIS, CAUDA EPIDIDYMIS AND SEMINAL VESICLE WITH MICROPHOTOGRAPHY OF SPERMATOZOA VIABILITY AND HOS

Δ^5, 3β-HSD and 17β-HSD Enzyme Activities in Testis: Testicular Δ^5, 3β-HSD and 17β-HSD enzyme activities were decreased significantly ($P < 0.05$) in TPHmLE treated group when compared with the matched control group **Fig. 2**.

Serum LH, FSH and Testosterone Levels: Serum LH, FSH and testosterone levels were diminished significantly ($P < 0.05$) in TPHmLE treated group when compared with control group **Fig. 2**.

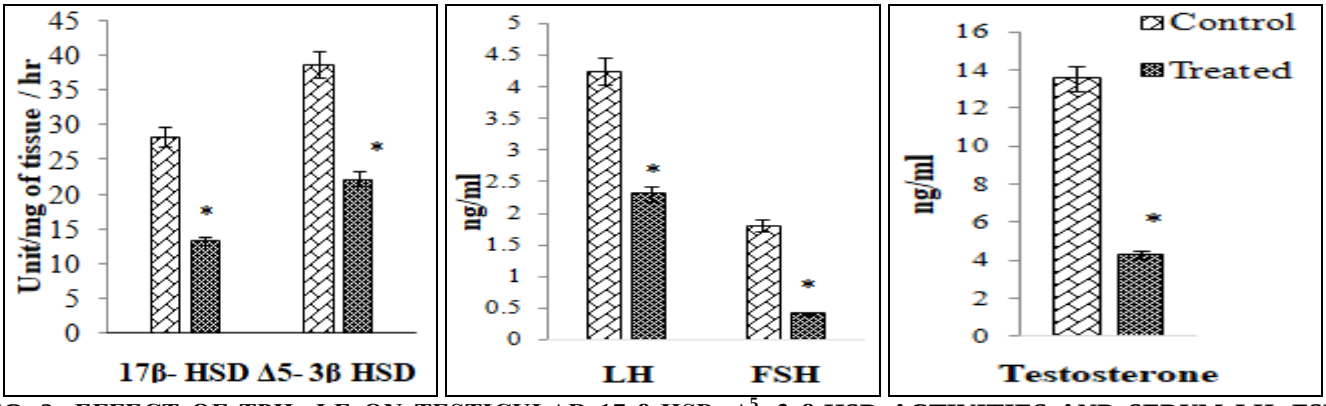


FIG. 2: EFFECT OF TPHmLE ON TESTICULAR 17 β-HSD, Δ^5, 3 β-HSD ACTIVITIES AND SERUM LH, FSH, TESTOSTERONE LEVELS. Data were represented as Mean ±SEM (n = 6). Two-tail t-test was performed. Asterisk (*) in each bar indicates significant difference ($P < 0.05$).

Activities of Testicular SOD and POD: In TPHmLE treated group, activities of testicular POD and SOD were decreased significantly ($P < 0.05$) in comparison with the control group **Fig. 3**.

TBARS and CD Levels in Testis: Testicular TBARS and CD levels were significantly elevated after the 28 days of treatment of TPHmLE in the rat with respect to the control group **Fig. 3.**

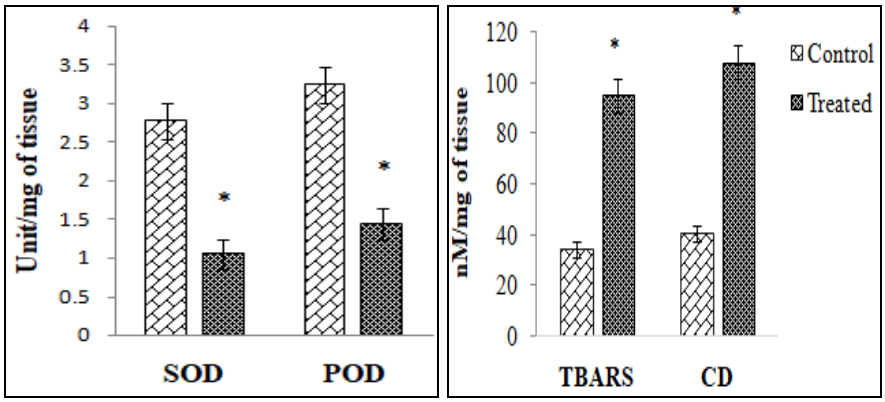


FIG. 3: EFFECT OF TPHmLE ON TESTICULAR OXIDATIVE STRESS RELATED BIO-SENSORS. Data were represented as mean ± sem (n = 6). Two-tail t-test was performed. Asterisk (*) in each bar indicates significant difference ($p < 0.05$).

Activities of SGPT, SGOT and Testicular ACP: There was no significant difference observed in SGPT, SGOT, and testicular ACP activities in TPHmLE treated group and control group **Fig. 4**.

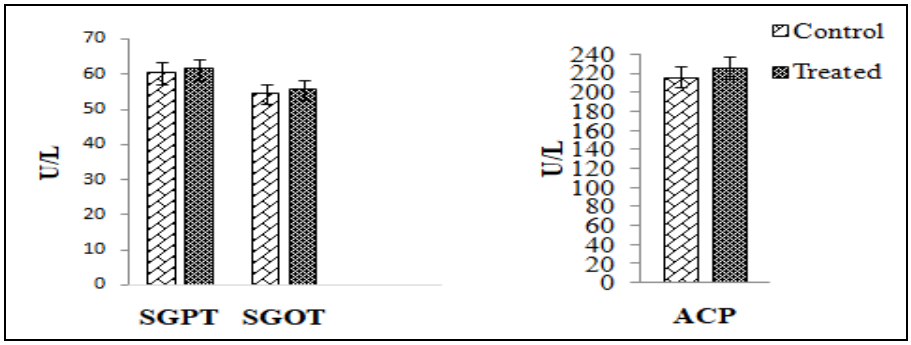


FIG. 4: EFFECT OF TPHmLE ON SGPT, SGOT AND TESTICULAR ACP ACTIVITIES. Data were represented as Mean ± SEM (n = 6). Two-tail t-test was performed. These data were not significantly differed from each other ($P > 0.05$).

Morphometrical and Histochemical Findings: Histopathological investigation based on HE staining has been observed that the control group has normal testicular morphology and spermatogenesis, containing adequate amounts of spermatogenic series of cells with regular distribution of spermatogonia, spermatocytes and spermatids with normal appearance of spermatozoa within the ST with high level of Johnsen score **Fig. 5**. In TPHmLE treated group, distribution pattern of the germinal cells layer was altered and the seminiferous tubular diameter, epithelial height, thickness of basement membrane and Johnsen score were significantly decreased **Table 3**. The interstitial Leydig's cell number was also decreased significantly in TPHmLE treated group when compared with control group **Table 3**, **Fig. 5.**

TABLE 3: EFFECT OF TPHmLE ON SEMINIFEROUS TUBULE DIAMETER (STD), GERMINAL EPITHELIAL HEIGHT, BASEMENT MEMBRANE THICKNESS, LEYDIG CELL NUMBER AND JOHNSEN'S SCORE

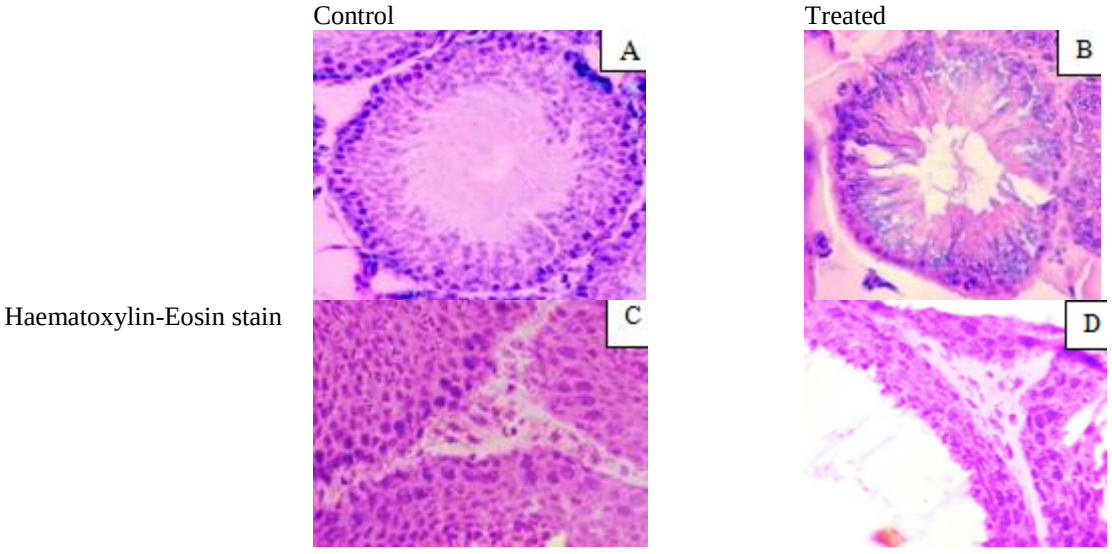
Group	Seminiferous tubule diameter (STD) (µm)	Germinal epithelial height (µm)	Basement membrane thickness (µm)	Leydig's cell number	Johnsen's score
Control	254.67 ± 5.49	108.33 ± 1.58	3.60 ± 0.09	30.16 ± 1.01	9.17 ± 0.30
Treated	181.83 ± 3.07*	26.17 ± 1.40*	2.05 ± 0.06*	11.66 ± 0.42*	4.33 ± 0.21*

Data represented as Mean ± SEM, n = 6. Two-tail t-test was performed, and the asterisk (*) in each vertical column indicates significant difference ($P < 0.05$).

Histochemical observation of Feulgen's reaction test reported that DNA content of spermatogenic cells decreased in TPHmLE-treated rats compared with control rats **FIG. 5**. In control group spermatogenic cells contain a sufficient amount of DNA and showed deep pink colour whereas, TPHmLE treated group spermatogenic cell showed fade pink colour **Fig. 5**.

The basement membrane thickness of STs with extracellular space of first three germinal cell layers along with Sertoli cells, and Leydig's cells showed normal PAS reaction in control group which indicated by sufficient amount of PAS-positive material present and developed pink colour. On the other hand, in treated group moderate to lower PAS reactions were observed in the STs and extracellular space so, faint PAS stains (pink) coloured cells with decreased thickness of basement membrane was observed **Fig. 5**.

Histochemical observations of Sudan black stain demonstrated that TPHmLE treated animals were increased cytoplasmic lipid accumulation in cell lineage of spermatogenesis as well as Leydig's cell. Accordingly in control group, the majority of spermatogenic cells and Leydig's cell were presented with less sudanophilic molecules because of proper utilization of this molecule by several biogenesis processes **Fig. 5.**



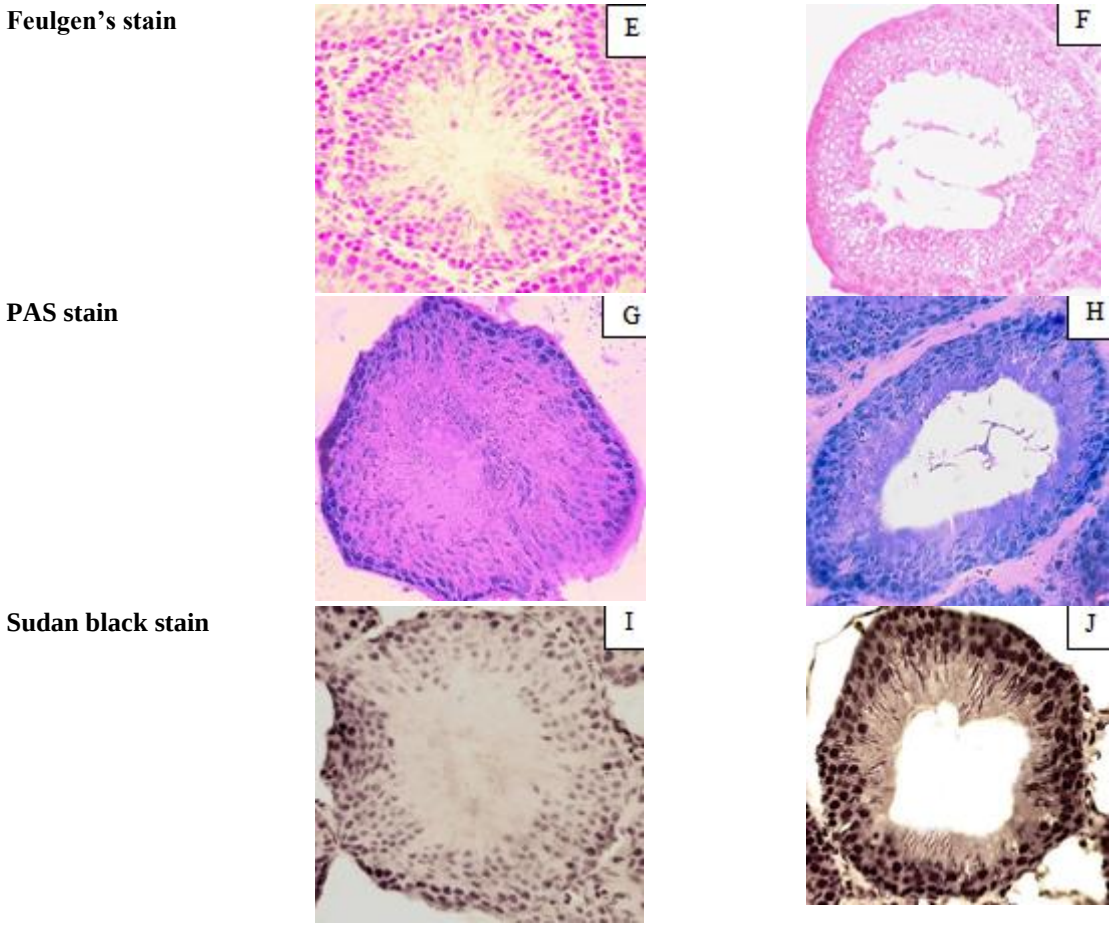


FIG. 5: HISTOLOGICAL INVESTIGATION OF TESTIS BASED ON HE, FEULGEN, PAS AND SUDAN BLACK STAINING (400X). [A] Normal architecture and regular distribution pattern of spermatogenic cells. [B] Disruption of germinal layer and irregular arrangement of spermatogenic cell. [C] Adequate number of Leydig's cell with homogenously distributed. [D] Lacking of Leydig's cell. [E] Spermatogenic cell contain sufficient amount of DNA and shows deep pink colour. [F] Spermatogenic cell contain less DNA which shows fade pink. [G] Sufficient amounts of PAS positive material present and developed pink colour. [H] Moderated to lower PAS reaction were observed and developed faint PAS stain [I] Less amount of Sudanophilic molecule indicated by light black colour. [J] High amount of lipid accumulated and developed dark colour.

DISCUSSION: The current research provides novel findings on the anti-gonadal efficacy of TPHmLE in male albino rats. Final body weight, testiculo-somatic, epididymo-somatic and seminalvesculo-somatic indices were significantly diminished by TPHmLE treatment that also attributed to the disruption of testosterone synthesis and secretion as the growth of accessory sex organs regulated by testosterone [38]. The bio-active ingredients present in the hydro-methanolic extract of *T. peruviana* significantly inhibit the gonadotropin (LH, FSH) [39] as well as testicular steroidogenic key enzymes (Δ5, 3β-HSD and 17β-HSD) [40, 41] activities in comparison to the control group. From the histological cross-sectional finding of testis the Leydig cells count in the interstitial space of ST significantly reduced in TPHmLE treated group which is one of the causes of lower testosterone level.

Deviation of semen quality in TPHmLE treated group may be due to excess oxidative free radical imposition in spermatozoa as well as damage of the antioxidant defence system in testis [42]. Reactive oxygen species (ROS) leads to damage of mitochondrial DNA that causes lower level of ATP currency and hampered spermatozoal motility and also decreased the activities of POD and SOD which may alter the membrane integrity of spermatozoa and other abnormalities that results in low sperm motility and cellular death [34]. The end products of free radicals like TBARS and CD levels were higher in TPHmLE treated group which may direct the low testosterone level [43]. This oxidative injury hampered the tuning mechanism of the pituitary testicular axis that was negatively deviated by extract-treated group [42]. LH and FSH levels were decreased significantly which directed lower testosterone level and reduced

spermatogenesis. Haematoxylin and eosin staining of the testicular section of treated group showed that reduced the number of spermatogonium as well as degenerated spermatocyte and spermatid which leads to decreased spermatogenesis [44]. Mucopolysaccharide and glycogen molecules provide the energy for capacitation, help in spermatogenesis, also regulate apoptosis, and increased the lysosomal activity in germ cells [45, 46]. Significantly diminution of mucopolysaccharide and glycogen level observed in PAS staining of testicular section.

DNA contains were studied by Feulgen's staining method and detected apoptotic changes in spermatogonium in STs of different groups [47]. This is attributed to sperm DNA damage. The spermatogonium of TPHmLE treated group looks light pink colour that indicated a lower amount of DNA in the nucleus than control which cause inhibited the cell cycle process. Therefore, elevated the apoptosis process in spermatogonium ultimately diminished spermatogenesis [48].

In Sudan black stained testicular section, it was observed that the lower level of Sudanophilic molecule was present in the control group where as higher lipids accumulation was noted in the spermatogenesis cells lineage of TPHmLE treated group. It can focus that lipase and other oxidative enzyme activities decreased which altered the testosterone biosynthesis in Leydig's cell and directly lowered spermatogenesis as well as energy fuel for spermatozoal motility [49].

CONCLUSION: The hydro-methanol (2:3) extract of leaves of *T. peruviana* shows significant antifertility effect directly by excess oxidative stress generation in the testis that altered the antioxidant enzyme activities leading to sperm DNA damage. On the other hand, it altered the pituitary-gonadal axis, suppress the gonadotrophins and androgen synthesis. Further research will explore the active ingredient(s) present in the extract and its actual mode of action for the anti-spermatogenic activity.

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Declarations:

CONFLICT OF INTEREST: The author declares that there is no conflict of interest.

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Author Contributions: PM: Animal treatment, semen analysis, performed histology, data analysis. PRB: Biochemical analysis. RM: Extract preparation, AR: Hormonal assay. CM: Supervision, writing-reviewing and editing, formal analysis. All authors revised and approved the final manuscript for publication.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon request.

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