Avicenna Journal of Phytomedicine (AJP)

Original Research Article

Evaluation of anti-gonadal activity of chloroform fraction of *Thevetia peruviana* leaves in male albino rats

Prabir Mondal[1], Sayandev Midya[1], Parag Ranjita Bera[1], Chhanda Mallick Mukherjee[1,*]

[1]*Department of Biomedical Laboratory Science and Management, Vidyasagar University, Midnapore-721102, West Bengal, India*

Article history:
Received: Dec 10, 2024
Received in revised form: Aug 06, 2025
Accepted: Aug 18, 2025
Epub ahead of print

**** Corresponding Author:***
Tel:+919733954520
Fax:275329
chhanda_nutri@mail.vidyasagar.ac.in

Keywords:
Phyto molecules
Sperm count
Catalase
17β-HSD
Androgen receptor
Anti-spermatogenic.

Abstract

Objective: Anti-gonadal activity of chloroform fraction of *Thevetia peruviana* leaves (Ch-TPHmLE) in male rat was evaluated in this study.

Materials and Methods: The Ch-TPHmLE (5 mg/100 g of body-weight (BW)/day) and standard antiandrogenic agent cyproterone acetate (CyPA) at the dose of 2.5 mg/100 g of BW were given to male rats for 28 days. Sperm profile, serum gonadotrophins and testosterone were measured. Testicular superoxide-dismutase, catalase activities and zymographic-expression, malondialdehyde, conjugated-diene, TNF-α levels and immunohistochemical markers were evaluated. Testicular steroidogenic enzyme activities and androgen receptor (*AR*), *StAR*, *HSD*, *Bcl2*, *P53*, *caspase-3* and *BAX* gene expressions were observed. LC-MS study was done for Ch-TPHmLE.

Results: Sperm profile, gonadotrophins, testosterone as well as testicular steroidogenic key enzyme activities were reduced significantly ($p<0.05$) and *StAR*, *AR*, *HSD* genes expressions were downregulated in Ch-TPHmLE and CyPA treated groups compared to the control group. Testicular anti-oxidant enzyme activities and zymographic-expression patterns were reduced significantly and malondialdehyde, conjugated-diene, TNF-α were increased significantly in the Ch-TPHmLE and CyPA treated groups. Testicular *caspase-3* gene and proapoptotic genes *P53*, and *BAX* expressions were upregulated but anti-apoptotic gene *Bcl2* was downregulated as confirmed by elevated number of apoptotic germ cells in immunostaining. The bio-active phyto-compounds γ-Sitosterol, Stigmasterol, Dehydroergosterol, β-Amyrin, Lupeol, and α-Amyrone were identified by LC-MS analysis of Ch-TPHmLE.

Conclusion: It is concluded that the synergistic action of these phyto-molecule(s) of chloroform fraction of *Thevetia peruviana* (TPHmLE) shows anti-gonadal activity by alteration of serum gonadotrophins, and androgen levels and generation of excess oxidative free-radicals that trigger testicular germ-cell apoptosis.

Please cite this paper as:
Mondal P, Midya S, Ranjita Bera P, Mukherjee Mallick C. Evaluation of anti-gonadal activity of chloroform fraction of *Thevetia peruviana* leaves in male albino rats. Avicenna J Phytomed, 2025. Epub ahead of print.

Introduction

Uncontrolled elevation of population imposes an extra burden to the community that affects the economic status, health and productivity of growing countries throughout the worldwide (Ghosh et al. 2022). The improvement of reproductive bio-medicine for good family planning with reduced maternal and infantile mortality are very essential (Muttreja and Singh 2018). Several synthetic hormonal contraceptive pills and methods are available for both male and female but these are not always safe for prolonged use, especially the birth control techniques for females, have high margins of life-threatening problems (Muttreja and Singh 2018).

Some male contraceptives act as Ca^{2+} channel blockers by hampering the lipid metabolism of the sperm and prevent fertilization (Coutinho 2002). A task force was established by the World Health Organization (WHO) to control the population explosion worldwide by developing herbal non-steroidal male contraceptives from phyto-molecule(s) of different medicinal plants without any adverse effects (WHO 1996; Ghosh et al. 2018). Molecular mechanisms of action of available herbal contraceptives are still unknown. Scientists are engaged in developing male contraceptive from herbal origin that are cheaper, eco-friendly, and has fewer health hazards (Ghosh et al. 2022). Gossypol derived from cotton seed prevent the spermatogenesis process (Coutinho 2002), but the primary drawback is change in libido, and development of cardiac arrhythmias and hypokalaemic paralysis due to change in electrolyte status (Porat 1990). Therefore, it is essential to develop an effective, reversible, and safe male contraceptive without affecting libido. Yellow oleander, scientifically known as *Thevetia peruviana* belonging to the Apocynaceae family, is a popular folk medicine. The bark extract of this tree has shown anti-spermatogenic activity in a rat model (Gupta et al. 2011).

In vitro spermicidal effect of *T. peruviana* leaves has been established by us in human sperm (Mallick and Mondal 2022; Mondal et al. 2022). It is also established that hydro-methanolic extract from the leaves of *T. peruviana* has promising effect on testicular damage (Mondal et al. 2024). On the basis of that study, we have gone through fractionation study of hydro-methanolic extract of *T. peruviana* by hexane (C_6H_{14}), chloroform ($CHCl_3$), ethyl acetate ($C_4H_8O_{2)}$, n-butanol ($C_4H_{10}O$) and residual water and studied the spermicidal efficacy on human sperm *in vitro*, and *in vivo* in a rat model. Among the above mentioned five fractions, chloroform showed the most promising spermicidal efficacy. Effective dose and effective duration of this fraction was selected in our earlier laboratory experiment. On that basis, we designed this *in vivo* model to identify active phytochemical of $CHCl_3$ fraction of the hydro-methanolic extract and determined the actual mode of action of this active compound(s) present in this fraction as compared with standard anti androgenic agent cyproterone acetate.

Materials and Methods

Preparation and collection of leaves fraction

T. peruviana leaves were collected (Midnapore, West Bengal) and confirmed (Specimen Id- VU/CM/104) by our institutional taxonomist from Department of Botany. The Leaves of *T. peruviana* were dried in shaded condition without any exposure of sunlight for 5 days. Then, the leaves were kept at 37^oC for a day and crushed. The crushed material was suspended in 50 g/L of hydro-methanol (2:3) and stirred intermittently for 3-4 days. Filtered and fractionations were done by different non-polar to polar solvents system of C_6H_{14}, $CHCl_3$, $C_4H_8O_2$, $C_4H_{10}O$, and water respectively (Ghosh et al. 2018). Chloroform ($CHCl_3$) fractionated part was dried in rotary evaporator and collected in glass container.

Chemical constituents

ELISA (enzyme-link immunosorbent-assay) kits of testosterone, luteinized hormone (LH), and follicle stimulating hormone (FSH) were acquired from Wuhan Fine Biological Technology Ltd. TRIzol-C reagent and Taq Mix (2x) kit supplied by SRL Pvt Ltd, India. cDNA synthesis kit from Himedia Lab. Pvt. Ltd, India. Calcium (Ca^{2+}), cholesterol, ACP (acid phosphatase), SGOT (serum glutamic oxaloacetate transaminase), ALP (alkaline phosphatase), and SGPT (serum glutamate pyruvate transaminase) assay kits were purchased from Coral, Tulip Diagnostic (Pvt) Ltd, India.

Animal treatment schedule

Adult fertile male albino rats (weighting, 120 ± 10 g) were kept and acclimatized (light: dark, 12:12 hr at 24 ± 1^oC) for 12 days with proper diet, and water *ad libitum*. This experiment was ratified by ethical committee of our institution (Registration No. VU/IAEC/CPCSEA/4/6/2022, Date-26/04/2022). Eighteen rats were equally circulated into three separate groups, so n=6 in each group (Charan and Biswas 2013):

Group I (Control): Six rats received 0.25 ml deionized-water (DW)/day.

Group II (Ch-TPHmLE): Rats of this group orally received single dose of Ch-TPHmLE at 5 mg dose/100 g bodyweight (BW)/day (Lohiya and Goyal 1992; Ghosh et al. 2018).

Group III (CyPA): Cyproterone acetate (CyPA) at the dose of 2.5 mg/100 g BW was given via oral route (Ghosh et al. 2017).

After the scheduled treatment (28 days), the final BW was written and rats were sacrificed by the euthanasia with carbon dioxide (CO_2) over dose at a flow rate displacing 30% to 70% of cage volume per minute. Testis, prostate, seminal vesicles, and epididymis weight were recorded. The right testis, seminal vesicles, and epididymis were taken for histological studies and left one was frozen (-20°*C*) for enzymatic study. Hormonal, enzymatic and biochemical tests were performed in serum and testis.

Epididymal spermatozoal profile assessment

Caudal epididymal spermatozoa were counted by Neubauer chamber under the microscope (400X) as per standard procedure (Mondal et al. 2024). Spermatozoal motility was assessed from single-drop (15 µl) of caudal suspension and result is expressed as percentage (Mallick and Mondal 2022). Spermatozoan viability was observed by eosin-nigrosine staining method (Mondal et al. 2022).

The hypoosmotic-swelling (HOS) test was executed for recognition of membrane integrity/intactness (Zeidan et al. 2018). HOS-test solution was added to epididymal semen at 10:1 ratio and incubated at 37 ^oC for an hour (Chauhan et al. 2011). Coiled tail sperms were counted under microscope (400X) and are expressed as percentage.

Estimation of reproductive parameters

Biochemical estimation of testicular Ca^{2+} (Kit Cat No. 1103010235), cholesterol (Kit Cat No. 1102040075) were done using kit (Coral Clinical System) (Allain et al. 1974; Soud et al. 2015), and the optical density was measured in semi-auto (Robonik, Prietest TOUCH, India PVT. Ltd., India) analyzer. The testicular 17β-HSD (hydroxysteroid dehydrogenase) activity was estimated by using 0.3 µmol testosterone, 25 mg % of bovine serum albumin (BSA), and 0.5 µmol nicotinamide adenine dinucleotide (NAD) at 340 nm against a blank in UV spectrophotometer (Thermo Fisher Scientific, Shanghai, China) (Jarabak et al. 1962). Standard method was applied for the assessment of testicular Δ^5,3β-HSD activity in a UV spectrophotometer (340 nm) by using dehydroepiandrosterone (DHEA), BSA and NAD (Talalay 1962). Testosterone and gonadotropins (LH and FSH) were analyzed by ELISA kits from the serum sample. Tests were executed by instruction of kit manufacturer company and ELISA

well reader (Robonik Ltd., India) (Mondal et al. 2024).

Oxidative stress marker analysis

For the spectrophotometric assessment of testicular superoxide dismutase (SOD) activity, tris buffer and pyrogallol was used at 420 nm of wavelength against blank (Marklund and Marklund 1974). The testicular catalase (CAT) activity was quantified by using of 30%-hydrogen peroxide (H_2O_2) and DW at 240 nm light path of UV-spectrophotometer (Sinha 1972). Malondialdehyde (MDA) was estimated in testicular homogenate using thio-barbituric acid (TBA)-trichloroacetic acid (TCA) by standard protocol at 540 nm (Ohkawa et al. 1979). Conjugated diene (CD) was quantified by the established biochemical method of Slater (1984). Methanol-chloroform (1:2) followed by spin for 6 min at 10000×g used for lipid extraction. Then evaporated chloroform under nitrogen stream and mixed with 1.5 ml of cyclo-hexane. Amount of formed hydro-peroxide was estimated by UV spectrophotometer at 233 nm and is expressed in nmol/mg of tissue.

Also, 100 mg testicular tissue was homogenized with phosphate buffer saline (PBS; pH 7.4) and 50 µg protein was run through native PAGE (12% polyacrylamide gel electrophoresis) for SOD expression analysis. PAGE was stained using tetramethyl-ethylenediamine (TEMED), nitroblue tetrazolium (NBT), and riboflavin for 12 min in dark conditions then exposed to bright light tilled achromatic bands appeared with blueish background (Dey et al. 2021). Similarly, 8% PAGE was used in electrophoresed for CAT enzyme detection by stained the gel using H_2O_2 solution and observed bright bands with blueish-green background (Dey et al. 2021).

Inflammatory marker assay

Testis samples were homogenized in phosphate buffer and then centrifuged to separate the supernatant. The resulting supernatant was collected, and levels of TNF-α were measured using ELISA kits, following the manufacturer's protocol (Ray Biotech Co., Peachtree Corners, USA) and the concentrations are reported in picograms per milliliter (pg/ml) (Theas et al. 2008).

Gene expression analysis

mRNA was isolated from testicular samples using RNA isolation kit (TRIzol-C, SRL, Batch No. 6451290) and converted cDNA using cDNA-synthesis kit (Himedia, MBT067-100R). 2X-PCR master-mix (SRL, Batch No. 9178375) and primers were used for cDNA amplification where GAPDH functioned as reference gene. The thermal cycles of polymerase chain reaction (PCR) are shown in Table 1 (Hwang et al. 2011; Dey et al. 2021; Ahmed et al. 2022). Finally, PCR products were electrophoresed in 1.8% agarose gel containing ethidium bromide (EtBr) and densitometric analysis of bands was documented through ImageJ software.

Table 1. Primer designed and thermal cycles of PCR. Abbreviation: F: Forward; R: Reverse.

Gene	Accession Number	Primer sequence	Denaturation (30 sec)	Annealing (30 sec)	Extension (40 sec)	No. of cycles
17-HSD	XM_027403120.2	**F** 5′ TTCTGCAAGGCTTTACCAGG3′ **R** 5′ACAAACTCATCGGCGGTCTT3′	94°C	57°C	75°C	30
3β-HSD	NM_001246700	**F** 5′ GCATTAACCCCACTCCCACT 3′ **R** 5′ GGACCCTGACCTCCTTCAGA 3′	94°C	59.3°C	72°C	33
StAR	NM_031558.3	**F** 5′ CACAGTCATCACCCATGAGC 3′ **R** 5′ AGCTCTGATGACACCGCTTT3′	94°C	57°C	75°C	30
AR	NM_012502.2	**F** 5′ TTACGAAGTGGGCATGATGA3′ **R** 5′ ATCTTGTCCAGGACTCGGTG3′	94°C	55°C	75°C	30
P53	NM_031781.4	**F** 5′ CTACTTCCCAGCAGGGTGTC 3′ **R** 5′AAAGTCTGCCTGTCGTCCAG3′	94°C	59.3°C	72°C	33
BAX	NM_017059.2	**F** 5′GATGCGTCCACCAAGAAG 3′ **R** 5′AGTTGAAGTTGCCGTCAG3′	94°C	57.5°C	72°C	32
Bcl2	NM_016993.2	**F** 5′ TGGCATCTTCTCCTTCCAGC 3′ **R** 5′ATCCCAGCCTCCGTTATCCT3′	94°C	57.5°C	72°C	32
Caspase 3	NM_001284409.1	**F** 5'GGTATTGAGACAGACAGTG3' **R** 5'CATGGGATCTGTTTCTTTGC3'	94°C	55°C	72°C	30
GAPDH	NM_017008.4	**F** 5′ GGGAAACCCATCACCATC3′ **R** 5′ CCCTGTTGCTGTAGCCAT 3′	94°C	56°C	75°C	30

DNA fragmentation assessment

DNA was prepared from testicular tissue using lysis buffer and spinning at 12,000×g in cold state for 30 min. DNA was isolated using phenol-chloroform (1:1), alcohol (ethyl), sodium acetate, and RNase. DNA was run in agarose gel (0.8%) with ethidium bromide (EtBr) at 100 V and bands were observed in gel doc (BioRed) (Garcia-Martinez et al. 1993).

Estimation of serum GOT, GPT, ACP, and ALP activities

Serum GOT (Cat. No. 1102200075) and GPT (Cat. No. 1102210075) were evaluated by kit method in semi-auto analyzer (Reitman and Frankel 1957). Serum ACP (Cat. No. 1102010102) and ALP (Cat. No. 1102020103) activities were determined using α-naphthyl phosphate (α-NPP) and p-nitrophenyl phosphate (p-NPP) respectively as a substrate (Soud et al. 2015).

Histomorphological, histochemical and immunohistochemical (IHC)/ terminal-deoxynucleotidyl-transferase-dUTP nick end labeling (TUNEL) study

Paraffin-embedded blocks of testis, epididymis, and seminal vesicle were sectioned at 5-6 µm thickness by microtome (Leica, Germany). Routine hematoxylin-eosin staining was performed for histoarchitectural study including diameter of seminiferous tubule (ST), germinal epithelium height, lumen length, and basal membrane thickness (Adibmoradi et al. 2015). Spermatogenesis along with spermiogenesis was studied using analysis of spermatogenesis index (SI), tubular differentiation index (TDI), and repopulation index (RI) (Hesari et al. 2015). To compute TDI index, the ratio of seminiferous tube with three or more differentiated germ cell lines from spermatogonia A. To compute SI index, ratio of seminiferous tube contain spermatid and lacking of spermatids. To calculate RI index, the ratio of spermatogonia-B to spermatogonia-A. Quantifying different generations of germ cells (spermatogonia-A (A-Sg), pre-leptotene-spermatocyte (pL-Sc), mid-pachytene-spermatocyte (mP-Sc), and spermatid (7Sd)) Sertoli cell and also Leydig's cell at stage-VII (Leblond and Clermont 1952). Immunostaining (TUNEL assay) was performed for the detection of germ cell apoptosis by the kit (2TdT-DAB Apoptosis finding kit, Cat No. 4810-30-K) guideline and alkaline-phosphatase stain (ALP stain) was performed by conventional method from testicular sections (Shi et al. 1997; Adibmoradi et al. 2015). Finally, sections were mounted and observed under computerized image analyzer microscope (Olympus CX21iLED-Magcam DC5).

LC-MS analyses of *T. peruviana* chloroform fraction

For this purpose, used Waters 2695 separation module attached with Quattro microTM API mass spectrometer (Waters, USA). 10 µl Ch-TPHmLE sample was injected into the LC system using C-18 separation column (internal diameter- 2.1 × 100 mm; particle size- 5 µm) at 25°C. The mobile phase consisted of 94 % aqueous, 2 % acetonitrile and 4 % methanol with flow rate 300 µl/min. The photodiode array (PDA) sensor (Model No. Waters 2998) detected all of the chemicals between 190 and 690 nm. Using the MS-scanning mode, the data was collected with a scan range of 100-600 (m/z); the interscan delay period was 0.1 sec, and the entire scan duration was 0.5 sec using the operating system Mass-Lynx 4 upgraded software (Pal et al. 2013).

Statistical analysis

Results are expressed as mean ± standard error of mean (Mean ± SEM) and ANOVA followed by multiple comparison two-tailed t test at $p<0.05$ using SPSS-16.0 in Microsoft Windows 10 system (Sokal and Rohle 2013).

Results

Body weight and organo-somatic indices (OSI)

Rate of BW gain was similar in Ch-TPHmLE-treated rats and control rats on day-to-day basis, no significant alteration was observed between the control and Ch-TPHmLE treated group. The OSI of testis, epididymis, prostate, and seminal vesicle were significantly decreased in *T. peruviana* treated group when compared with the control group. Whereas, in CyPA treated group final BW and above mentioned OSI were significantly decreased as compared to the Ch-TPHmLE group and control group (Figure 1 and Table 2).

Epididymal spermatozoan profile

Epididymal spermatozoan count, percentage of viable, motile, and HOS-positive (coiled tail) spermatozoa were significantly lower in the Ch-TPHmLE and CyPA treated rats than the control rats after 28 days of treatment. More significant diminution of all the said parameters was observed in the CyPA induced group than Ch-TPHmLE group (Table 2). Tail-to-tail and head-to-head agglutinations were also observed in both Ch-TPHmLE and CyPA treated epididymal spermatozoa in comparison to the controls (Figure 2).

Testicular Ca^{2+} and cholesterol

In Ch-TPHmLE and CyPA treated rats, testicular Ca^{2+} was reduced whereas testicular cholesterol level elevated significantly as compared to the control rats. Most significant alteration of Ca^{2+}, cholesterol were noted in CyPA fed rats compared to Ch-TPHmLE group (Table 2).

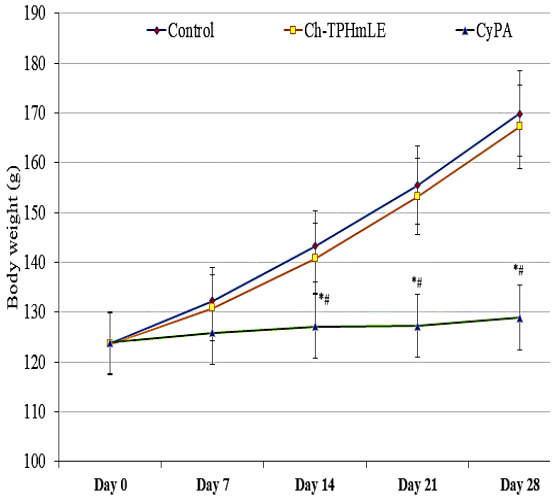

Figure 1. Effect of Ch-TPHmLE and CyPA on body weight, Mean±SEM, n=6. ANOVA followed by two-tail t-test was performed. Asterisk (*) indicates significant difference compared with the control ($p<0.05$) and hashtag (#) indicates significant difference ($p<0.05$) compared with the Ch-TPHmLE.

Table 2. Effect of Ch-TPHmLE and CyPA on organo-somatic indices (g%), epididymal sperm count, motility, viability, HOS, testicular Ca^{2+} and cholesterol levels. Data are presented as Mean ± SEM, n = 6. ANOVA followed by two-tail t-test was performed, and the asterisk (*) in each horizontal rows indicates significant difference ($p<0.05$) compared with the control and hashtag (#) indicates significant difference ($p<0.05$) compared with the Ch-TPHmLE.

	Parameters	Control	Ch-TPHmLE	CyPA
Organo-somatic index (g%)	Testis	1.81±0.12	1.26±0.13*	0.97±0.04*#
	Epididymis	0.87±0.07	0.32±0.01*	0.27±0.02*#
	Prostate	0.24±0.01	0.14±0.01*	0.09±0.01*#
	Seminal vesicle	0.81±0.04	0.23±0.03*	0.15±.02*#
Sperm profile	Count (million/ml of epididymal fluid)	51.75±2.07	14.03±0.77*	7.88±0.46*#
	Motility (%)	87.13±0.67	26.88±1.52*	20.88±0.83*#
	Viability (%)	89.87±0.64	29.79±1.41*	22.13±0.46*#
	HOS (%)	88.66 ± 0.73	28.66±1.28*	21.88±0.99*#
Testicular	Ca^{2+} (mg/dl)	4.73 ± 0.27	1.67±0.19*	0.86±0.05*#
	Cholesterol (mg/dl)	34.88 ± 0.74	57.75±1.55*	69.63±0.95*#

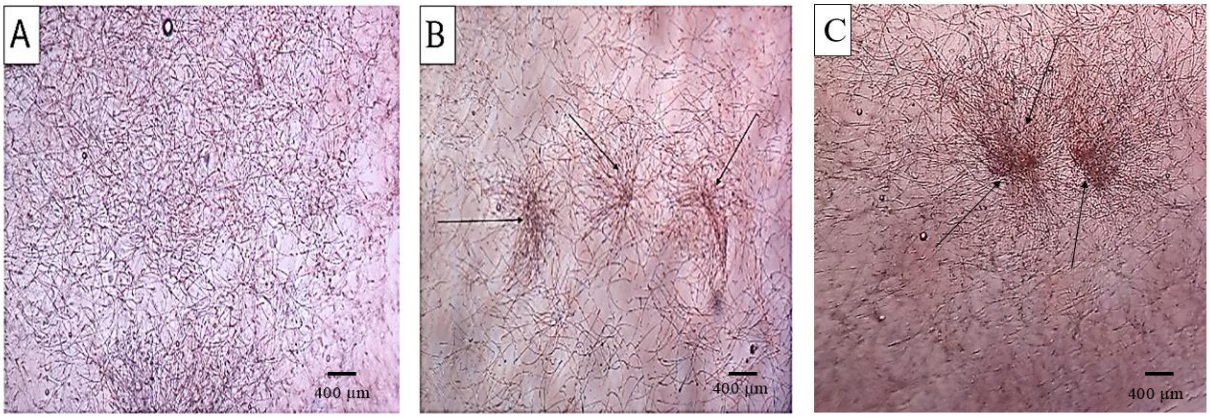

Figure 2. Microphotography (100X) of spermatozoan agglutination. A: Control group, B: Ch-TPHmLE treated group, C: CyPA treated group. Arrow (⟶) indicate agglutination of spermatozoa.

Androgenic key enzymes, testosterone and gonadotrophins

17β-HSD and Δ^5,3β-HSD enzyme activities in testis, and serum testosterone, LH, and FSH levels were decreased significantly ($p<0.05$) in Ch-TPHmLE and CyPA-treated groups compared to control rats. More significant diminution observed in 17β-HSD and Δ^5,3β-HSD enzyme activities in testis and serum testosterone, LH, and FSH levels in CyPA treated group compared to 5 mg dose of Ch-TPHmLE treated group (Figure 3).

Oxidative stress marker

Testicular redox equilibrium was monitored by evaluating the antioxidant enzymes (SOD and CAT) activities and their electro-zymographic expressions pattern and both were significantly reduced ($p<0.05$) whereas MDA and CD were significantly elevated after 28 days of Ch-TPHmLE and CyPA treatment compared to the control. But more significant alteration (elevation of SOD and CAT and reduction of MDA and CD) was observed in CyPA treated animals compared to Ch-TPHmLE animals (Figure 4a and b).

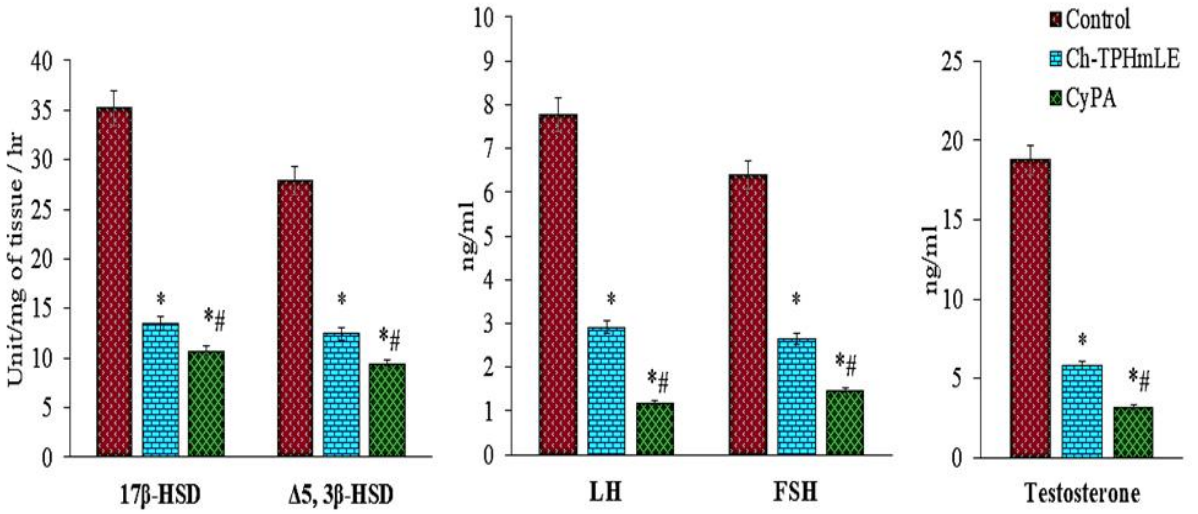

Figure 3. Effect of Ch-TPHmLE and CyPA on testicular 17β-hydroxylsteroid dehydrogenase (17β-HSD), Δ^5,3β-hydroxysteroid dehydrogenase (Δ^5,3β-HSD) and luteinizing hormone (LH), follicle stimulating hormone (FSH) and testosterone levels in serum. Data are presented as Mean±SEM (n=6). ANOVA followed by two-tail t-test was performed. Asterisk (*) in each bar indicates significant difference compared with the control ($p<0.05$) and hashtag (#) indicates significant difference ($p<0.05$) compared with the Ch-TPHmLE.

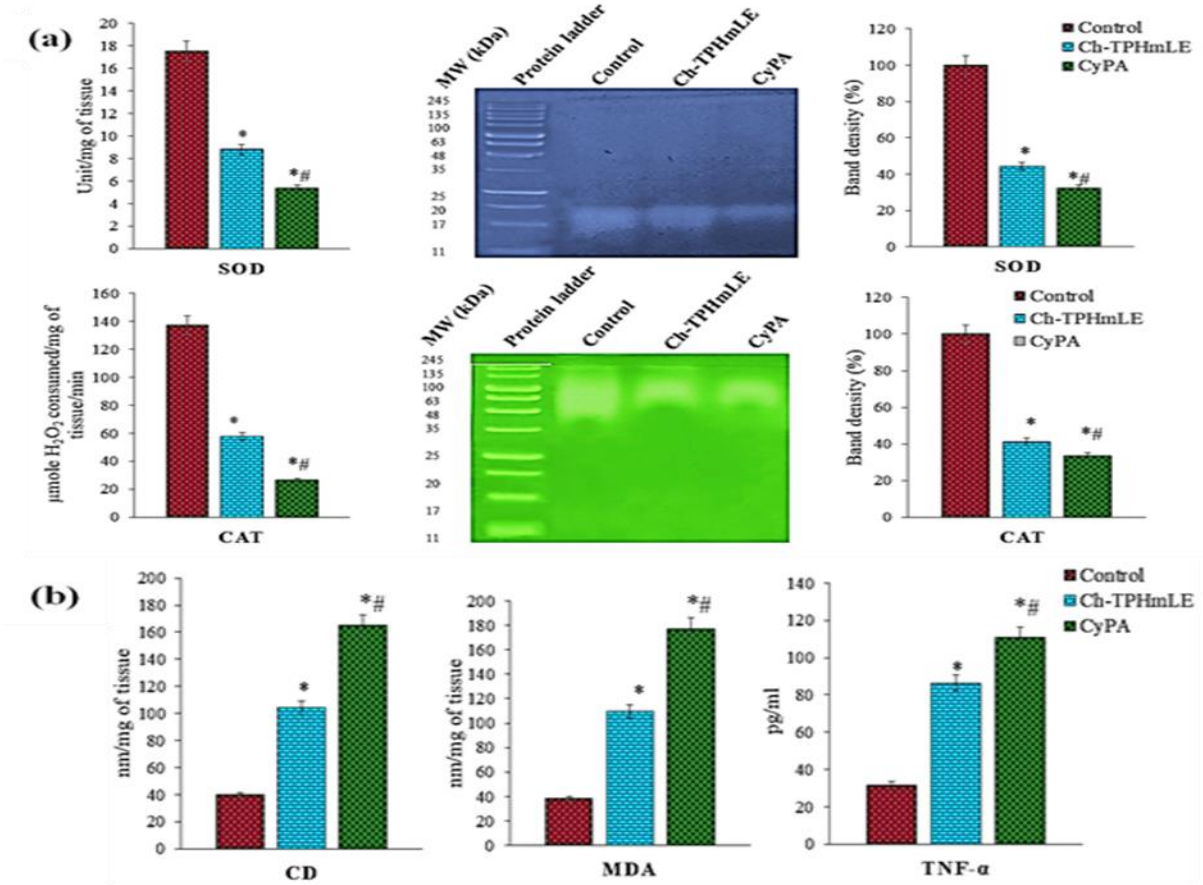

Figure 4. (a) Effect of Ch-TPHmLE and CyPA on testicular superoxide dismutase (SOD), catalase (CAT) activities and expressions. (b) Effect of Ch-TPHmLE and CyPA on testicular malondialdehyde (MDA), conjugated diene (CD) and tumor necrosis factor-α (TNF-α). Data are presented as Mean±SEM (n=6). ANOVA followed by two-tail t-test was performed. Asterisk (*) in each bar indicates significant difference compared with the control ($p<0.05$) and hashtag (#) indicates significant difference ($p<0.05$) compared with the Ch-TPHmLE.

Inflammatory marker assay

TNF-α level in testis was significantly elevated in Ch-TPHmLE and CyPA treated groups compared to the control. But more significant elevation of TNF-α was observed in CyPA treated animals compared to the Ch-TPHmLE treated animals (Figure 4b).

Expression status of testicular genes

After treatment with Ch-TPHmLE and CyPA, semi-quantitative gradient of PCR analysis showed downregulated expression of testicular *17β-HSD, 3β-HSD*, *StAR* and *AR* genes compared to the controls. Upregulated expression of *caspase-3* gene along with pro-apoptotic *P53* and *BAX* genes and, downward expression status of anti-apoptotic gene *Bcl-2* compared with the controls were observed (Figure 5).

DNA fragmentation grades

In Ch-TPHmLE and CyPA treated rats (lane-2 and lane-3), high rate of DNA degradation was noted compared with the control rats. For both Ch-TPHmLE and CyPA exposed rats, far migration of DNA with faded intense band was visible (Figure 6a).

Activities of serum GPT, GOT, ACP, and ALP

In Ch-TPHmLE treated group and control group, no noticeable variation was observed in serum GPT, GOT, ACP, or ALP activities. The activities of these enzymes were significantly ($p<0.05$) elevated in the CyPA group than the control and Ch-TPHmLE treated group (Figure 6b).

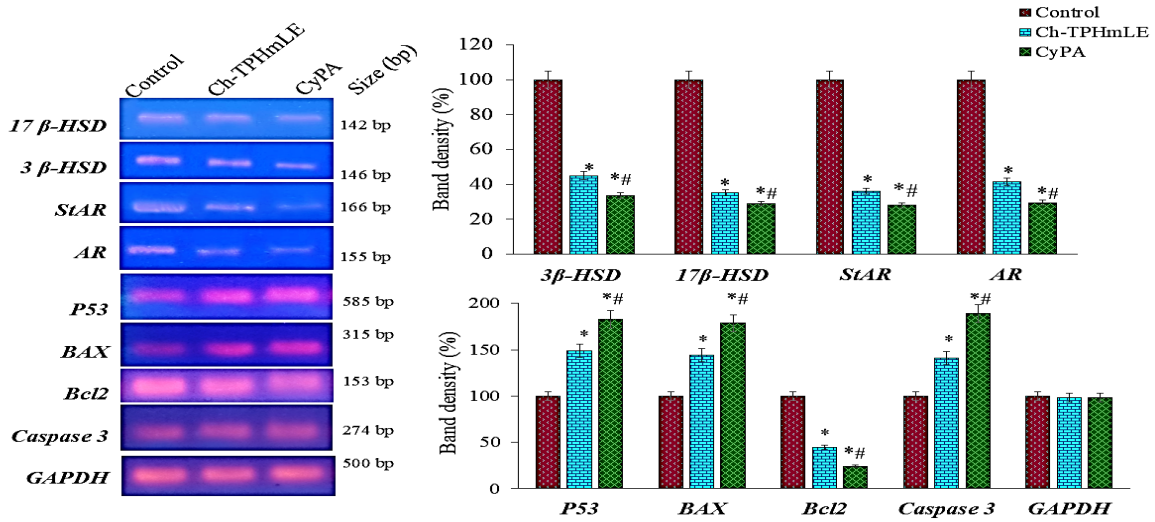

Figure 5. The effect of Ch-TPHmLE and CyPA on testicular gene expressions of 17β-hydroxylsteroid dehydrogenase *(17β-HSD)*, 3β-hydroxysteroid dehydrogenase *(3β-HSD)* steroidogenic acute regulatory *(StAR)*, *androgen receptor (AR)* and on tumor protein 53 (*P53)*, Bcl2 associated X protein *(BAX)* and B-cell lymphoma 2 (*Bcl2*) along with *caspase-3* gene expressions status by PCR study where *GAPDH* was considered as a principle gene. Data are presented as Mean±SEM (n=6). ANOVA followed by two-tail t-test was performed. Asterisk (*) in each bar indicates significant difference compared with the control ($p<0.05$) and hashtag (#) indicates significant difference ($p<0.05$) compared with the Ch-TPHmLE.

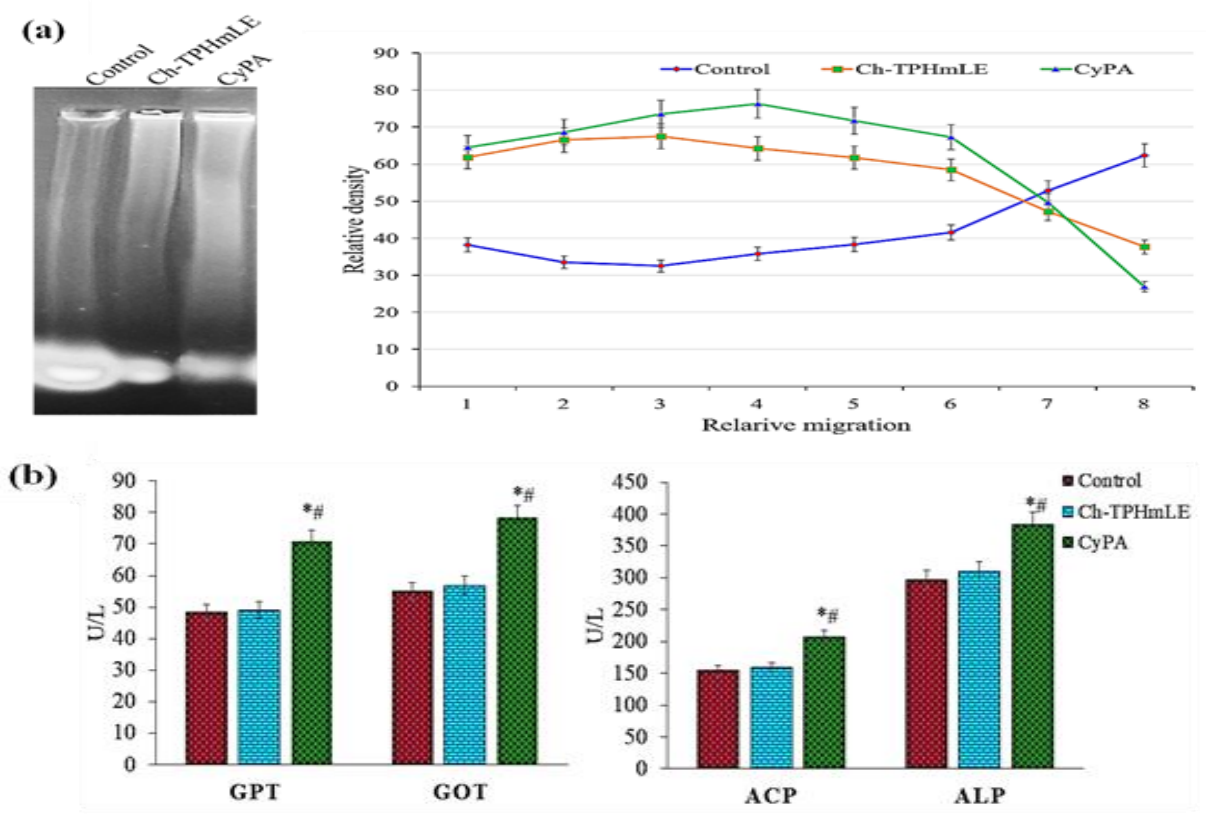

Figure 6. (a) Effect of Ch-TPHmLE and CyPA on DNA fragmentation in testicular cells. Graphical representation of the band intensity of DNA at its different positions on the agarose gel was evaluated by Image(J) software, expressed in relative/ normalized values. (b) Effect of Ch-TPHmLE and CyPA on serum glutamate pyruvate transaminase (GPT), glutamic oxaloacetate transaminase (GOT), acid phosphatase (ACP), and alkaline phosphatase (ALP) activities. Data are presented as Mean±SEM (n=6). ANOVA followed by two-tail t-test was performed. Asterisk (*) in each bar indicates significant difference compared with the control ($p<0.05$) and hashtag (#) indicates significant difference ($p<0.05$) compared with the Ch-TPHmLE.

Morphometrical, histochemical, and immunohistochemical findings

Histopathological investigation of the testis, epididymis (cauda), and seminal vesicle in the control group shows normal architecture with normal distribution of spermatogenic cells within the ST.

In Ch-TPHmLE and CyPA treated groups, number and distribution pattern of the spermatogenic cells, Sertoli cells and Leydig cells were altered in ST. So, the TDI, SI and RI indices were decreased significantly (Table 3 and Figure 7). The spermatozoal population decreased in epididymis in Ch-TPHmLE and CyPA treated rats compared with control rats (Figure 7).

Muscular layer of seminal vesicular was thickened and prominent disruption of cytoarchitecture of lamina propria distribution pattern was observed in the Ch-TPHmLE and CyPA groups compared to the controls (Figure 7).

Testicular TUNEL positive cells number was elevated in the Ch-TPHmLE and CyPA treated groups compared to control (Figure 7). In the Ch-TPHmLE and CyPA treated groups, moderate to high levels of ALP activity was observed in testicular germ cells indicated by dark brown color compared to the control where faint brown colored cells were observed (Figure 7).

LC-MS analysis of *T. peruviana* (Ch-TPHmLE)

Six bio-active compounds were identified from Ch-TPHmLE through LC-MS analysis along with their tentative names, mole formula, mole weight (m/z) are tabulated (Table 4). The bio-active phyto-compounds are γ-Sitosterol, Stigmasterol, Dehydroergosterol, β-Amyrin, Lupeol, and α-Amyrone at different retention time (Figure 8).

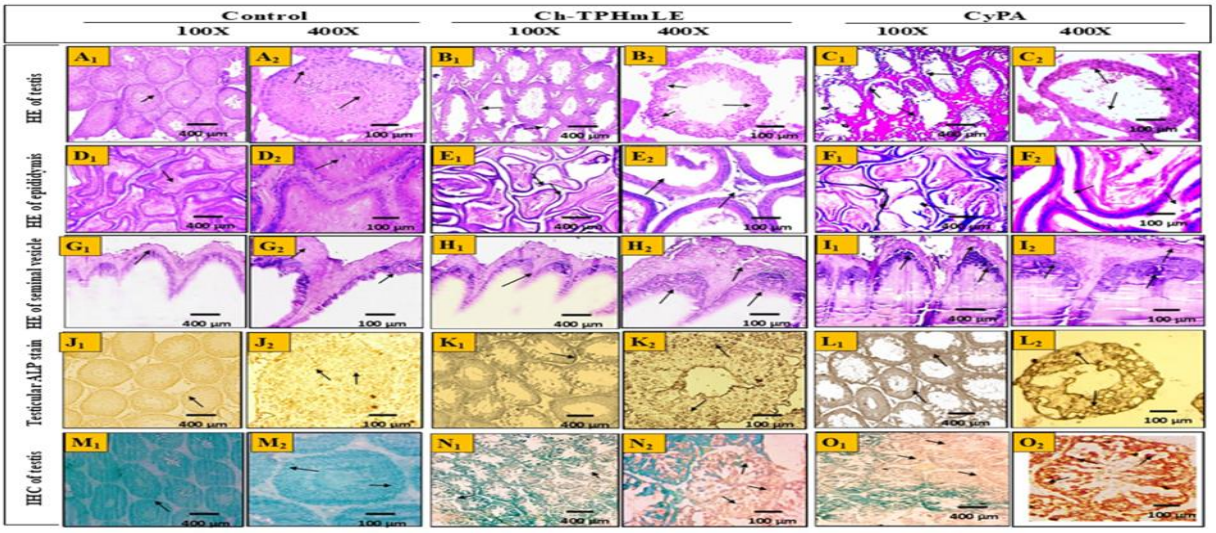

Figure 7. Microphotography (100X and 400X) of hematoxylin-eosin staining of testis, epididymis, and seminal vesicle along with immunostaining and alkaline phosphatase (ALP) staining in testis. A_1 & A_2: H-E stain of control rats display seminiferous tubules containing all stages of spermatogenesis in sequence. B_1 & B_2: In Ch-TPHmLE treated rats, reduction in the size of seminiferous tubules, characterized by fewer germ cells and an absence of spermatozoa in the lumen. C_1 & C_2: In CyPA treated rats maximum degeneration of germ cell layer and spermatozoa. D_1 & D_2: Histological observation of epididymal regions revealed an adequate spermatozoan population in control rats. E_1 & E_2: In Ch-TPHmLE treated group decreased spermatozoan population was observed in epididymis. F_1 & F_2.: In CyPA treated group very few spermatozoan population was observed in epididymis. G_1 & G_2: Normal architecture of seminal vesicular and proper lamina propria distribution. H_1 & H_2: Disruption of seminal vesicular muscularis thickness and architecture as well as altered lamina propria distribution pattern in Ch-TPHmLE group. I_1 & I_2: High level of disruption in thickness and architecture of seminal vesicular muscularis layer as well as altered lamina propria distribution pattern in CyPA group. J_1 & J_2: In control faint brown color developed in ALP stain. K_1 & K_2: Moderate to high levels of ALP activity were observed in spermatogenic cells in STs and developed dark brown color. L_1 & L_2: In CyPA group high levels of ALP activity were detected in spermatogenic cells and developed dark brown to black color. M_1 & M_2: Germ cells in ST showed green color indicating no apoptotic changes. N_1 & N_2: Brown color apoptotic cells were present in seminiferous tubule of testis. O_1 & O_2: Promising apoptotic change in testicular germ cell in CyPA treated group.

Table 3 Effect of Ch-TPHmLE and CyPA on histomorphometric indices and spermatogenic cells in seminiferous tubule. Data are presented as Mean±SEM, n=6. ANOVA followed by two-tail t-test was performed, and the asterisk (*) in each horizontal rows indicates significant difference ($p<0.05$) compared with control and hashtag (#) indicates significant difference ($p<0.05$) compared with the Ch-TPHmLE.

Parameters	Control	Ch-TPHmLE	CyPA
Seminiferous tube diameter (µm)	694.325±19.30	297.38±10.01*	241.88±3.79*#
Germinal epithelial height (µm)	172.13±7.99	52.13±4.45*	41.50±4.18*#
Lumen height (µm)	450.25±29.23	145.25±4.05*	134.50±3.57*#
Basal membrane thickness (µm)	13.63±0.61	7.63±0.78*	4.00±0.62*#
Spermatogonia [ASg]	7.13±0.51	4.38±0.44*	2.63±0.30*#
Preleptotene spermatocyte [pLSc]	48.38±3.32	34.50±2.86*	27.63±1.17*#
Mid pachytene spermatocyte [mPSc]	63.38± 3.34	35.00±1.51*	29.88±1.45*#
Spermatid [7Sd]	195.13±3.47	7.25±0.84*	3.87±0.63*#
Sertoli's cell	8.88±0.70	5.88±0.51*	3.75±0.68*#
Leydig's cell	16.38±0.78	8.63±0.69*	5.75±0.61*#
Tubular differentiation index [TDI] (%)	87.93±1.38	22.55±1.60*	17.75±0.72*#
Spermatogenesis index [SI] (%)	86.24±1.45	17.56±1.82*	13.50±0.79*#
Repopulation index [RI] (%)	89.10±1.41	81.15±1.24*	75.88±1.68*#

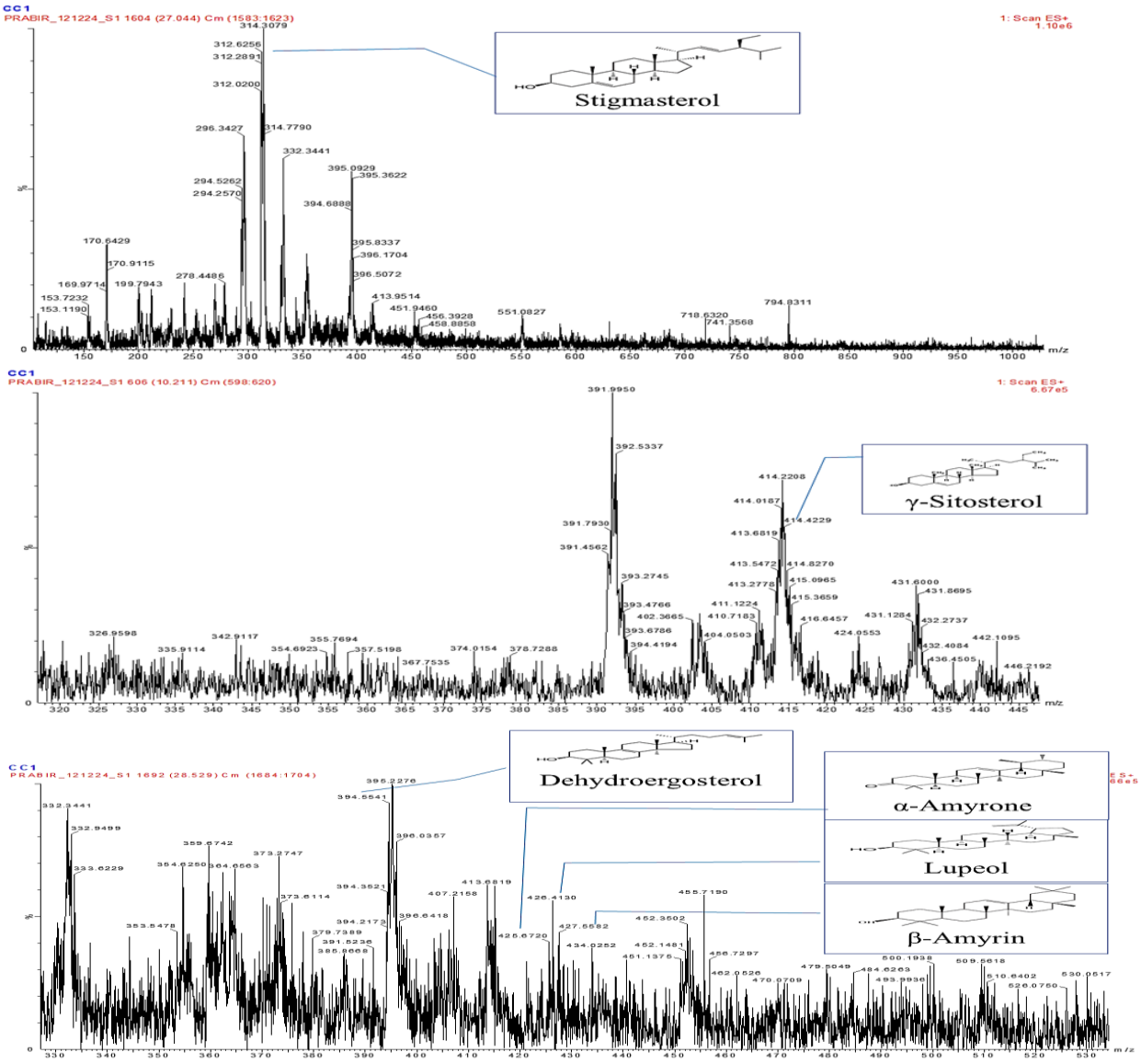

Figure 8. Phytoconstituents isolated by LC-MS analysis of chloroform fraction of *T. peruviana* (Ch-TPHmLE).

Table 4. Tentatively identified phytoconstituents by LC-MS analysis of chloroform fraction of *T. peruviana* (Ch-TPHmLE).

Sl. No.	RT (min)	Ion mode	Observed m/z	Reference m/z	Mol. Formula	Proposed/ Tentative compound
1.	10.211	$[M+H^+]$	414.42	414.70	$C_{29}H_{50}O$	γ-Sitosterol
2.	27.044	$[M+H^+]$	413.95	412,69	$C_{29}H_{48}O$	Stigmasterol
3.	28.5	$[M+H^+]$	427.67	426.7	$C_{30}H_{50}O$	β-Amyrin
4.	28.51	$[M+H^+]$	426.41	426.7	$C_{30}H_{50}O$	Lupeol
5.	28.52	$[M+H^+]$	425.67	424.7	$C_{30}H_{48}O$	α-Amyrone
6.	28.52	$[M+H^+]$	394.55	394.6	$C_{28}H_{42}O$	Dehydroergosterol

Discussion

Medicinal plant research has established an up-growing interest of research to control overpopulation. These herbal components either act as spermicidal agent or have a role in alteration of spermatogenesis process (Shaik et al. 2017). We have already established the spermicidal efficacy of *T. peruviana* leaves in an *in vitro* study (Mallick and Mondal 2022; Mondal et al. 2022). The present *in vivo* experiment was designed to study the male contraceptive efficacy of the most effective fraction i.e. chloroform fraction of *T. peruviana*. In this study, no significant alteration of body weight was observed between the control and Ch-TPHmLE treated group, whereas in CyPA treated group, body weight was significantly decreased. Testis and other accessory sex organs weight and their somatic indices significantly decreased in both *T. peruviana* and CyPA treated groups. This may be due to the growth inhibitory effect of Ch-TPHmLE and CyPA on reproductive organ through inhibition of testosterone synthesis as testosterone is one of the prime regulator of sex organs growth and development (Ghosh et al. 2017). The testicular inhibitory effect of Ch-TPHmLE and CyPA supported by the significant diminution of the gonadotropins (LH and FSH) directed lower testosterone levels. Significant diminution of epididymal sperm profiles was observed which ultimately reduced spermatogenesis process (Ghosh et al. 2018). Testicular haematoxylin-eosin staining of Ch-TPHmLE-treated and CyPA groups showed that significant reduction of A-Sg, pL-Sc, mP-Sc, and 7Sd germ cells count at stage VII spermatogenic cycle. Significant reduction of TDI, RI, and SI indices and seminiferous tubular diameter (STD) along with Leydig's cell and Sertoli's cell count (Adibmoradi et al. 2015), which may also support the diminution in testosterone synthesis as Leydig's cell, has a crucial role in testosterone synthesis (Gupta et al. 2011). Low Sertoli cell count can lead to reduced secretion of androgen-binding protein (ABP) which is directly proportional to the expression of *AR* gene and resulting in alteration of androgen action to the cellular level (Hwang et al. 2011). This, in turn, can negatively impact the development and maturation of spermatids and secondary spermatocytes, ultimately affecting spermatogenesis (Shaik et al. 2017).

Steroidogenic enzymes activity and expressions in testis were significantly decreased in Ch-TPHmLE- and CyPA-treated groups which in turn, leads to inhibition in testicular androgenesis as Δ^5, 3β-HSD and 17β-HSD are the key enzymes for androgenesis (Mondal et al. 2024). The inhibition of testicular androgenesis was also reflected here from the testicular cholesterol because it is the precursor of androgens and testosterone (Ghosh et al. 2022). Elevation in testicular cholesterol reflects improper utilization of cholesterol (Ghosh et al. 2022). Testosterone synthesis and release in testis depend on a series of rate-limiting enzymatic processes (Jeyakumar et al. 1995). To initiate this

process in Leydig's cell, mitochondrial StAR protein helps in the rapid transfer of cholesterol from outer membrane to inner mitochondrial membrane where cholesterol translates into pregnenolone which is then transformed to testosterone by the action of steroidogenic enzymes (Meena et al. 2012). So, the significant diminution of *StAR* gene expression causes low StAR-protein expression that is directly connected with inhibition of testosterone production (Tilbrook and Clarke 2001). Lower concentration of testosterone hampered spermatogenesis that was reflected by lower sperm count. Inhibition of spermatozoan motility in both treated groups may cause of spermatozoan agglutination (Siegel et al. 1986).

To determine the impact of oxidative stress on testicular function, we measured SOD and CAT activity as well as the quantity of end products of free radicals (CD and MDA) levels. Oxidative stress parameters provide additional evidence supporting the influence of Ch-TPHmLE and CyPA on alteration of androgenesis and spermatogenesis processes through the mediation of increased inflammation that expressed by high level of TNF-α and apoptosis. *Bcl-2* family are important anti-apoptotic marker which play a crucial role in determining a cell's susceptibility to apoptosis (Dey et al. 2021). Following pro-apoptotic marker genes *P53*, *BAX* and *caspase-3* gene, which are play vital roles in promoting germ cells apoptosis. Overexpression of *BAX*, *P53*, *caspase-3* and down regulation of *Bcl2* gene were observed in Ch-TPHmLE and CyPA treated groups which also supported by increased TUNEL positive germ cell numbers in testicular tissue. The mechanism of action of apoptosis is illustrated in Figure 9. Ch-TPHmLE has anti-androgenic, oxidative degenerative or apoptotic activity like cyproterone acetate (Ghosh and Mallick 2020). So, we compare the toxicity profile of Ch-TPHmLE and CyPA with control. Serum ACP, ALP, GOT and, GPT activities were elevated in CyPA group but these toxic markers remain unaltered in Ch-TPHmLE group. So, in therapeutic dose, Ch-TPHmLE has no metabolic toxicity compared to CyPA.

Chloroform fraction of *T. peruviana* leaves contains phytosterol i.e. γ-Sitosterol, Stigmasterol, Dehydroergosterol (Samanta et al. 2021) and triterpenes like β-Amyrin, Lupeol and, α-Amyrone identified from the LC-MS data that are responsible for male fertility regulatory compounds (Leite et al. 2008; Gupta et al. 2011; Khan et al. 2024).

Findings can be inferred that Ch-TPHmLE contain the above-mentioned active phytochemicals that exhibit anti-testicular properties. Two potential hypotheses could be explained the anti-testicular activity of Ch-TPHmLE. Firstly, the active ingredient(s) may modify the pituitary-testicular hormonal balance, therefore inhibiting the testicular activities. Secondly, the effective ingredient(s) could trigger oxidative stress in testicular tissue, leading to the production of free radicals that impede sperm functions through the elevation of inflammatory markers and activation of germ cells apoptosis. Further studies are required to validate the hypotheses or mechanism of action of active ingredient(s) from Ch-TPHmLE in this concern.

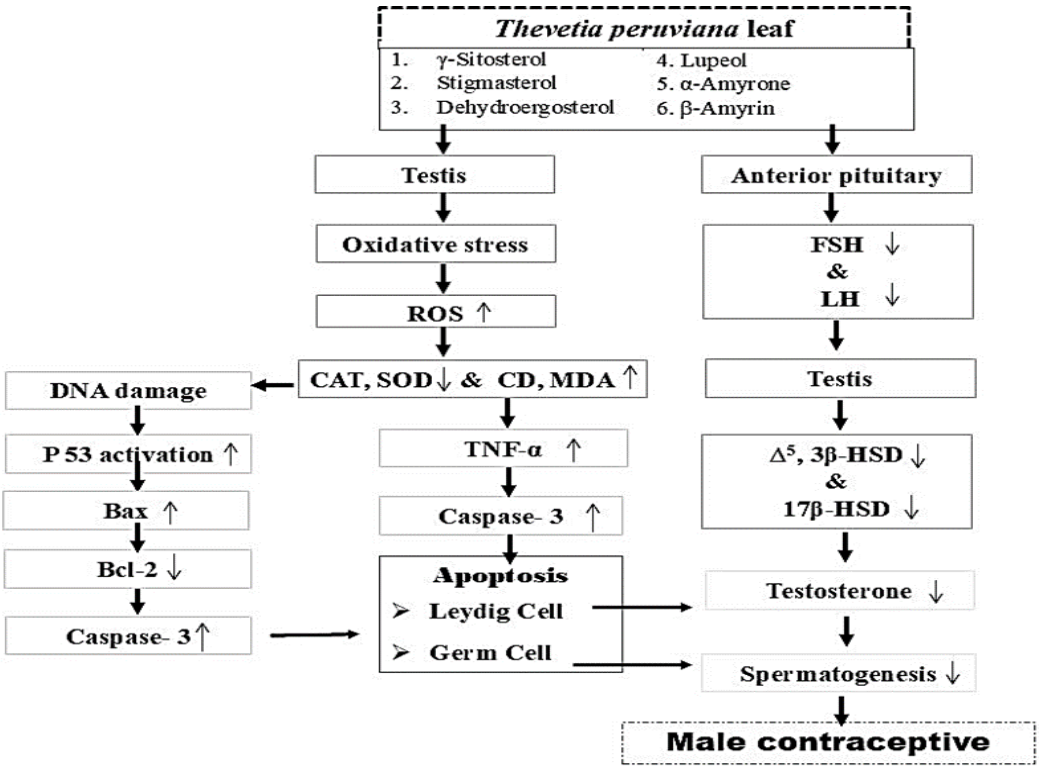

Figure 9. Schematic diagram of male contraceptive efficacy of *T. peruviana* through oxidative stress dependent apoptosis and hormonal alteration. SOD: Superoxide dismutase; CAT: Catalase; MDA: Malondialdehyde; CD: Conjugated diene; TNF-α: Tumor necrosis factor-α; ROS: Reactive oxygen species; LH: Luteinizing hormone; FSH: Follicle stimulating hormone; 17β-HSD: 17β-hydroxylsteroid dehydrogenase; Δ^5,3β-HSD: Δ^5,3β-hydroxysteroid dehydrogenase; DNA: Deoxyribonucleic acid; P53: Tumor protein 53; BAX: Bcl2 associated X; Bcl2: B-cell lymphoma 2.

Acknowledgment

We are thankful to Vidyasagar University authority for providing us with research facilities to conduct this work.

Conflicts of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical Considerations

This experiment was ratified by proper housing and care in our institutional animal house. The study followed regulatory guideline of CPCSEA.

Code of Ethics

This experiment was conducted by the permission of ethical committee of our institution, Registration No. VU/IAEC/CPCSEA/4/6/2022, Date-26/04/2022.

Authors' Contributions

PM: Animal treatment, semen analysis, hormonal assay, performed histology, data analysis. **SDM:** Extract preparation, **PRB**: Biochemical analysis. **CMM:** Supervision, writing-reviewing and editing, formal analysis. All authors revised and approved the final manuscript for publication.

References

Adibmoradi M, Morovvati H, Moradi H R et al (2015) Protective effects of wheat sprout on testicular toxicity in male rats exposed to lead. Reprod Syst Sex Disord 4:1-9. DOI: 10.4172/2161-038X.1000156

Ahmed MM, Hussein MMA, Saber T et al (2022) Palliative effect of resveratrol against nanosized iron oxide-induced oxidative stress and steroidogenesis related genes dysregulation in testicular tissue of adult male rats. Int J Environ Res Public Health 19:8171. https://doi.org/10.3390/ijerph19138171

Allain CC, Poon LS, Chan CSG et al (1974) Enzymatic determination of total serum cholesterol. Clin Chem 20:470-475. https:// doi.org/10.1093/clinc hem/20.4. 470.

Charan J, Biswas T (2013) How to calculate sample size for different study designs in medical research?. Indian J Psychol Med 35:121-126. https://doi.org/10.4103/0253-7176.116232

Chauhan NS, Sharma V, Dixit VK (2011) Effect of *Asteracantha longifolia* seeds on the sexual behaviour of male rats. Nat Prod Res 25:1423-1431. https://doi.org/10.1080/14786410802588493

Coutinho EM (2002) Gossypol: a contraceptive for men. Contraception 65:259-263. https://doi.org/10.1016/s0010-7824(02)00294-9

Dey A, Perveen H, Khandare AL et al (2021) Arsenic-induced uterine apoptotic damage is protected by ethyl acetate fraction of *Camellia sinensis* (green tea) via Bcl-2-BAX through NF-κB regulations in Wistar rats. Environ sci pollut r 28:41095-41108. https://doi.org/10.1007/s11356-021-13457-w

Garcia-Martinez V, Macias D, Gañan Y et al (1993) Internucleosomal DNA fragmentation and programmed cell death (apoptosis) in the interdigital tissue of the embryonic chick leg bud. J Cell Sci 106:201-208. https://doi.org/10.1242/jcs.106.1.201

Ghosh A, Pakhira BP, Tripathy A et al (2017) Male contraceptive efficacy of poly herbal formulation, contracept-TM, composed of aqueous extracts of *Terminalia chebula* fruit and *Musa balbisiana* seed in rat. Pharm Biol 55:2035-2042. https://doi.org/10.1080/13880209.2017.1357734

Ghosh C, Mallick C (2020) Protective effect of ethanolic extract of *Hygrophila auriculata* seeds in cyproterone acetate-induced sexual dysfunction in male albino rats. Andrologia 52:e13482. https://doi.org/10.1111/and.13482

Ghosh P, Gupta P, Tripathy A et al (2018) Evaluation of hypotesticular activities of different solvent fractions of hydro-methanolic extract of the fruit of *Terminalia chebula* in Wistar strain adult albino rat: Genomic and flow cytometric approaches. J Appl Biomed 16:394-400. http://dx.doi.org/10.1016/j.jab.2018.08.003

Ghosh P, Tripathy A, Gupta P et al (2022) Preventive role of human chorionic gonadotrophin (hCG) or Vitamin E or Vitamin C on ethyl acetate fraction of *Terminalia chebula* mediated antitesticular activities. Int J Food Sci Nutr 11:1167-1179.

Gupta R, Kachhawa JB, Gupta RS et al (2011) Phytochemical evaluation and antispermatogenic activity of *Thevetia peruviana* methanol extract in male albino rats. Hum Fertil 14:53-59. https://doi.org/10.3109/14647273.2010.542230

Hesari AK, Shahrooz R, Ahmadi A et al (2015) Crocin prevention of anemia-induced changes in structural and functional parameters of mice testes. J Appl Biomed 13:213-223. https://doi.org/10.1016/j.jab.2015.02.001

Hwang TI, Liao TL, Lin JF et al (2011) Low-dose testosterone treatment decreases oxidative damage in TM3 Leydig cells. Asian J Androl 13:432. https://doi.org/10.1038/aja.2010.159

Jarabak J, Adams JA, Williams-Ashman HG et al (1962) Purification of a 17β-hydroxysteroid dehydrogenase of human placenta and studies on its transhydrogenase function. J Biol Chem 237:345-357. https://doi.org/10.1016/s0021-9258%2818%2993926-8

Jeyakumar M, Suresh R, Krishnamurthy HN et al (1995) Changes in testicular function following specific deprivation of LH in adult male rabbit. J Endocrinol 147:111-120. https://doi.org/10.1677/joe.0.1470111

Khan RA, Afzal S, Aati HY et al (2024) Phytochemical characterization of *Thevetia peruviana* (lucky nut) bark extracts by GC-MS analysis, along with evaluation of its biological activities, and molecular docking study. Heliyon 10:e33151.

https://doi.org/10.1016/j.heliyon.2024.e33151

Leblond CP, Clermont Y (1952) Definition of the stage of the cycle of seminiferous epithelium in the rat. Ann New York Acad Sci 55:548-573. https://doi.org/10.1111/j.1749-6632.1952.tb26576.x

Leite CQ, Sato DN, Higuchi CT et al (2008) Antimycobacterial activity of *Byrsonima crassa* Nied leaf extracts. Rev Bras Pl Med10:63-66.

Lohiya NK, Goyal RB (1992) Antifertility investigations on the crude chloroform extract of *Carica papaya* Linn. seeds in male albino rats. Indian J Exp Biol 30:1051-1055.

Mallick C, Mondal P (2022) Spermicidal efficacy of *Thevetia Peruviana* leaves by modulating DNA damage and antioxidant status. Andrology S2:001. DOI:10.35248/2167-0250.22.S2.001.

Marklund S, Marklund G (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 47:469-474. https://doi.org/10.1111/j.1432-1033.1974.tb03714.x

Meena R, Misro MM, Ghosh D et al (2012) Complete sperm suppression induced by dienogest plus testosterone undecanoate is associated with down-regulation in the expression of upstream steroidogenic enzyme genes in rat testis. Contraception 86:163-171. https://doi.org/10.1016/j.contraception.2011.11.020

Mondal P, Bera PR, Maity R et al (2024) Anti-spermatogenic effect of *Thevetia peruviana* leaves in albino rat. Int J Pharm Sci & Res15:177-186. doi: 10.13040/IJPSR.0975-8232.15(1). 177-86.

Mondal P, Maity R, Mallick C (2022) *In vitro* spermicidal effect of *Thevetia Peruviana* leaves on human spermatozoa. Andrologia 54:e14323. https://doi.org/10.1111/and.14323

Muttreja P, Singh S (2018) Family planning in India: The way forward. Indian J Med Res 148: S1. https://doi.org/10.4103%2Fijmr.IJMR_2067_17

Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351-358. https://doi.org/10.1016/0003-2697(79)90738-3

Pal A, Misra B, Das S et al (2013) Antidiabetic effect of *Cephalandra indica* Q in diabetic rats. Ind J Res in Homoe 7:81-90. https://doi.org/10.4103/0974-7168.116632

Porat O (1990) Effects of gossypol on the motility of mammalian sperm. Mol Reprod Dev 25:400-408. https://doi.org/10.1002/mrd.1080250414

Reitman S, Frankel S (1957) A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am J Clin Pathol 28:56-63. https://doi.org/10.1093/ajcp/28.1.56.

Samanta J, Bhattacharya S, Rana AC (2021) Evaluation of ethyl acetate, chloroform and toluene fractions of *Thevetia peruviana* (Pers.) K. Schum methanolic leaf extract for uterotonic activity. Am J Heterocycl Chem 7:7-10. https://doi.org/10.11648/j.ajhc.20210701.12

Shaik A, Yalavarthi P, Bannoth C (2017) Role of anti-fertility medicinal plants on male & female reproduction. J complement altern med res 3:1-22. http://dx.doi.org/10.9734/JOCAMR/2017/34632

Shi SR, Cote RJ, Young LL et al (1997) Antigen retrieval immunohistochemistry: practice and development. J Histotechnol 20:145-154. http://dx.doi.org/10.1177/002215549704500301

Siegel I, Dudkiewicz AB, Friberg J et al (1986) Inhibition of sperm motility and agglutination of sperm cells by free fatty acids in whole semen. Fertil Steril 45:273-279. https://doi.org/10.1016/S0015-0282(16)49167-3

Sinha AK (1972) Colorimetric assay of catalase. Anal Biochem 47:389-394. https:// doi.org/ 10. 1016/ 0003- 2697(72)90132-7

Slater TF (1984) Overview of methods used for detecting lipid peroxidation. InMethods in enzymology. Academic Press 105:283-293. https://doi.org/10.1016/s0076-6879(84)05036-9

Sokal RR, Rohle FJ (2013) Biometry: The principles and practice of statistics in biological research; Introduction to analysis of variance, 4th Edn. WH Freeman

and Company, New York, pp179-206.

Soud P, Gupta HL, Kumar P et al (2015) Estimation and comparison of levels of alkaline phosphatase (ALP), acid phosphatase (ACP), calcium (Ca) and potassium (K) in serum of subjects with and without periodontal disease (PD). Int J Appl Dent Sci 1:90-93. https://doi.org/10.3390/ijerph19138171

Talalay P (1962) Hydroxysteroid dehydrogenases: Hydroxysteroid+ DPN+(TPN+)⇄ Ketosteroid DPNH (TPNH)+ H+. InMethods in enzymology. Academic Press 5:512-526. https://doi.org/10.1016/S0076-6879(62)05269-6

Theas MS, Rival C, Jarazo-Dietrich S et al (2008) Tumour necrosis factor-α released by testicular macrophages induces apoptosis of germ cells in autoimmune orchitis. Hum reprod 23:1865-1872. https://doi.org/10.1093/humrep/den240

Tilbrook AJ, Clarke IJ (2001) Negative feedback regulation of the secretion and actions of gonadotropin releasing hormone in males. Biol Reprod 64:735-742. https://doi.org/10.1095/biolreprod64.3.735

WHO (1996) Task Force on Methods of Regulation of Male Fertility. Contraception efficacy of testosterone induced azoospermia and oligospermia in normal men. Fertil Steril 65:821-829.

Zeidan A, El-Sharabassy A, Mekkawy Y et al (2018) Assessment of the functional integrity of equine spermatozoal membrane using hypo-osmotic swelling test. Suez Canal Vet Med J 23:157-168. https://dx.doi.org/10.21608/scvmj.2018.60499