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AMELIORATIVE AND PHYTO-THERAPEUTIC POTENTIAL OF ANCIENT MEDICINAL PLANTS TO MANAGE REPRODUCTIVE HEALTH IN MALE ALBINO RATS

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Abstract:
The use of nicotine is increasing particularly in young generation which leads to the development of different diseases. Reproductive problem in male and female is also one of them because nicotine directly interferes with antioxidant mechanism, reproductive hormonal axis and destroyed the reproductive organ histology. This research was planned to explore the ameliorative and phytotherapeutic potential of selected medicinal plants including *M. pruriens*, *C. behen* and *P. embilica* hydroethanolic extracts preparation as Polyherbal preparation (PHP) in albino male rats. PHP was evaluated for phytochemicals, antioxidant activities, *In-vitro* spermatozoa parameters and *In-vivo* study in male albino rats after induction of infertility by nicotine. The results revealed the presence of wide range phytomolecules including tannins, flavonoids, saponins, alkaloids, triterpenes, and glycosides; TPC (56.13 ± 1.39 mgGAE/g), and TFC (18.915±1.417 µgCE/g dry). HPLC results showed different important phenolic compounds while FTIR analysis reveals many peaks for the presence of functional molecules. The results of hemolytic and thrombolytic activities explored significant ($p<0.05$) protective role of selected medicinal plants. *In-vitro* spermatozoa parameters study revealed that PHP has significant ($p<0.05$) potential on the motility and viability of sperms. The results of in-vivo study explored that PHP treatment significantly ($p<0.05$) restore the antioxidant status after intoxication with nicotine. A significant ($p<0.05$) improvement in the hepatic like ALT, AST and renal parameters including urea, creatinine and uric acid was also found. Moreover, the results of reproductive hormones including LH, FSH, testosterone and histological study of testis tissues explored significant ($p<0.05$) therapeutic impact of PHP treatment as compared to control groups. It could be concluded that selected medicinal plants have significant antioxidant as well as reproductive problems management properties that can help potential uses in traditional medicines as a therapeutic agent. However, trial at high level then clinical trials is required to declare this formation as a safe drug.

Keywords: Nicotine, oxidative stress, infertility, reproductive problems, polyherbal preparation, therapeutic agent

Introduction:

Nicotine was found as a naturally formed alkaloid that occurs primarily in the solanaceous plant family, mainly in the tobacco plant (*Nicotiana tabacum*). Numerous negative effects of nicotine have been documented in both humans and lab animals, and it has been demonstrated to impact various targets (Gopumadhavan et al., 2008). It has been demonstrated that long-term smokers suffer psychological effects. In addition, it has been found that nicotine interferes with rats' antioxidant defense mechanisms (Mirunalini & Krishnaveni, 2010). Cotinine is the main byproduct of the C-oxidation route of the nicotine biotransformation, one of the several metabolic pathways that metabolize nicotine (Gopumadhavan et al., 2008). Although the liver is thought to be the primary location for nicotine biotransformation, the kidneys and lungs are also involved in metabolism (Ahmad et al., 2021). Numerous studies have been conducted on the effects of nicotine on humans, animals, and diverse cell structures (Lanka, 2016). It has long been known to cause oxidative stress in both *in vivo* and *in vitro* settings. The main cause of the high toxicity effect is nicotine. Nicotine destroyed sertoli and spermatogenic cells resulting in impaired spermatogenesis. Nicotine also targets male accessory sex organ such as prostate which is not secure from damaging its secretory functions (Gottfredsen et al., 2013).

Despite the innovations triggered by the development of new synthetic medicines and antimicrobial agents, medicinal plants still hold an important role in today's medical system (Shahid et al., 2022; Atta et al., 2023). According to the World Health Organization (WHO) report, more than 80% of people in underdeveloped countries rely on herbal remedies because they are unable to afford allopathic treatments. Several Asian countries are seeing an increase in the usage of medicinal herbs (Rahman et al., 2022). Natural antioxidants found in medicinal herbs are highly abundant and scavenge free radicals produced during the process of oxidative stress. Reactive oxygen species (ROS) produced by oxidative stress as well as due to the failure of antioxidant defense mechanism, which can lead to hemostatic collapse and destruction of connective tissue operations. This may ultimately end up in a variety of disorders like high blood pressure, diabetes, cirrhosis of the liver, and fertility problems (Riaz et al., 2016).

Mucuna pruriens (*M. pruriens*) Lin. plant has historically been known by various names, including "Nescafe, Cowhage, and velvet bean (Fatima et al., 2022). Native to tropical regions of Asia and Africa; and is widely recognized for its numerous uses in agriculture and traditional medicine as a source of bioactive compounds (Onigemoa et al., 2022). The Mucuna plant possesses a wide range of biological properties, such as antioxidant, anti-infertility, anti-microbial, antiinflammatory, liver-protective, and cardioprotective effects (Murthy & Mishra, 2016). *M. Pruriens* is a therapeutic plant because of its richness in flavonoids, phenolics, and various proteins in its seeds (Zahra et al., 2022). Mucuna seeds have been shown to be a free radical scavenger (Deli et al., 2020). *Phyllanthus Emblica* (*P. Emblica* L.) is extensively found in tropical and subtropical regions of India, China, Indonesia, and Malaysia and commonly known as amla. *P. emblica* has an effective antioxidant capacity that may be attributed in part to the presence of flavonoids and various derivatives of gallic acids, such as epigallocatechin gallate and includes minerals, vitamins, amino acids, and vitamin C as well (Sharma et al., 2019). It is referred to as a brain and cardiac tonic in Unani medicine. Many Ayurvedic and Unani medicinal preparations contain the fruits of the Amla plant (Saini et al., 2022). They are applied in the management of coronary artery disease and leucorrhea. Moreover, amla is used to treat a number of stomach conditions, such as dyspepsia (Saini et al., 2022). *Centaurea behen's* root, known as behman safed, has been traditionally employed in herbal remedies for centuries. *C. behen* is an evergreen plant with a height of roughly one meter. *C. Behen* is employed in conventional herbal therapy after being dried and processed into a fine powder due to its therapeutic potential (Albayrak et al., 2017). Several active phytochemical components like sesquiterpene lactones, augerin B, guaianolides, grosshemin, solistitialin and Crystalline alkaloid bahamine, taraxasterol, acetate etc. (Chougule et al., 2012). Reactive oxygen species (ROS) aree produced by nicotine and reproductive

tissues are very sensitive towards ROS. So this research aimed to determine that the hydroethanolic extracts of *P. embilica, M. pruriens* and *C. behen* may have therapeutic potential to manage oxidative and reproductive problems in male albino rats induced by nicotine.

Materials and Methods:

Plant material and place of work

The seeds of *M. pruriens* L., roots of *C. behen* and seeds of *P. embilica* were obtained commercially from local market in Faisalabad, Punjab, Pakistan. The specimens were identified and authenticated from the Department of Eastern Medicine, Government College University, Faisalabad, Pakistan. The research work was performed at Department of Eastern Medicine and animal trail was run at Department of Pharmacy, Government College University, Faisalabad, Pakistan after ethical approval committee. Hydroethanolic extract (70%) was prepared following the protocol of Munir et al., (2022) of all selected plant materials and then were mixed in equal amounts (1:1:1) to make polyherbal preparation (PHP) for *in vitro* and *in vivo* studies.

Phytochemical analysis

Qualitative analysis for several phytochemicals in the hydroethanolic extracts of PHP was done according to the Munir et al., (2022) and phytochemicals includes alkaloids, flavonoids, tannins, saponins, glycosides, steroids, and triterpenoids.

Total Phenolic Contents Estimation

PHP was processed to estimate the total phenolic contents (TPC) using the Folin-Ciocalteu method described by Jain et al., (2014) Gallic acid was used to construct the standard curve, and a reading at 765 nm wavelength was taken to measure the intensity of the color complex. Total phenolic contents present in extracts was measured as mg equivalent to gallic acid (GAE) per mL of extract using the following formula; Total phenolic contents = C×V/M
where C = concentration of gallic acid (mg/mL) taken from standard; V = volume of extract used in mL; M = weight of plant extract in grams.

Total Flavonoids Contents

The quantification of total flavonoids contents (TFC) was done following the protocol described by Pranuthi et al., (2014) and optical density was measured using 510 nm wavelength. The TFC present in the hydroethanolic extract was calculated using a linear regression curve of Catechin (CE) as μg CE/g of dried plants material.

High Performance Liquid Chromatography (HPLC) of PHP

The identification of the selected phenolic compounds in the PHP was done using HPLC system (Perkin Elmer, USA). The analysis was conducted with a C18 column (250 x 4.6 mm, 5micrometer film width) maintained at thirty degrees Celsius. A Chromera HPLC system (Perkin Elmer, USA) with a Flexer Binary LC pump and a UV/Vis LC Detector was employed. Solvent A (70:30), composed of acetonitrile: methanol, respectively, and solvent B consisted of double distilled water with 5% glacial acetic acid, were used as mobile phase. Wavelength 275 nm was used to identify different phytochemical compounds using standards to compare the retention times and spiking (Munir et al., 2020).

Fourier Transform Infrared Spectroscopy (FTIR)

An FTIR spectrometer was used to examine the chemical bonds in the isolated phytochemicals. The protocol of Munir et al., (2020) was followed, and the FTIR spectrometer used was the Bruker Platinum ATR model with a diamond crystal.

Antioxidant Activities Total Antioxidant Capacity (Phosphomolybdenum Method)
Munir et al., (2022) protocol was used to determine the TAC of hydroethanolic extracts using Phosphomolybdenum assay. TAC results were represented in ascorbic acid (used as standard) equivalents mg/g of the dry plant and for reference control Butylated hydroxytoluene (BHT) was used.

Free Radicals Scavenging Activity
H_2O_2 scavenging activity was determined following the protocol of Keser et al., (2012). At 230 nm, absorption was determined right after ten minutes of incubation at 37 degrees Celsius. Ascorbic acid, or vitamin C, served as a positive control while phosphate buffer saline (PBS) was used as negative control (Keser et al., 2012). Calculation was done as following where 'AS' represent the absorbance in the presence of the extract sample or standard.

H_2O_2 scavenging (%) = {1-AS}/AS x 100
Then 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging potential of PHP was investigated according to the Marinova & Batchvarov, (2011) using Vitamin C and BHT as standard.
Calculation was done as following where 'Ao' and 'AS' represent the absorbance of blank and absorbance of sample or standard, respectively.

DPPH scavenging (%) = {Ao-AS}/Ao x 100

Evaluation of Reducing Power Activity
To evaluate the antioxidant potential of the selected medicinal plants, the ferric reducing power assay (FRAP) method was used in which the reducing potential of substance Fe3+ (CN)6 into Fe2+ (CN)6 by direct electron donation was measured. Yadav et al., (2014) protocol was used to determine the reducing potential of plant extract. Different concentrations of PHP as 25, 50, 100, 150, 200, 250, 300, 350, and 400 (µg/mL) were used to determine the dose response effect using 700 nm wavelength.

***In-vitro* assessment of Cytotoxicity in *M. pruriens* Extracts Hemolytic Assay for Evaluating the Cytotoxic Potential of PHP**
We employed a modified version of the Tsamesidis and Kalogianni, (2023) technique to assess the cytotoxicity of a hydroethanolic plant extracts (PHP). In triplicates, a red blood cell (RBC) solution was used at a concentration of 7.0 x 10^8 RBCs/mL. The results were calculated as follows:

$$Percent\ Hemolysis = \frac{(Ae-Ap)}{(Ad-Ap)} \times 100$$

Testing Thrombolytic Potential through Clot Lysis assay
To assess the potential of PHP to dissolve blood clots, we followed the procedure described by Munir et al., (2020). Approval was obtained from the Institutional Scientific Scrutiny Group (Ref. No. GCUF/DAS/19/1534) to recruit six healthy subjects. The clot-dissolving activity was determined using PBS as the negative control and a tube of streptokinase (1,500,000 IU) as the positive control, where W_I is initial clot weight and W_L is clot weight after lysis.
$Clot\ Dissolving\ Activity$ (%) = $\{W_I\text{-}W_L/W_I\} \times 100$

***In vitro* Spermatozoa Parameters**.
Healthy volunteers (time-to pregnancy (TTP) 12 month) (n = 05) were chosen for the semen samples collection after taking written informed consent and semen samples were processed according to World Health Organization (WHO) protocol.18 and sperms motility total (%), progressive motility (%) and viability (%) were measured according to the protocol of Munir et al., (2022).

In vivo Experimentation in male albino rats Cytotoxic Studies

The cytotoxic studies were conducted on rats to assess the toxicity of hydroethanolic extracts preparations. The dose was increased from 500 mg/kg/body weight to 750 mg/kg/body weight to 1000 mg/kg/body weight. At a dosage of 1000 mg/kg/body weight, it was noted that toxic symptoms developed after 12 hours of the medication administration. As a result, the highest dose chosen for the trial was less than one-fourth (200mg/kg/body weight) of the toxic level drug.

Grouping of animals with different plant extract dosages

For *in-vivo* experiments, albino male rats weighing between 190-200 grams at between eight and ten weeks of age were utilized as the animal model. There was total 6 groups and each group included 6 animals (n= 6). Group-1: Control group (normal diet and water only); Group-2: Intoxicated control (20 percent Nicotine 1 mg/Kg/body weight intraperitoneally (IP) (daily);
Group-3: Positive control (Nicotine 1 mg/Kg/body weight IP (daily) and administered Vitamin-E 100 mg/Kg/body weight orally (daily); Group-4: treatment group (Nicotine 1 mg/Kg/body weight IP daily) and administered PHP (50 mg/kg/body weight as low dose) (orally daily); Group5: Nicotine one mg/Kg/body weight given IP daily and PHP as 100 mg/Kg/body weight Group6: Nicotine one mg/kg/body weight and PHP as 200 mg/Kg/body weight as the high dose of hydroethanolic extracts on daily basis. After approval by the institutional ethical review panel, all animal groups were kept in the animal house for six weeks with a standard diet, a cycle of 12 hours of light and darkness, and a normal husbandry atmosphere.

Measurement of body weight change, Blood parameters and reproductive hormones To evaluate the impact of treatments on experimental rats each animal was weighed before starting the trial and then after 07 days to observe any change in the body weight. Then pre-clinical trial total weight of every group was subtracted from the post-clinical trial body weight to calculate the gain in weight (in grams). After forty-two (42) days, blood samples were taken using the heart puncture technique to determine specific antioxidants like total antioxidant status (TAS), total oxidant status (TOS) by Sahreen et al (2013) protocol; and blood parameters including creatinine and uric acid by Swanson et al. (1993), urea by Burtis and Ashwood (1994), liver enzymes like ALT and AST by Panteghini & Ceriotti, (2000). Then standard Enzyme-Linked Immunosorbent
Assay (ELISA) based kit method was used to measure the reproductive hormones, which includes testosterone by Chen et al., (1991), LH by Frank et al., (1996), and FSH by Qiu et al., (1998) in all male rats.

Histological Analysis Of Testicular Tissues

At the end of trial animals were dissected, testis tissue samples were obtained and processed following the protocol as described by Munir et al., (2020) to make thin sections of tissues for histological changes and stained by hematoxylin & eosin stain.

Statistical Data Analysis

Values were expressed as Mean ± SEM (standard error of means). To evaluate the results one-way analysis of variance (ANOVA) and Tukey test were used, and significance was defined as $p < 0.05$.

Results

The plants that were chosen for the research study have many active chemicals that have been linked to a variety of biological processes and may function as natural pharmaceuticals in the medical area. The crude hydroethanolic extracts preparation showed a variety of significant phytoconstituents, such as Alkaloids, carbohydrate, flavonoids, glycosides, phenols, saponin, tannins and terpenoids (table 1). The substance may possess a range of biological, antioxidant, and scavenging qualities.

Table 1: Result of Qualitative analysis of polyherbal preparation (PHP) prepared by mixing the Extracts for different Phytochemicals.

Phytoconstituents/ Plant	PHP prepared from Extract
Alkaloids by Dragendorff's test	++
Carbohydrate by Fehling test	++
Flavonoids by Shinoda's test	+++
Glycosides by Molisch's test	+++
Phenols by Liebermann-burchardt test	+++
Saponin by Frothing test	++
Tannins by Iron chloride test	++
Terpenoids	++

+ = low amount, ++ = Intermediate amount, +++ = high amount

Total Flavonoid & Phenolic Contents and Antioxidant Activities

The results of Total flavonoid content (TFC), and total phenolic content (TPC) of hydroethanolic extracts in form of PHP represent that the selected medicinal plants have significantly ($p < 0.05$) adequate amount of these therapeutically active Phyto molecules (Mean ± SD) (Table 2). **Table 2:** Total flavonoid content (TFC), antioxidant operations and total phenolic content (TPC) of hydroethanolic extracts of PHP (Mean ± SEM)

Phytochemicals/Plants	PHP	Vitamin C
TPC (mg GAE/g dry plant materials)	561.3 ± 13.9	--
TFC (µg CE/g dry plant materials)	189.15 ± 14.17	--
H2O2 Scavenging Activity (%)	16.39 ± 4.00	39.89 ± 3.31
DPPH Scavenging Activity (%)	62.10 ± 5.0	78.34 ± 4.38

High Performance Liquid Chromatography (HPLC) Studies

The PHP' HPLC chromatogram displays several peaks, which denotes the presence of a wide range of phytochemicals in the Selected includes Quercitin, Gallic Acid, Vanillic Acid, Benzoic Acid, Chlorogenic Acid, Syringic Acid, p-Coumeric Acid, m-Coumeric Acid, Ferulic Acid Cinamic Acid and Sinapic Acid. Polyphenols, a class of plant-based chemicals with antioxidant characteristics, are quantified using different standards (Figure 1 and Table 3). The existence of additional peaks may be due to other phytochemicals, which include flavonoids, alkaloids, and glycosides which may be responsible for several therapeutic activities of selected medicinal plant.

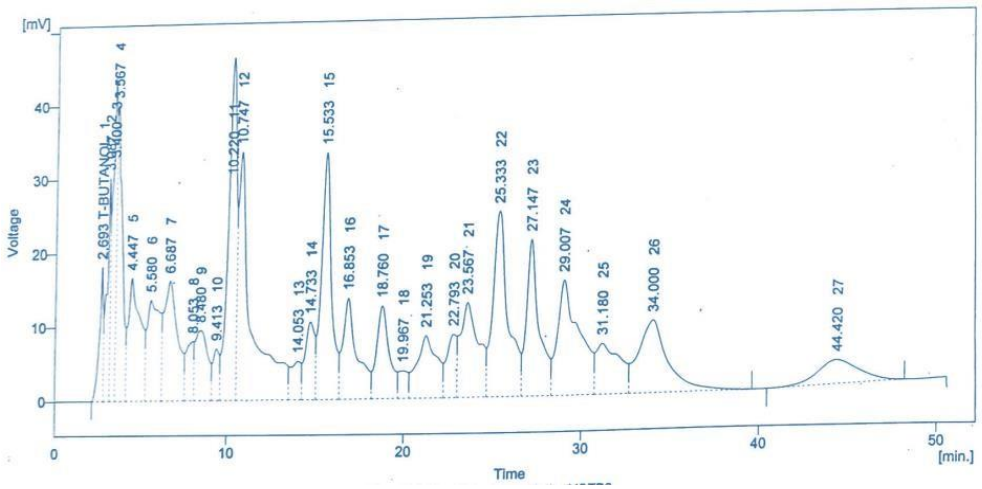

Figure 1: The HPLC chromatogram of the plant extract with a C18 column (250 x 4.6 mm, 5 micrometer film width) maintained at thirty degrees Celsius using Chromera HPLC system (Perkin Elmer, USA) with a Flexer Binary LC pump and a UV/Vis LC Detector.

Table 3: The HPLC studies revealed the presence of different phenolic coumpounds in the selected medicinal plants extracts in the form of PHP.

Phytomolecules	Retention time (min)	Area (mV.s)	Area (%)	Concentration of molecules (ppm)
Quercitin	2.967	4.547	0.1	1.59
Gallic Acid	4.813	133.701	2.3	4.81
Vanillic Acid	13.480	23.694	0.4	1.46
Benzoic Acid	14.653	25.659	0.4	2.71
Chlorogenic Acid	15.560	45.662	0.8	3.56
Syringic Acid	16.660	35.945	0.6	0.89
p-Coumeric Acid	18.020	77.467	1.3	1.16
m-Coumeric Acid	20.473	193.526	3.4	2.32
Ferulic Acid	21.933	55.061	1.0	3.96
Cinamic Acid	25.240	67.013	1.2	2.34
Sinapic Acid	26.627	265.355	4.6	3.44

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy revealed absorption maxima at 3,274.5 cm/sec, 1,716 cm/sec, 1,611 cm/sec, 1,447 cm/sec, 1,314 cm/sec, 1,206 cm/sec, and 1,025 cm/sec (Figure 2 and Table 4) which explored wide range of functional groups in the PHP might be responsible for the biological and therapeutic activities.

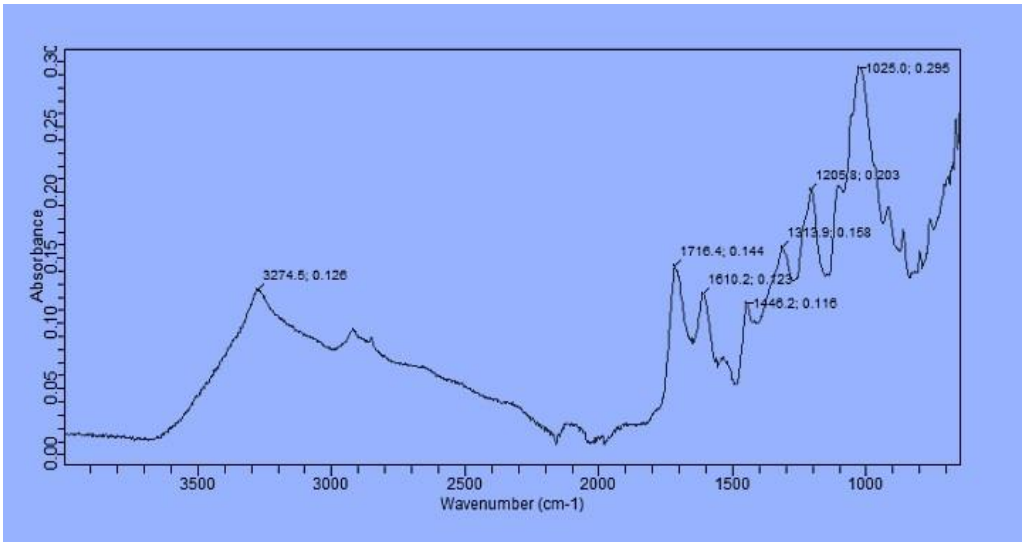

Figure 1: The hydroethanolic extracts PHP infrared spectra show that a variety of functional groups are present.

Table 4: Different functional groups and possible phytochemical constituents identified using FTIR in hydroethanolic extracts PHP.

Wave number (cm^{-1})/ Possible functional groups	Polyherbal Preparation (PHP)
4,000 to 2,500/cm O-H, N-H and C-H Stretch vibration	3,274.5
2000 to 1500/cm C=C, C=O, C=N	1,716 1,611
Stretch vibration	
1500 to 400 /cm C-C, C-N, C-O, C-Cl, C-I, S-S and N=O* Stretch, Bend or scissoring, rock vibration	1,447 1,314 1,206 1,025

In-Vitro Antioxidant Activity

PHP capacity to scavenge H2O2 was measured and found to be 16.39 % and moreover *PHP* also have potential to DPPH free radicals when tested using the DPPH assay (62.10 %) given in Table 2. Reducing power, in general, is a measurement of a substance's capacity to give electrons. As antioxidants can give electrons to free radicals and prevent the cellular structures from harming effect of oxidant agents might be present in cigarette smoke or produce as metabolites of these molecules. The results of FRAP assay and TAC by phosphomolybdenum method explored that with increasing the concentration of plant extract their antioxidant response also increased on comparing with ascorbic acid as control (Figure 3a and 3b).

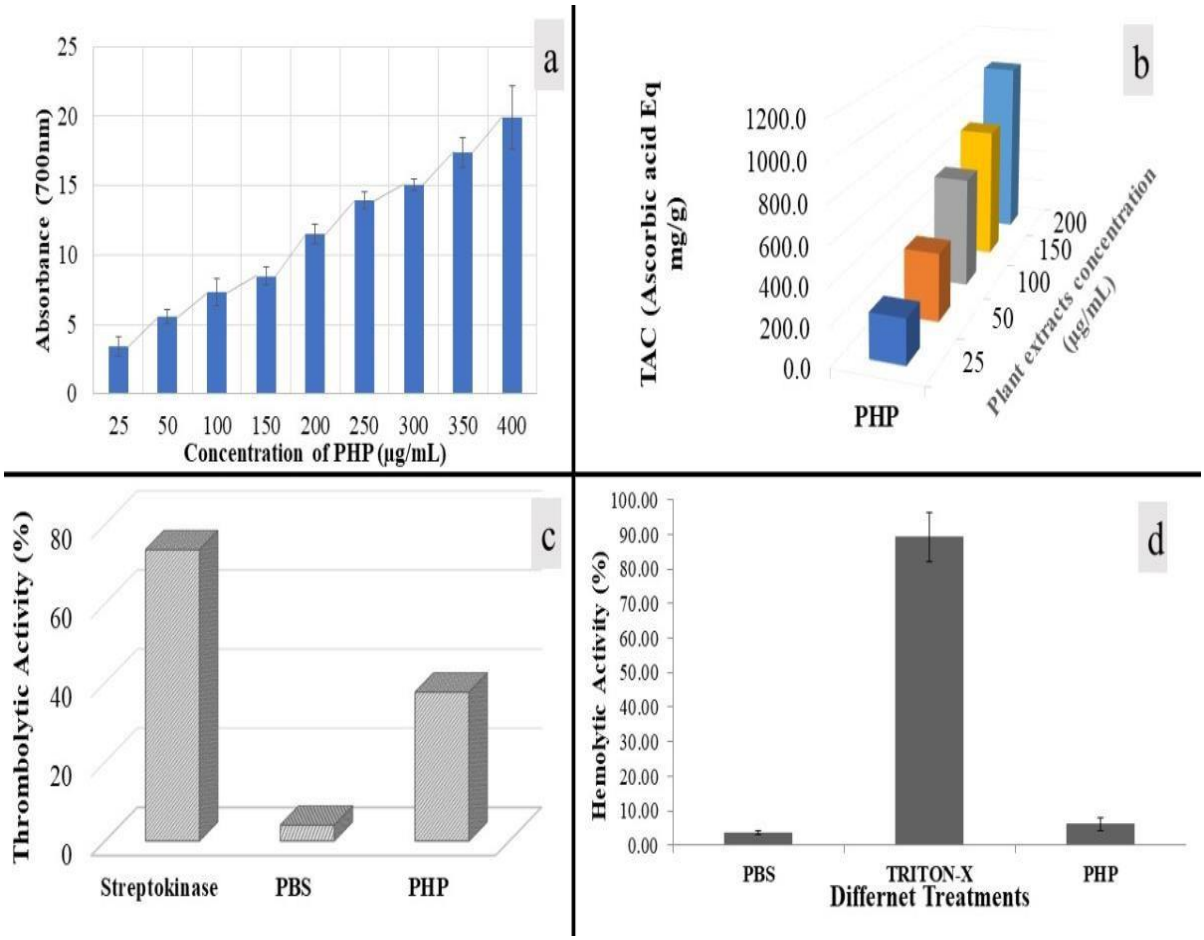

Figure 3: (a) PHP Reducing potential using different amounts of PHP by FRAP method, **(b)** PHP TAC by Phosphomolybdate assay **(c)** PHP thrombolytic activity determined against streptokinase as positive control and PBS as negative control **(d)** PHP hemolytic activity determined against Triton-X as positive control and PBS as negative control.

Hemolytic and Thrombolytic Activities

The results of thrombolytic assay showed that PHP have clot lysis potential (37.6 ± 4.3) compared able to the streptokinase (73.6 ± 7.7) used as positive control (Figure 2c). It was found on incubating the selected plants extracts preparation with RBCs that these medicinal plants cause non-significant hemolysis as compared to positive control Triton-X (89.31 ± 7.053) and negative control PBS (3.65 ± 0.675) (Figure 2d).

In vitro Spermatozoa Parameters

The results of i*n vitro* spermatozoa parameters explored that PHP different doses (25µg/mL, 50µg/mL, 100µg/mL) have significant ($p<0.05$) impact on the preservation of morphology, viability (%), and motility (%) of sperms as compared to control (normal saline) and plant extract (Figures 4).

Moreover, PHP did not show any significant ($p>0.05$) spermicidal potential while mixed with semen and incubated for 120 min at 37°C (Figures 4C, D and E).

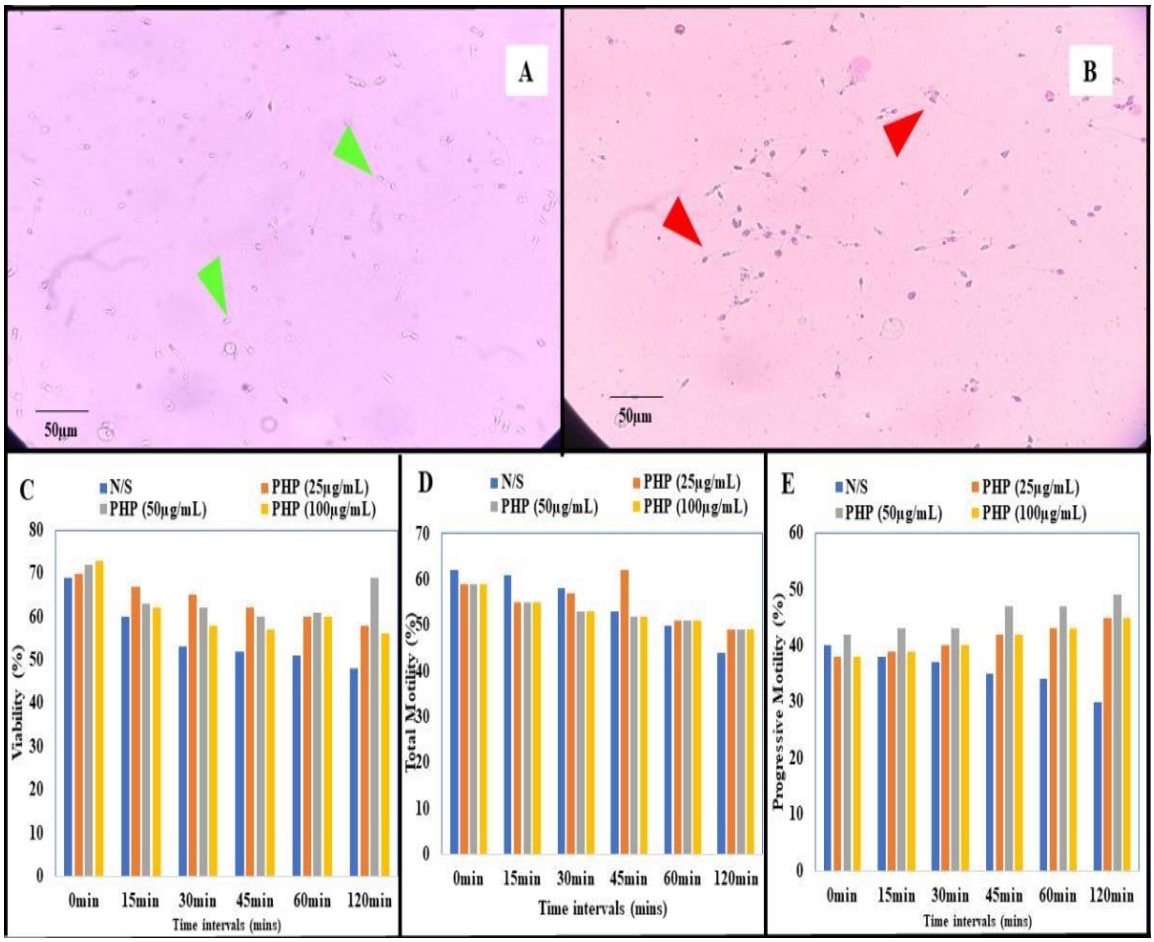

Figure 4: *In vitro* Spermatozoa Parameters after incubating with the PHP prepared from selective medicinal plants at different concentrations (A) Green arrow heads showed viable sperms stained with 0.5% eosin Y (B) Red arrow heads showed non-viable (pink color) sperms (C) Viability (%) of sperms at different time intervals (D) Total motility (%) of sperms at different time intervals and (E) Progressive motility (%) of sperms at different time intervals.

In Vivo Hydroethanolic Extract' Therapeutic Response

Renal Funtion Tests (RFTs), Liver Funtion Tests (LFTs), TOS and TAS

It was observed that the induction of nicotine in rats resulted in a significant ($P < 0.01$) reduction in TAS and a rise in TOS, whereas daily ingestion of plant extracts notably ($P < 0.01$) restored these biochemical parameters which not only improved the TAS and TOS but also represent the improvement of health status (Table 5). The PHP has Reno as well as hepatoprotective potential. Since most medicinal products have a variety of health benefits, assessment of drug toxicity has become one of the main concerns in drug development. After being intoxicated with nicotine, the statistical analysis showed a significant ($P < 0.01$) rise in liver enzymes which represent the liver damage. While on the other hand, administration of various doses of selected plants hydroethanolic extracts resulted in a dose-dependent improvement in the liver enzymes (Table 5). The results of renal parameters explored that intoxication caused using nicotine greatly ($p<0.05$) affects functioning of kidneys as measured by blood urea, creatinine, and uric acid levels. However, the administration of PHP significantly ($p<0.05$) restores these renal parameters in a dose-dependent manner (Table 5).

Table 5: Selective biochemical parameters in different treatment groups measured at the end of animal trials.

Parameters	G-1	G-2	G-3	G-4	G-5	G-6	P-value
TAS	0.788 ± 0.145^{CD}	1.13 ± 1.034^{F}	1.9 ± 1.2^{E}	0.920 ± 1.026^{C}	1.574 ± 1.054^{B}	2.603 ± 1.138^{A}	<0.01
TOS	14.578 ± 2.09^{BC}	27.649 ± 4.11^{A}	22.663 ± 2.121^{A}	19.156 ± 2.498^{B}	17.9C05 ± 1.633^{BC}	12.280 ± 1.379^{C}	<0.01
Urea (mg/dL)	28 ± 3.23C	44.25 ± 6.3A	41.67 ± 4.97A	43.18 ± 5.55A	34.18 ± 4.07B	30.17 ± 3.3C	<0.05
Creatinine (mg/dL)	0.67 ± 0.03B	1.1 ± 0.07A	1.0 ± 0.06A	1.0 ± 0.05A	0.8 ± 0.03B	0.7 ± 0.04B	<0.05
Uric Acid (mg/dL)	3.99 ± 0.8B	4.82 ± 0.9A	4.75 ± 1.0A	4.63 ± 0.71A	4.60 ± 0.85A	3.82 ± 0.59B	<0.05
ALT (IU/L)	22.67D	41.66A	37.5B	34.83C	25.166D	22.5D	<0.05
AST (IU/L)	15.33DE	53.5A	37.33B	30.51C	33.23C	24.66D	<0.05

G-1: Control group (normal diet and water only); G-2: Intoxicated control (20 percent Nicotine 1 mg/Kg/body weight intraperitoneally
(IP) (daily); G-3: Positive control (Nicotine 1 mg/Kg/body weight IP (daily) and administered Vitamin-E 100 mg/Kg/body weight orally (daily); G-4: treatment group (Nicotine 1 mg/Kg/body weight IP daily) and administered PHP (50 mg/kg/body weight as low dose) (orally daily); G-5: Nicotine one mg/Kg/body weight given IP daily and PHP as 100 mg/Kg/body weight; G-6: Nicotine one mg/kg/body weight and PHP as 200 mg/Kg/body weight as the high dose of hydroethanolic extracts preparation on daily basis.

TAS (Total antioxidant status), TOS (Total oxidant status), ALT (Alanine transaminase), AST (Aspartate transaminase). Values are mean + SEM (standard error) of means of the study groups. Different alphabets in the columns indicate significant group mean differences.
The $p<0.05$ considered statistically significant while $p<0.01$ indicates highly significant.
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Therapeutic effects on reproductive hormones and body weight

The statistical test results showed that, when compared to normal rats, rats exposed to nicotine had substantially ($p<0.01$) lower body weight and male reproductive hormones such as LH, FSH, and testosterone. A significant ($p<0.05$) improvement in the body weight was also observed at the end of experimental trial (Table 6 and Figure 5) in groups treated with standard drug as well as with different doses of PHP. Significant ($p<0.01$) restoration of the hormones has been reported on the use of various doses of hydroethanolic extracts preparation of selected medicinal plants when compared with positive control animals given Vitamin E.

Table 6: Mean ± SEM values of change in body weight of all animals under different treatments.

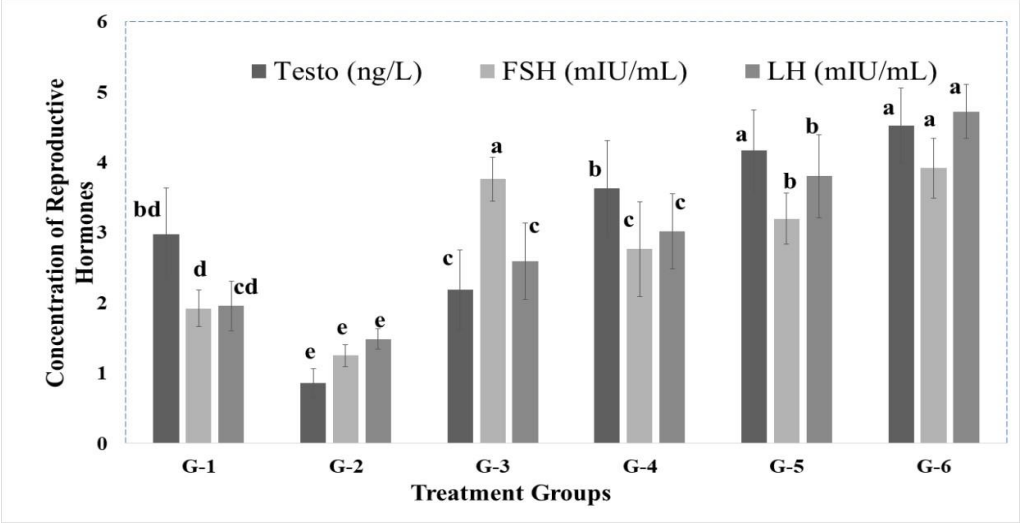

Figure 5: Mean ± SE values of the reproductive hormones in different experimental groups. Column share the same alphabets indicate non-significant ($p > 0.05$) differences.

Histological examination of testicular tissues

Histological analysis of the testicles showed that, in comparison to the standard control group, the rats' nicotine intoxication severely damaged the spermatogenic cells in the seminiferous tubes (Figure B1 & B2). Testis from normal group showed several seminiferous tube cells with germinal tissue (4-6 layers) at different spermatogenic stages and mature sperm cells in the central lumen.

In a normal testis, there were also enough other cells, such as Leydig and Sertoli cells (Figure 6A1 & A2). After nicotine intoxication, the histo-architecture of the areas from the positive controls and rats given high dose of PHP demonstrated significant improvement in the histological structure as compared to intoxicated group (Figure 6C & 6D).

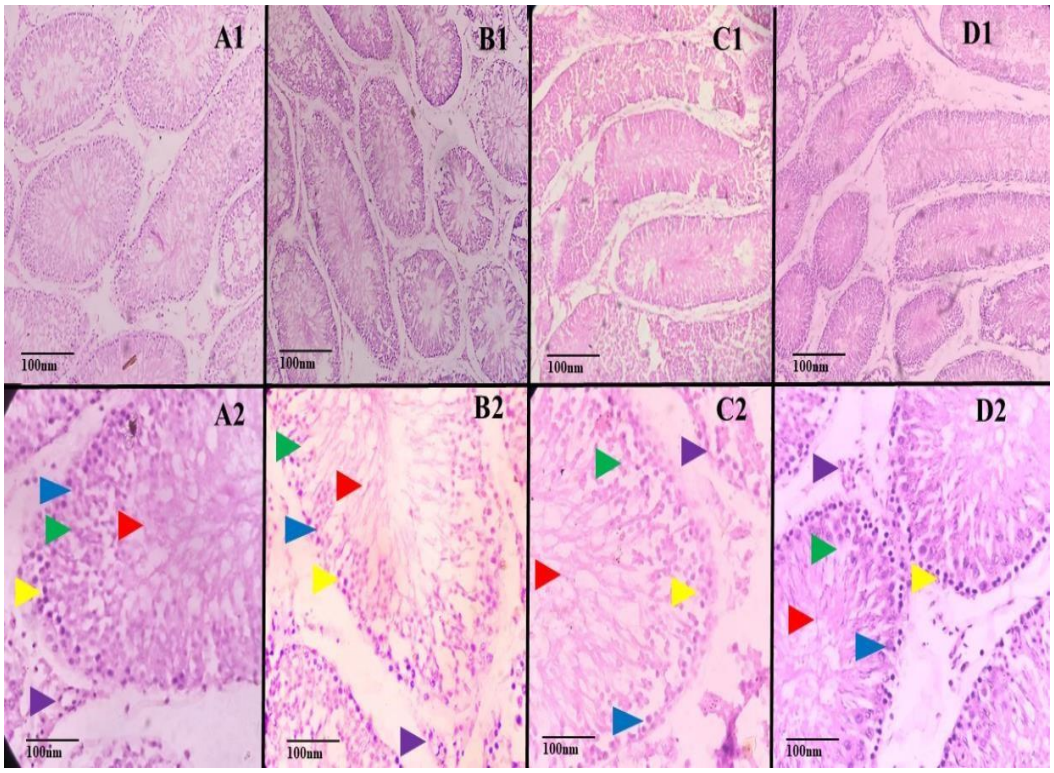

Figure 6: Rat testis histological structure as determined by sections of paraffin. stained with hematoxylin and eosin (A1-D1 at 100x and A2-D2 400x). (A1 & A2) Sections from G-1 Control group (normal diet and water only); (B1 & B2) Sections from G-2 Intoxicated control (20 percent Nicotine 1 mg/Kg/body weight intraperitoneally (IP) (daily); (C1 & C2) Sections from G-3: Positive control (Nicotine 1 mg/Kg/body weight IP (daily) and administered Vitamin-E 100 mg/Kg/body weight orally (daily); and (D1 & D2) Sections from G-6: Nicotine 1 mg/kg/body weight and PHP as 200 mg/Kg/body weight as the high dose of PHP. Seminiferous tubes are shaped normally and are arranged normally in sections A, C and D, with Sertoli cells (SCs) (Blue arrowhead) and spermatogonia (Sg) laying on an undamaged basement wall (yellow arrowhead). Primary spermatocytes (Ps), round (RS), elongated (ES), (Green arrowhead) and tubules lumen fill with mature spermatozoa (Red arrowhead) in sections A, C and D and normal distribution of Leydig cells in sections A, C and D (Purple arrowhead).

Discussion

Infertility in couples want to conceive is prevalent about 15% and 50% of the male factors contributed in this. Oxidative stress (OS) as a major contributor in male infertility caused sperm dysfunction, metabolic stress, reproductive hormonal disturbance and even damage the testicular structure (Agarwal et al., 2014). According to Variya et al. (2016), the main ingredient in smoking cigarettes that affects lung development and reduces pulmonary function is nicotine (Yadav et al., 2017). According to Owen et al., (1998), smoking is an important risk factor for more than twenty diseases,

reproductive problem is one of them. The results of current research work also explored that intoxication of albino rats with nicotine significantly ($p<0.05$) affects different stress markers as well as blood parameters. Moreover, a significant change in histological structure of testis was observed in animal groups injected with nicotine. According to literature review it was found that we are the first time used compound of three plants including *M. pruriens* L., *C. behen* and *P. embilica* hydroethanolic extracts in the form of polyherbal preparation (PHP). It was found that these plants contain a wide range of biologically active compound in the extracts and our finding agree with Concessao *et al.*, (2023) and Boniface et al., 2024 who reported that *M. pruriens* seeds contain alkaloid, carbohydrate, flavonoids, saponins etc. It was also reported that *P. embilica* have more than 70 organic compounds including tannin, vitamin-C, carbohydrates, polyphenol etc (Acharya, 2016; Acharya et al., 2021). Fatima et al., 2019 reported that *C. behen* plant contain many pharmacologically and therapeutically important compounds including crystalline alkaloid bahamine, taraxasterol and its acetate, myristate, inulin and a glucoside (Yousefi et al., 2023). Our finding also revealed the strong antioxidant properties of PHP prepared by mixing the *M. pruriens* L., *C. behen* and *P. embilica* hydroethanolic extracts which revealed that this therapeutic preparation has capacity to cope with free radicals and to improve the health status of their consumers. Such activities may enhance the reproductive health by neutralizing the oxidative stress (Quranayati et al., 2023; Acharya et al., 2021; Celikezen et al., 2019; Iamsaard et al., 2023; Lapyuneyong et al., 2022). Furthermore, it was also reported that the presence of such phytochemical compounds induces antioxidant potentials which further emphasized that these compounds possess antioxidant properties due to their ability to scavenge free radicals (Munir et al., 2022).

Due to the high antioxidant capacity and diversity of physiologically active constituents in selected herbs, their hydroethanolic extracts were evaluated *in vitro* for their effect on spermatozoa parameters. The beneficial properties of selected medicinal plants on selective spermatozoa parameters might be due to their potent antioxidant potential. Moreover, the presence of phytochemical constituents found in HPLC and FTIR has made PHP importance in treating infertility problems. The results of in vitro spermatozoa parameters explored the protective effect on the viability and motility of sperms while incubated with different concentrations of extracts (Concessao et al., 2023; Ashidi et al., 2019; Acharya et al., 2021; Thasmi et al., 2022). Our results also agree with the findings of Ahmed et al., (2023) and Munir et al., 2022 who used medicinal plants and reported the improvement in motility and the stabilization of sperm membranes.

Therefore, such medicinal plants preparation can be used as natural preservative for the spermatozoa used in intrauterine insemination (IUI) and in vitro fertilization (IVF).
Hypothalamus regulates the secretion of LH and FSH from anterior pituitary, by releasing the gonadotrophin releasing hormone (GnRH). FSH has function in the spermatogenesis regulation while on the other side LH regulate the synthesis and secretion of testosterone from the Leydig cell in the testis, male reproductive organ. Testosterone then act on the spermatogonia cells to stimulate the spermatogenesis and for the development of prostate as well as testis in the male (Rudolph et al., 2016). The normal level of testosterone in the male also maintain the male secondary characteristics like hair growth, endocrine function, muscles mass and bone density (Munir et al., 2020). The current study agrees with findings of Arun et al., 2018 who found that *P. emblica* leaf extract have potential to ameliorate the testis in male rats with chronic stress. It was also reported that the *M. pruriens* seed extract has male reproductive improvement role in chronic stressed male rats by improving the testosterone level (Lapyuneyong et al., 2022) and it was also reported that the used of root extract of Red and White Bahman in the infertile couple induce the fertility (Faghihi et al., 2018). Oxidative stress in the experimental rats caused the testicular toxicity in rats and caused the atrophy of testicular tissue, germinal layer degeneration, lowered the serum level of gonadotropins (FSH and LH) and testosterone in male rat (Khan and Ahmed, 2009). In addition, it was also reported that many hormonal fluctuations like lower level of serum cortisol, testosterone, FSH, and insulin and impairment of

prolactin secretion are associated with liver disorders in males (Riaz et al., 2016; Khan et al., 2009). Our medicinal preparation (PHP) administration also improve the hepatic and renal parameters in treatment groups due to the hepatic and Reno protective potential of selected medicinal plants (Quranayati et al., 2023; Concessao et al., 2023).The results of current research work explored that after the administration of different doses of PHP not only improve the synthesis and secretion of reproductive hormones but also improve the histological structure of male reproductive tissue i.e. testis, maintain the body weight as well as restore the spermatogenesis in dose dependent manner as compared to nicotine intoxicated animal groups and standard group (Figure 5 and Figure 6). Lapyuneyong et al., (2022) and Iamsaard et al., (2023) reported that the administration of *M. pruriens* seeds extract prevents the testicular structure, spermatogenesis and semen quality in male rats under chronic stress. Our results are also in coherent of the findings of Acharya et al., (2021) who reported that the administration of *P. emblica* L.to male Swiss mice improved the testis architecture and improve the reproductive efficacy. The results of Arun et al., (2018) explored that on giving the extract of *P. emblica* to male rats significantly ($p<0.05$) enhanced the testosterone and sperm quantity while on other hand lower the cortisol level, MDA levels, abnormalities of spermatozoa as well as reduced the histopathology of testicular sections taken from male rats.

Conclusion

The use of nicotine is increasing particularly in young generation which leads to the development of different diseases. Reproductive problem in male and female is also one of them because nicotine directly interferes with antioxidant mechanism, reproductive hormonal axis and destroyed the reproductive organ histology. The uses of tradition medicines for the management of different diseases are increasing due to Neutra and Pharmaceutical properties with no well reported side effects. The findings of current research work also revealed the ameliorative and phyto-therapeutic potential of selected medicinal plants including *M. pruriens, C. behen* and *P. embilica* hydroethanolic extracts preparation as Polyherbal preparation (PHP) in albino male rats. Phytochemical analysis, antioxidant studies, hemolytic, thrombolytic, spermatozoa parameters and animal trial explored significant results. Moreover, reproductive hormones and histological study of testis explored the therapeutic activities of PHP. It could be concluded that selected medicinal plants have significant antioxidant as well as reproductive problems management properties that can help potential uses in traditional medicines as a therapeutic agent. However, trial at high level then clinical trials is required to declare this formation as a safe drug.

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