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Myricetin-mediated Ameliorative Effect of a Super-critical Fluid Extract of *Myrica esculenta* Against Cyclophosphamide-induced Oligospermia in Sprague Dawley Rats

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Abstract

Background: Oligospermia contributes to male infertility through oxidative stress, impairment of spermatogenesis and disruption of steroidogenic function. *Myrica esculenta* is a medicinal plant reported to exhibit anti-oxidant and anti-inflammatory activities. **Aim:** To evaluate the protective effect of Supercritical fluid Extract of *M. esculenta* (SEME) against cyclophosphamide-induced oligospermia. **Methods:** The SEME was prepared and GC-MS profiling was conducted to confirm the presence of myricetin in SEME. Subsequently, thirty-six Sprague Dawley rats were assigned to six groups. Except for the normal control group, all animals were administered cyclophosphamide (30 mg/kg; intraperitoneally) once daily for five consecutive days to induce oligospermia. SEME was administered orally to three groups at doses of 100, 200, and 300 mg/kg, respectively, for 15 days. Sexual behaviour parameters, including Mount Latency (ML) and Frequency (MF), Intromission Latency (IL) and Frequency (IF), Ejaculation Frequency (EF), Hesitation Time (HT), PostEjaculatory Latency (PEL), and Penile Erection Index (PEI) were recorded. On Day 15, testicular index and Blood Glucose Levels (BGLs) were measured. Subsequently, sperm parameters (count, motility, viability, and morphology), oxidative stress markers (glutathione and catalase), and serum biochemical indices (testosterone, 3β-hydroxysteroid dehydrogenase [3β-HSD], and 17β-HSD) were assessed. **Results:** Treatment with SEME significantly reduced ML ($P < 0.05$), IL ($P < 0.05$), HT ($P < 0.05$), and PEL ($P < 0.05$), while increasing IF, EF, MF, and PEI ($P < 0.05$). SEME treatment restored testicular index and significantly reduced elevated BGLs ($P < 0.05$). It also improved sperm count, motility, viability, and morphology ($P < 0.05$), elevated glutathione and catalase levels ($P < 0.05$), and regulated testosterone, 3β-HSD, and 17β-HSD levels ($P < 0.05$). **Conclusion:** SEME demonstrated a protective role against cyclophosphamide-induced oligospermia through mitigation of oxidative stress restoration of spermatogenic and steroidogenic functions.

Major Findings: Treatment with SEME exhibited protective effect against cyclophosphamide-induced oligospermia through improving sexual behaviour, sperm parameters, and testicular index. This protective effect was associated with reduction of oxidative stress parameters and regulation of steroidogenic enzymes.

Keywords: Myricetin, *M. esculanta*, Oligospermia, Sprague Dawley Rats, Supercritical-fluid Extract

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1. Introduction

Oligospermia is defined as a reduction of sperm count in the ejaculate resulting in male infertility[1]. Diminished spermatogenesis and sperm quality attributes to the prognosis of oligospermia[2]. Globally, oligospermia is responsible for approximately 50% cases of male infertility[3]. Oligospermia is majorly driven

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by oxidative-stress mediated sperm DNA damage[4], mitochondrial dysfunction[5], lipid peroxidation[6], and consequent apoptosis of germ cells[7]. Subsequently, the associated mechanisms lead to disruption of sperm count, viability, motility, and morphology. Development of oligospermia is associated with intrinsic molecular pathways, including Nuclear factor-E2-related factor 2 (Nrf2), p53, Bax/Bcl2, NF-κB, StAR, and Hydroxysteroid Dehydrogenase (HSD) signalling[8-13]. These pathways correspond to key pathological events, including oxidative stress, mitochondrial dysfunction, DNA damage, apoptosis, inflammation, and steroidogenic dysregulation, which collectively impairs spermatogenesis and reduce sperm output[13]. Chemotherapeutic agents, including cyclophosphamide exhibits similar molecular dysregulation to impair spermatogenesis[14]. Existing treatment options against oligospermia produces transient restoration of spermatogenesis and sperm viability[15]. Therefore, medicinal plants exhibiting anti-oxidant, anti-apoptotic, and cytoprotective effects may exhibit therapeutic potential against oligospermia.

In recent years, flavonoids containing medicinal plants have garnered interest as potential protective agents against oligospermia based on to their antioxidant, and anti-inflammatory properties[16,17]. *M. esculenta* is a flavonoid rich medicinal plant reported to exhibit anti-oxidant, anti-microbial, anti-inflammatory, and neuroprotective effect[18-20]. Myricetin is a flavonol phytoconstituent abundantly found in plants including, *M. esculenta*, *M. rubra*, *Cameria sinensis*, and *Allium cepa*[21-24]. Myricetin is reported to restore Hypothalamic-Pituitary-Testicular (HPT) axis and Leydigcell steroidogenesis to maintain hormonal balance essential for spermatogenesis[25,26]. Myricetin also attenuates oxidative stress inflammation, and germ-cell apoptosis through downregulating Nrf2 pathway, TLR-4/NF-kB pathway, and Bax/Bcl-2 pathways, respectively[27,28]. Myricetin enhances sperm motility, viability, capacitation, and metabolism through PI3K/AKT-dependent signalling[29]. This study involves the Supercritical Fluid (SCF) extraction of *M. esculanta* to prepare a myricetin-rich phytochemical extract against oligospermia.

The study hypothesised the myricetin-associated protective potential of the Supercritical fluid Extract of *M. esculanta* (SEME) against cyclophosphamide induced oligospermia through regulation of oxidative stress, apoptosis of germ cells, and preserving spermatogenesis. Oligospermia was induced in Sprague Dawley (SD) rats through the administration of cyclophosphamide. In this study, the presence of myricetin was validated through the Gas-Chromatography Mass Spectroscopy (GC-MS) method. Subsequently, the therapeutic effect of SEME against oligospermia was assess through evaluating behavioural parameters (Mount Latency [ML], Mount Frequency [MF], Intromission Latency (IL), Intromission Frequency [IF], Hesitation Time [HT], Ejaculation Frequency [EF], Post-Ejaculatory Latency [PEL], and Penile Erection Index [PEI]) and seminal parameters sperm count, sperm viability, sperm motility, and abnormal sperm). Additionally, protective effect of SEME on oxidative stress parameter (glutathione [GSH] and catalase), biochemical parameters (serum testosterone, 3βhydroxysteroid dehydrogenase [3β-HSD], and 17β-HSD), Blood Glucose Levels (BGL) and testicular index were studied.

2. Materials and Methods

2.1 Plant Procurement and Authentication

Bark samples of *M. esculenta* Buch.-Ham were sourced from the Ayurveda Department's plant repository at Parul University, Vadodara. Botanical identification and authentication of the specimen were carried out by the Department of Botany, Parul University, Vadodara (plant authentication number: PU/PIA/DGcerti625)

2.2 Extract Preparation

SEME was prepared following the procedure outlined by Dobariya *et al*[30]. The bark was finely ground and passed through a 40-mesh sieve before extraction. Supercritical fluid extraction was conducted using a 2 TECH-L/S system. Once temperature equilibrium was established in both the extraction and separation chambers, carbon dioxide was supplied at 25 L/h under 8 MPa pressure. The supercritical extract obtained was collected and stored at -20° C until further analysis.

2.3 GC/MS Analysis

GC-MS analysis of SEME was carried out using a Shimadzu QP2020 NX equipped with a 70 eV electron-ionisation source. Chromatographic separation employed a fused-silica capillary column (30 m × 0.25

mm, 0.25 µm film thickness). The sample was prepared in methanol prior to injection. The oven program initiated at 75° C, followed by a ramp to 160° C at 4° C/min with a 3-min hold, then increased to 210° C at 3° C/min for 4 minutes, and finally elevated to 290° C at 5° C/min with a 12-minute hold. Injector and interface temperatures were set at 260° C and 270° C, respectively. Helium was used as the carrier gas at a flow rate of 1.2 mL/min. A 1 µL volume was injected in split mode (12:1). Data were acquired in full-scan mode over m/z 35-650, with the ion source and quadrupole temperatures maintained at 240° C and 155° C, respectively.

2.4 Experimental Animals

Approval for the use of 54 SD rats (36 male rats and 18 female rats) was granted by the Institutional Animal Ethics Committee (IAEC) of Parul Institute of Pharmacy (IAEC No.: PIPH 42/24) on 11 December 2024. Female rats were used exclusively for sexual behaviour assessment as receptive partners. A total of 36 male rats were included in experimental grouping and biochemical evaluations. Animals were acclimatised for 14 days prior to the study under standard conditions in accordance with CCSEA guidelines[31]. Throughout the acclimation and experimental period, rats had unrestricted access to standard feed and water.

2.5 Experimental Design and Treatment Regimen

Experimental procedure was conducted following the ARRIVE 2.0 guidelines[32]. A total of 36 male rats were randomly assigned to six groups, with six animals in each group. All rats, except those in NC group, received cyclophosphamide (30 mg/kg, intraperitoneally [*i.p.*]) from Day 1 to Day 5 for the induction of oligospermia. The NC and Disease Control (DC) groups were administered 0.1% CMC orally (1 mg/kg/day) from Day 1 to Day 15. The Clomiphene Citrate (CC) group was treated with clomiphene citrate at 0.25 mg/kg (i.p.) for the same duration. The three SEME-treated groups received oral doses of 100 mg/kg (SEME-L), 200 mg/kg (SEMEM), or 300 mg/kg (SEME-H). The experimental design and dosing schedule are illustrated in Table 1.

2.6 Tissue Sample Collection

Approximately, 1mL of blood samples were obtained from each animal through the retroorbital plexus under anaesthesia using pentobarbital sodium (50 mg/kg; *i.p*)[33]. The blood samples were subsequently subjected to centrifugation at 12000 × g for 10 minutes at 4° C to collect the serum. Animals were subjected to *i.p.* administration of ketamine (190 mg/kg) and xylazine (10 mg/kg) for euthanasia[34]. Subsequently, the testes were excised, weighted and subjected to homogenisation in PBS at 1:10 w/v ratio using a mechanical homogeniser. The homogenate was subjected to centrifugation at 10,000 × g for 10 minutes at 4° C in a cooling centrifuge, and the supernatant was collected for biochemical analysis.

2.7 Sexual Behavioural Parameters

Sexual behaviour in male rats was assessed in the presence of a sexually receptive female, which was introduced from one side of the testing chamber to serve as the stimulus. All behavioural parameters were video-recorded.

2.7.1 Mount and Intromission Parameters

Parameters including, ML and IL were recorded as the time elapsed from the introduction of the female to occurrence of the first mount and first intromission, respectively. Similarly, MF and IF were recorded as number of mounts and intromission[35].

2.7.2 Hesitation Time

HT was evaluated following a standard protocol[36]. Briefly, the time taken by male rat to cross toward the receptive female was recorded.

2.7.3 Ejaculation Frequency and Post-ejaculatory Latency

EF was monitored and the PEL was recorded as the duration from the ejaculation event to the next subsequent intromission[37].

2.7.4 Penile Erection Index

Penile erection was evaluated on day 28 following the protocol described by Guo *et al*[38]. Animals were then placed in observation cages and monitored for 30 minutes. A PE event was noted when a rat bent down to lick its erect penis. The PEI was calculated following the equation provided below.

$$Penile\ Erection\ index = \%\ Erection\ in\ Rats \times Mean\ Number\ of\ Erections$$

Table 1. Experimental design and treatment regimen

Experimental Groups (N = 36)	Treatment Dose (mg/kg)	Route of Treatment Administration	Treatment Duration (Days)
Normal control (n = 6) 0.1% CMC	-	Oral	15
Disease control* (n = 6) 0.1% CMC + CFM (30 mg/kg)	-	Oral	15
clomiphene citrate (n = 6) clomiphene citrate + cyclophosphamide (30 mg/kg)*	0.25	*i.p.*	5
SEME-L (n = 6) SEME (100 mg/kg) + cyclophosphamide (30 mg/kg)*	100	Oral	15
SEME-M (n = 6) SEME (200 mg/kg) + cyclophosphamide (30 mg/kg)*	200	Oral	15
SEME-H (n = 6) SEME (300 mg/kg) + cyclophosphamide (30 mg/kg)*	300	Oral	15

* Intraperitoneal injection of CFM (30 mg/kg) was supplied to each animal from Day 1 to Day 5.
N = total number of animals; n = number of animal in each group; SEME = supercritical extract of *M. esculanta*

2.8 Glucose Levels and Testicular Index

The BGLs in the experimental groups were recorded according to Trinder's method[39]. Excised tissues were cleaned and weighed. Testicular index was measured following the equation provided below.

$$Testicular\ Index = \frac{Weight\ of\ testis}{Weight\ of\ rat}$$

2.9 Seminal Parameters

2.9.1 Sperm Count

Sperm count was determined following the standard protocol[40]. A concise portion of the right cauda epididymis was placed in 5 mL of 0.9% normal saline at 24° C and allowed to release spermatozoa for 2-5 minutes. The resulting fluid was designated as suspension A. For total sperm count, 100 µL of suspension A was diluted with 1.9 mL of normal saline. A well-mixed aliquot was then loaded onto a haemocytometer and allowed to equilibrate for 2-3 minutes. Sperm cells were counted in the White Blood Cell (WBC) squares under a 10× objective, and total sperm concentration was calculated using the standard haemocytometer formula.

$$Sperm\ count\left(\times\frac{10^6}{mL}\right) = \frac{Total\ sperm\ counted \times dilution\ volume}{0.4}$$

2.9.2 Sperm Viability

The sperm viability was evaluated following the standard protocol[41]. Sperm viability was assessed by placing 10 µL of suspension A on a clean slide, followed by the addition of 10 µL of 0.9% saline and 10 µL of trypan blue. The mixture was gently blended using a micropipette tip, covered with a coverslip, and examined under a 40× objective. Viable sperm appeared unstained, whereas non-viable sperm showed pink to light-red staining. A total of 200 spermatozoa were counted, and viability (%) was calculated following the equation provided below.

$$Sperm\ Viability(\%) = \frac{Count\ of\ viable\ sperm}{Total\ count\ of\ sperm} \times 100$$

2.9.3 Sperm Motility

Sperm motility was evaluated by loading 10 µL of suspension A onto a haemocytometer and examining the sample under a 40× objective[41]. Motile and non-motile spermatozoa present within the four WBC counting squares were recorded. Sperm motility was calculated following the equation provided below.

$$Sperm\ motility = \frac{Count\ of\ motile\ sperm}{Total\ count\ of\ sperm} \times 100$$

2.9.4 Abnormal Sperm

Sperm morphology was assessed by mixing 100 µL of suspension A with 1 mL of formalin (10%) in a micro-centrifuge tube, followed by gentle mixing[41]. Subsequently, 2-3 drops of 1% eosin were added, and the mixture was allowed to stain at room temperature for 30-45 minutes. A small aliquot of the stained sample was then placed on a clean slide, covered with a coverslip, and examined microscopically. At least 100 spermatozoa across 4-5 fields were evaluated for morphological abnormalities such as tailless, headless,

unhooked head, double tail, coiled tail, double head, and bent tail forms and recorded as abnormal sperm. The percentage of abnormal sperm was calculated accordingly.

$$Abnormal\ Sperm(\%) = \frac{Count\ of\ abnormal\ sperm}{Total\ count\ of\ sperm} \times 100$$

2.10 Oxidative Stress Parameters

2.10.1 Glutathione

A 100 µL aliquot of tissue homogenate was combined with 400 µL of phosphate buffer (0.2 M, pH 7.4), followed by the addition of 1.5 mL of DTNB solution (0.1 mM)[42]. The reaction mixture was incubated for 10 minutes at room temperature, and absorbance was subsequently measured at 412 nm. Total GSH levels were calculated from a standard curve using linear regression and expressed as µM per mg of protein. Background absorbance was corrected using a reagent blank containing no sample or GSH.

2.10.2 Catalase

A 0.1 mL portion of tissue homogenate was mixed with 1.9 mL of PBS, followed by the addition of 1 mL of H_2O_2 solution[43]. The decline in absorbance at 240 nm was monitored over 1 minute at 3-minute intervals. A reference cuvette containing 0.1 mL homogenate and 2.9 mL PBS served as the sample control. Catalase activity was calculated using a molar extinction coefficient of 43.6 and expressed as mM of H_2O_2 decomposed per minute per milligram of protein at 25° C.

2.11 Biochemical Parameters

2.11.1 Serum Testosterone

Serum testosterone concentrations were quantified using a Chemiluminescence Immunoassay (CLIA) technique[44].

2.11.2 3β-Hydroxysteroid Dehydrogenase

Tissue was homogenised at 4° C to achieve a concentration of 100 mg/mL in 20% spectroscopic-grade glycerol containing 5 mM potassium phosphate and 1 mM EDTA, and the homogenate was centrifuged at 10,000 g for 30 minutes at 4° C[45]. The reaction mixture consisted of 1 mL of the resulting supernatant, 1 mL of 100 µM sodium pyrophosphate buffer (pH 8.9), 0.04 mL of ethanol containing 30 µg dehydroepiandrosterone, and 0.96 mL of 25 mg% bovine serum albumin. Enzyme activity was initiated by adding 0.1 mL of 0.5 µM NAD and monitored spectrophotometrically at 340 nm. Activity was determined relative to a control lacking NAD, where a change in absorbance of 0.001/min at 340 nm corresponded to one unit of enzyme activity.

2.11.3 17β-Hydroxysteroid Dehydrogenase

Testicular 17β-HSD Levels was determined biochemically using the same supernatant prepared for the 3β-HSD assay[45]. For the reaction mixture, 1 mL of the supernatant was combined with 3 mL of incubation solution containing 0.96 mL of 25 mg% BSA, 0.04 mL of ethanol with 0.3 µM testosterone, and 1 mL of 0.44 mM sodium pyrophosphate buffer (pH 10.2). The reaction was initiated by adding 0.1 mL of 0.5 µM NAD, and absorbance was monitored at 340 nm. Enzyme activity was calculated relative to a control lacking NAD, with a change of 0.001 absorbance units per minute at 340 nm representing one unit of activity.

2.12 Statistical Analysis

Statistical evaluation of all assay parameters was conducted using one-way ANOVA followed by Tukey's post hoc test for intergroup comparisons. Analyses were carried out in GraphPad Prism 10, and results are expressed as mean ± standard error of the mean (SEM).

3. Results

3.1 GC/MS Profiling

A summary of the GC/MS profiling of the SEMA is presented in Table 2. Trifluoroguanidine appeared at 7.080 min, followed by milbemycin B (7.130 min) and glycerin (7.288 min). A prominent peak corresponding

Table 2. GC/MS analysis of supercritical fluid extract of *M. esculenta*

Sr. No.	Name of Compound	Retention Time (min)
1	Trifluoroguanidine	7.080
2	Milbemycin B	7.130
3	Glycerin	7.288
4	Myricetin	7.583
5	Methyl nitrite	7.647

to myricetin was detected at 7.583 min. additionally, methyl nitrite was identified at 7.647 min. The representative GC/MS chromatogram highlighting the peak corresponding to myricetin is shown in Figure 1.

3.2 Sexual Behavioural Parameters

The DC group exhibited a significant increase (*P*-value < 0.05) in ML (275.4 ± 11.8 s) compared to the NC group (175.1 ± 12.6 s). Treatment with CC (145 ± 15.2 s), SEME-M (176.4 ± 12.8 s), SEME-H (164 ± 18.1 s) resulted in a significant reduction (*P*-value < 0.05) in latency. The IL increased significantly (*P*-value < 0.05) in the DC group (471.6 ± 14.5 s) compared to the NC group (325.2 ± 17.5 s). CC group (274.5 ± 14.6 s), SEME-M group (395.5 ± 16.5 s), and SEME 300 mg/kg group (348.1 ± 11.5 s) significantly reduced intromission latency. A significant increase (*P*-value < 0.05) in PEJ was observed in the DC group (802.5 ± 20.1 s) compared with the NC group (511.7 ± 22.1 s). CC group (545.5 ± 24 s), SEME-M group (685 ± 19.5 s), and SEME-H group 601.7 ± 17 s) exhibited a significant (*P*-value < 0.05) reduction in the PEJ. The HT increased significantly (*P*-value < 0.05) in the DC group (578 ± 15 s) compared with the NC group (347 ± 10.6 s). Significant reductions (*P*-value < 0.05) were observed following CC (378 ± 14 s), SEME-M (478 ± 10 s), and SEME-H (412 ± 13 s) treatment. The IF decreased significantly (*P*-value < 0.05) in the DC group (2.3 ± 0.2) relative to the NC group (7.1 ± 0.2). Treatment with CC (6.8 ± 0.4), SEME-M (5.7 ± 0.1), and SEMEH (8.2 ± 0.3) resulted in a statistically significant (*P*-value < 0.05) increase in the IF. The DC group exhibited a significant reduction (*P*-value < 0.05) in the EJ (0.2 ± 0.01) compared with the NC group (3.5 ± 0.08). Treatment with CC (3.1 ± 0.04), SEME-M (2.5 ± 0.06), and SEME-H (3.0 ± 0.03) significantly (*P*-value < 0.05) restored the EJ. The MF was significantly reduced (*P*-value < 0.05) in the DC group (4.4 ± 0.08) compared with the NC group (16.1 ± 1.8). Treatment with CC (14.2 ± 0.4), SEME-M (10.4 ± 0.6), and SEME-H (12.7 ± 0.3) produced significant increases (*P*-value < 0.05) in MF. The PEI was significantly reduced (*P*-value < 0.05) in the DC group (6.5 ± 0.8) compared with the NC group (22.5 ± 1.1). Treatment with CC (18.4 ± 1.4), SEMEM (13.4 ± 1.6), and SEMEH (17.1 ± 1.3) produced significant increase in the PEI. The SEME-L group did not exhibit a statistically significant differences in the sexual behavioural parameters. A graphical comparison of sexual behaviour parameters across the treatment groups is presented in Figure 2.

3.3 Testicular Index and Glucose Levels

The BGL increased significantly (P-value < 0.05) in the DC group (237.5 ± 3.2 mg/dL) compared to the NC group (112.5 ± 2.5 mg/dL). Treatment with CC (115.4 ± 1.5 mg/dL), SEME-L (178.4 ± 4.4 mg/dL), SEME-M (156.7 ± 2.8 mg/dL) and SEME-H (136.8 ± 2.5 mg/dL) produced significant reduction (*P*-value < 0.05) in the BGL. The DC group showed a significant decline (*P*-value < 0.05) in the testicular index (0.42 ± 0.025) compared to the NC group (0.68 ± 0.021). Treatment with CC (0.62 ± 0.015), while SEME-M (0.52 ± 0.021) and SEME-H (0.57 ± 0.013) significantly restored (*P*-value < 0.05) the testicular index compared to the NC group. A graphical comparison of the BGLs and testicular index across experimental groups is presented in Figure 3.

3.4 Sperm Parameters

The DC group showed a significant reduction (*P*-value < 0.05) in sperm count (30.25 ± 4.86 ×10^6/mL) compared

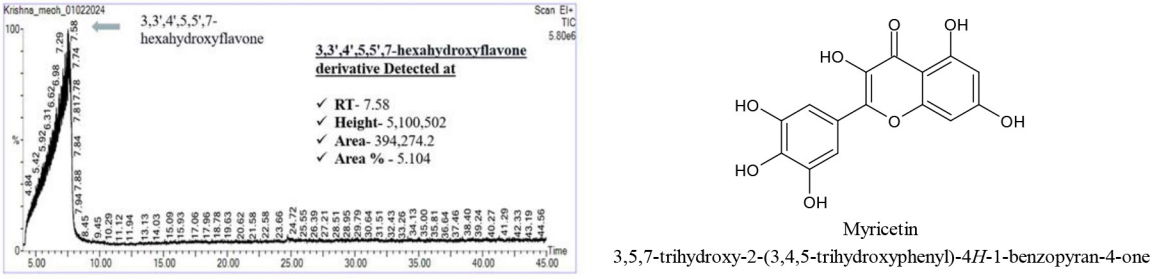
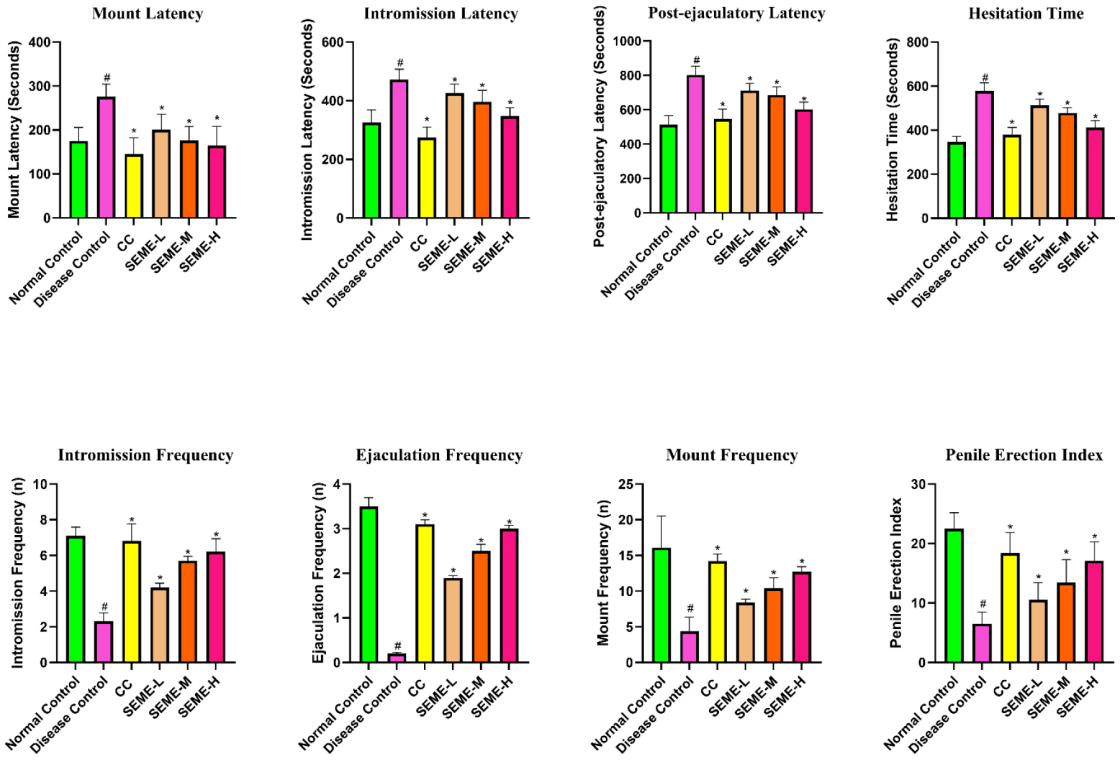
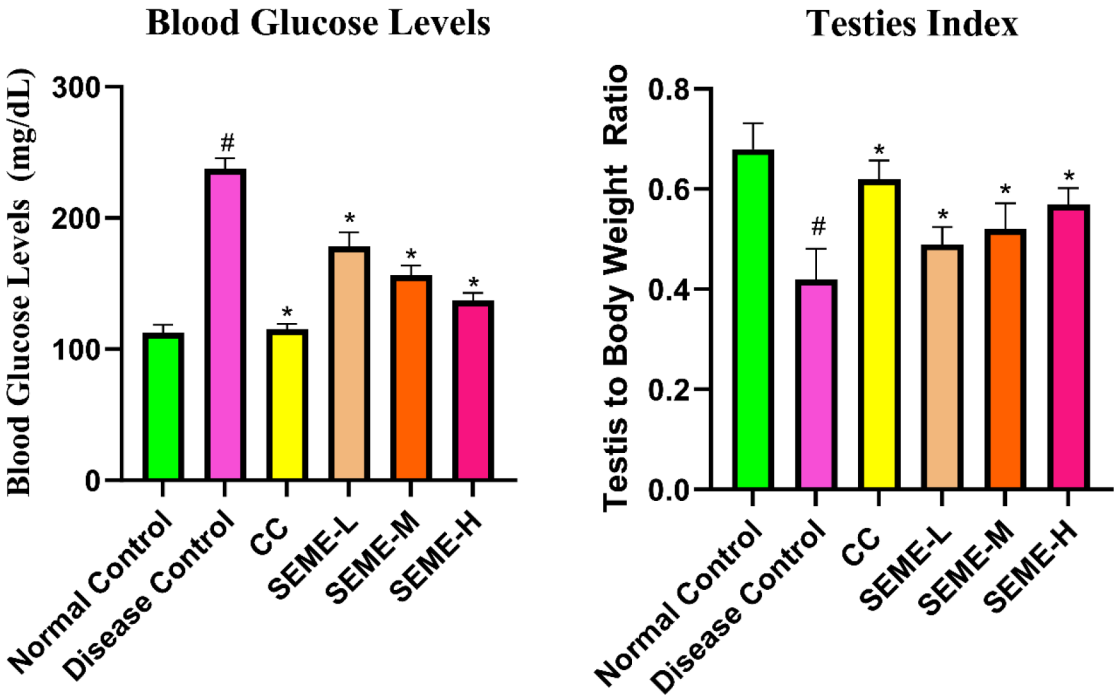


Figure 1. Chromatogram of supercritical fluid of *M. esculanta*.



(* indicates statistically significant difference in the treatment groups from the disease control group with P-value < 0.05; # indicates statistically significant difference in the disease control group from the normal control group with P-value < 0.05; CC = clomiphene citrate (0.25 mg/kg); SEME-L = SEME (100 mg/kg); SEME-M = SEME (200 mg/kg); SEME-H = SEME (300 mg/kg)

Figure 2. Graphical comparison of sexual behaviour parameters across the treatment groups.



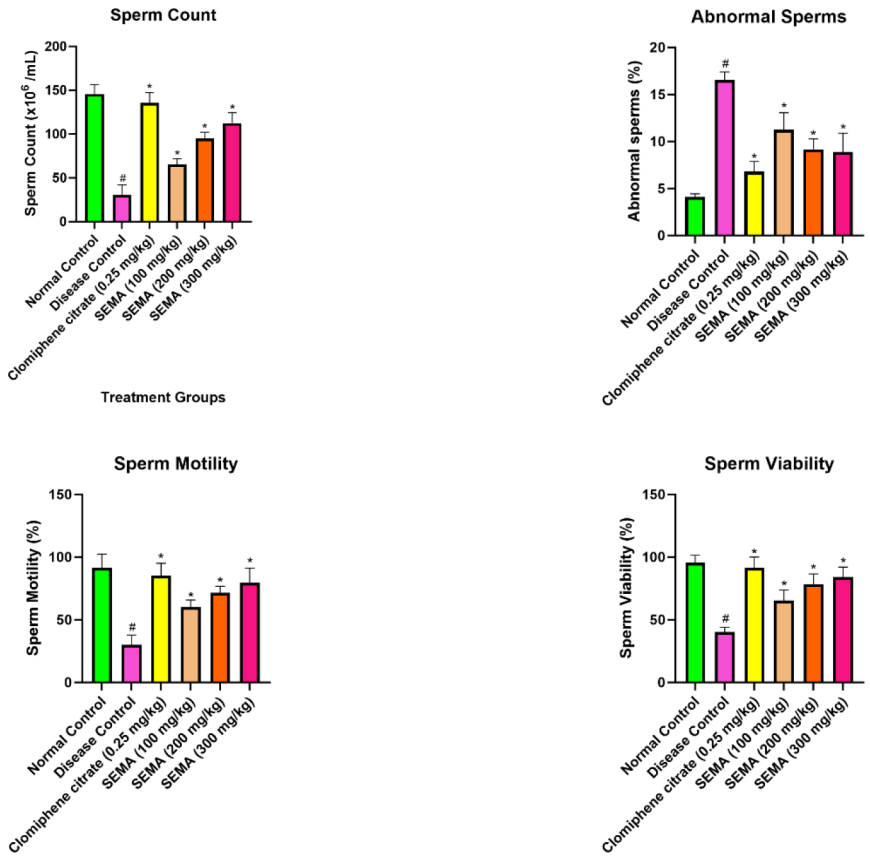
(* indicates statistically significant difference in the treatment groups from the disease control group with *P*-value < 0.05; # indicates statistically significant difference in the disease control group from the normal control group with *P*-value < 0.05; CC = clomiphene citrate (0.25 mg/kg); SEME-L = SEME (100 mg/kg); SEME-M = SEME (200 mg/kg); SEME-H = SEME (300 mg/kg)

Figure 3. Graphical comparison of blood glucose levels and testicular index across the treatment groups.

to the NC group (145.57 ± 4.51 ×10^6/mL). Treatment with CC (135.54 ± 4.81 ×10^6/mL), SEME-L (65.25 ± 2.74 ×10^6/mL), SEME-M (94.87 ± 2.96 ×10^6/mL), and SEME-H (112.49 ± 4.90 × 10^6/mL) exhibited statistically significant (*P*-value < 0.05) increase in sperm count in comparison with the DC group. A significant increase (*P*-value < 0.05) in abnormal sperm percentage was observed in DC group (16.54 ± 0.35 %) compared to the NC group (4.11 ± 0.13 %). Compared to the DC group, treatment with CC (6.79 ± 0.44 %), SEME-L (11.25 ± 0.74 %), SEME-M (9.15 ± 0.46 %), and SEMEH (8.89 ± 0.82 %) exhibited a significant decrease (*P*-value < 0.05) in the abnormal sperm percentage compared to the DC group. The sperm motility decreased significantly (*P*-value < 0.05) in the DC group (30.05 ± 3.23 %) relative to the NC group (91.25 ± 4.51 %). Treatment with CC (85.00 ± 4.10 %), SEME-L (60.20 ± 2.27 %), SEME-M (71.54 ± 2.08 %), and SEME-H (79.41 ± 4.82 %) significantly (*P*-value < 0.05) increased the sperm motility compared to the DC group. Sperm viability was significantly reduced (*P*-value < 0.05) in the DC group (40.25 ± 1.58 %) compared to the NC group (95.57 ± 2.40%). Treatment with CC (91.41 ± 3.54 %), SEME-L (65.26 ± 3.44 %), SEME-M (78.15 ± 3.41 %), and SEME-H (84.15 ± 3.22 %) exhibited significant increase (*P*-value < 0.05) increase the sperm viability. A graphical comparison of sperm count, abnormal sperm percentage, sperm motility, and sperm viability across treatment groups is demonstrated in Figure 4.

3.5 Oxidative Stress Parameters

A significant decrease (*P*-value < 0.05) in GSH levels was observed in the DC group (0.11 ± 0.011 µM) compared to the NC group (0.45 ± 0.016 µM). Treatment with CC restored glutathione concentrations to 0.35 ± 0.012 µM. SEME



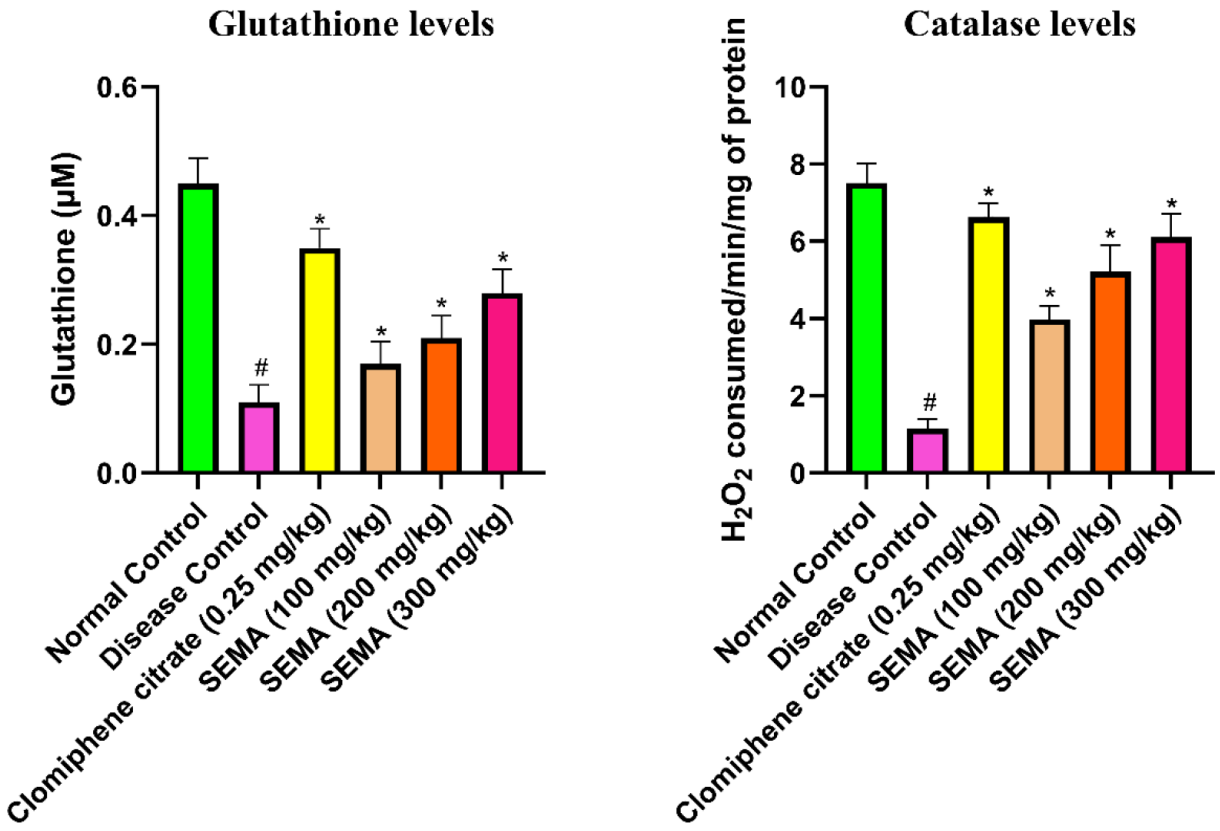
(* indicates statistically significant difference in the treatment groups from the disease control group with P-value < 0.05; # indicates statistically significant difference in the disease control group from the normal control group with P-value < 0.05; CC = clomiphene citrate (0.25 mg/kg); SEME-L = SEME (100 mg/kg); SEME-M = SEME (200 mg/kg); SEME-H = SEME (300 mg/kg)

Figure 4. A graphical comparison of sperm count, abnormal sperm percentage, sperm motility, and sperm viability across treatment groups.

administration produced a significant increase (*P*-value < 0.05) in SEME-L (0.17 ± 0.014 µM), SEME-M (0.21 ± 0.014 µM), and SEME-H (0.28 ± 0.015 µM) groups. Catalase levels was significantly reduced (*P*-value < 0.05) in the DC group compared to the NC group. Treatment with CC, SEME-L, SEME-M, and SEME-H significantly elevated catalase levels with P-value < 0.05. A graphical comparison of glutathione and catalase levels across treatment groups is presented in Figure 5.

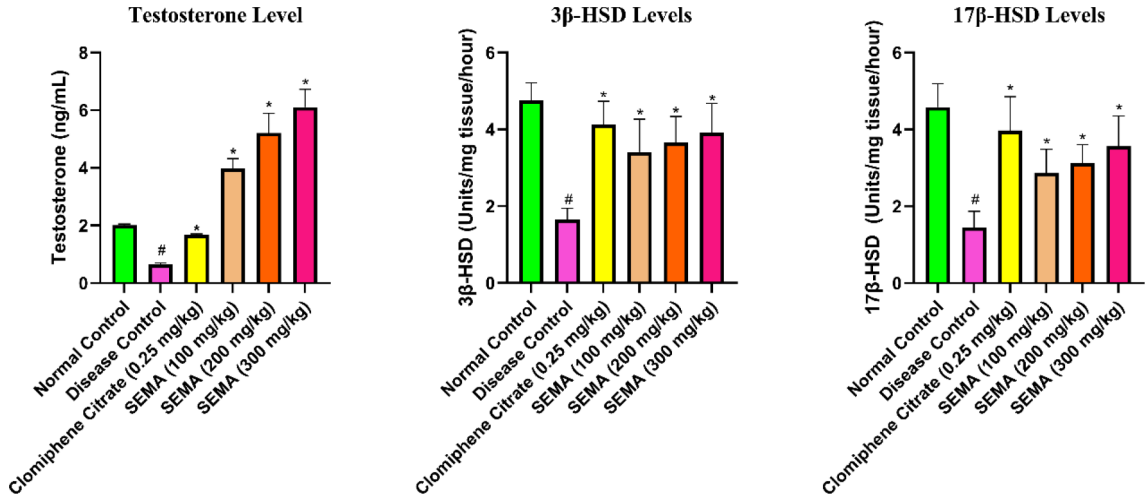
3.6 Biochemical Parameters

A substantial decrease (P-value < 0.05) in testosterone levels was observed in the DC group (0.85 ± 0.023 ng/mL) when compared to the NC group (2.01 ± 0.015 ng/mL). Treatment with CC (1.67 ± 0.015 ng/mL), SEME-L (3.98 ± 0.14 ng/mL), SEME-M (3.98 ± 0.14 ng/mL), and SEME-H (6.11 ± 0.25 ng/mL) significantly (*P*-value < 0.05) restored testosterone levels compared to the NC group. Levels of 3β-HSD was significantly (*P*-value < 0.05) reduced in the DC group (1.65 ± 0.12 U/mg tissue/hour) relative to the NC group (4.75 ± 0.19 U/mg tissue/hour). Treatment with CC restored enzyme activity to 4.12 ± 0.25 U/mg tissue/hour. SEME treatment significantly increased 3β-HSD activity across all doses: 3.41 ± 0.35 (SEME-L), 3.65 ± 0.28 (SEME-M), and 3.92 ± 0.31 U/mg tissue/hour (SEME-H). 17β-HSD levels decreased significantly (*P*-value < 0.05) in the DC group (1.45 ± 0.17 U/mg tissue/hour) compared to the NC group (4.58 ± 0.25 U/mg tissue/hour). Significant increases (*P*-value < 0.05) in 17β-HSD levels were observed after treatment with CC (3.97 ± 0.36 U/mg tissue/hour), SEME-L (2.87 ± 0.25 U/mg tissue/hour), SEME-M (3.12 ± 0.20 U/mg tissue/hour), and SEME-H (3.57 ± 0.32 U/mg tissue/hour) when compared with the NC Group. A comparative graphical representation of testosterone, 3β-HSD, and 17β-HSD levels across treatment groups is provided in Figure 6.



(* indicates statistically significant difference in the treatment groups from the disease control group with *P*-value < 0.05; # indicates statistically significant difference in the disease control group from the normal control group with *P*-value < 0.05; CC = clomiphene citrate (0.25 mg/kg); SEME-L = SEME (100 mg/kg); SEME-M = SEME (200 mg/kg); SEME-H = SEME (300 mg/kg)

Figure 5. A graphical comparison of glutathione and catalase levels across treatment groups.



(* indicates statistically significant difference in the treatment groups from the disease control group with P-value < 0.05; # indicates statistically significant difference in the disease control group from the normal control group with P-value < 0.05; CC = clomiphene citrate (0.25 mg/kg); HSD = hydroxysteroid dehydrogenase; SEME-L = SEME (100 mg/kg); SEME-M = SEME (200 mg/kg); SEME-H = SEME (300 mg/kg)

Figure 6. A graphical comparison of testosterone, 3β-HSD, and 17β-HSD levels across treatment groups.

4. Discussion

A pivotal causative factor in oligospermia is exposure to gonadotoxic agents[46]. Cyclophosphamide is a prescription drug being used against cancer and autoimmune disorders[47]. Exposure to cyclophosphamide may induces testicular damage, reduction in sperm count and disruption of hormonal homeostasis. Cyclophosphamide produces testicular toxicity through the generation of reactive oxygen species, oxidative stress, and DNA damage in germ cells[48]. Cyclophosphamide also leads to disruption of sertoli-germ cell interactions and apoptosis in spermatogenic cells. Cyclophosphamide metabolises to phosphoramide mustard and acrolein, which produce oxidative stress and apoptotic damage in testes. Produced ROS attack membrane lipids and DNA to cause DNA fragmentation, mutagenesis, and activation of intrinsic apoptosis pathways[49]. Additionally, cyclophosphamide disrupts testicular pro-/anti-apoptotic balance through upregulating Bax and suppressing Bcl-2 in spermatogenic cells, which subsequently triggers caspase-mediated germ cell death[50]. The net result is oxidative damage, inflammation, and apoptosis in the seminiferous epithelium, ultimately resembling oligospermia with impaired sperm parameters. Cyclophosphamide induced oligospermia involves direct testicular effects and central endocrine disruption[51]. Investigational compound, SEME was anticipated to counteract these mechanisms by targeting the aforementioned molecular pathways.

Supercritical fluid extraction has become a technique in pharmaceutical and nutraceutical research due to its efficiency in isolating thermolabile phytoconstituents while preserving their bioactivity and eliminating the need for organic solvents[52]. Numerous studies have reported the therapeutic potential of supercritical extracts across various disease models. The supercritical extract of *Acacia auriculiformis* has demonstrated notable cytotoxic and anti-mitotic activities[30]. The three SEME doses were selected for this study based on previously reported toxicity and efficacy data for *M. esculenta*[53]. Clomiphene citrate was selected as the standard drug based its ability to restore HPA axis function through enhancing gonadotropin release[54]. This mechanism aligns with the myricetin-mediated stimulation of testosterone production and spermatogenesis observed with SEME.

The GC-MS analysis validated the presence of myricetin in SEME, which is reported mitigate testicular oxidative injury through reducing oxidative stress and modulating inflammatory pathways[27]. Therefore, GC/MS provided a mechanistic basis of assessing the myricetin containing SEME against cyclophosphamide induced oligospermia. Sexual behavioural were measured as indices of libido and mating performance. Diminished sexual behaviour in animals treated with cyclophosphamide may resulted from the disruption of androgen levels. Improved sexual behaviour parameters

in SEME-treated rats may indicates restoration of androgen levels[55]. Additionally, suppressed mounting and intromission in DC animals may have resulted due to cyclophosphamide induced hypogonadism[56]. The regulation of mounting and intromission in SEME-treated may indicate a therapeutic effect in the reproductive axis. The testicular index in animals was compared as an indicator of testicular atrophy. A decline in testis weight is a hallmark of oligospermia, which reflects the loss of germ cells and seminiferous tubule mass[57]. Regulation of testicular index in SEME-treated animals indicates attenuation of structural degeneration in the testes. The BGLs were measured to monitor systemic metabolic disturbances leading to male fertility. Dysregulation of seminal parameters in animals of the DC group may indicate oligospermia, asthenozoospermia, and teratozoospermia due to dysregulated sperm count, morphology, and motility respectively[58]. The dysregulation of seminal parameters may have resulted due to oxidative stress and apoptotic loss of germ cells. A significant improvements of seminal parameters in treated animals indicates protective potential of SEME through antioxidant and anti-apoptotic effect in the testis[58]. Cyclophosphamide reduce the levels of GSH and catalase to produce oxidative stress on germ cells through dysregulation of Nrf2 pathway[59]. Restoration of the oxidative stress parameters indicates Nrf2-mediated antioxidant potential of SEME. Testosterone, 3βHSD, and 17β-HSD are responsible to regulate steroidogenesis and functioning of Leydig cells[60]. Additionally, 3β-HSD and 17β-HSD stimulate the biosynthesis of androgen biosynthesis[61]. In oligospermia, depletion of testosterone, 3β-HSD, and 17β-HSD leads to impaired steroidogenesis and endocrine insufficiency[62]. Treatment with SEME protected the steroidogenic cells from cyclophosphamide induced damage in testis and may have stimulated the endocrine axis to increase the testosterone levels. Additionally, myricetin is reported to upregulate the PI3K/Akt and ERK signalling pathways to enhance the functioning of steroidogenic acute regulatory protein responsible for mediating steroidogenesis[63]. Increased levels of 3β-HSD and 3β-HSD in SEME-treated animals may have resulted through the same mechanism.

The improvement in testosterone suggests activation of AMP-activated protein kinase, protein kinase B, mammalian target of Rapamycin, and extracellular signal-regulated kinases[64]. Additionally, the reduction in inflammation and oxidative stress implies upregulation of Nrf2/HO-1 and suppression of TLR4/NF-κB signalling[65]. However, the quantitative estimations of these genes were not conduced in this study. Therefore, molecular mechanism of SEME against oligospermia can be studied through the genetic estimation of pro-survival kinases and inflammatory signalling pathways. Additionally, the glucose lowering potential of SEME in disease condition can be considered as a potential mechanism against diabetes-induced male infertility.

3. Conclusion

Treatment with SEME exhibited significant protective effects against cyclophosphamide-induced oligospermia through modulation of oxidative stress and preservation of steroidogenic and spermatogenic functions. Animals treated with SEME demonstrated significant improvement in sexual behavioural parameters, including mount and intromission latency/frequency, ejaculation frequency, hesitation time, post-ejaculatory latency, and penile erection index, suggesting recovery of androgenic function. Restoration of testicular index and normalisation of blood glucose levels further indicate systemic protective effects. Additionally, treatment with SEME significantly regulated seminal parameters, including sperm count, viability, motility, and morphology, indicating preserved spermatogenesis. Concurrently, SEME restored oxidative stress markers (glutathione and catalase) and upregulated serum testosterone, 3β-HSD, and 17β-HSD levels, indicating protection of Leydig cell steroidogenesis. The efficacy and protective nature of SEME may be further investigated in other types of oligospermia or male infertility models involving oxidative stress, mitochondrial dysfunction, or steroidogenic disruption, including diabetes-induced or age-related infertility, by exploring target-specific mechanisms such as PI3K/Akt, Nrf2/HO-1, and TLR4/NF-κB signalling.

4. Ethics Approval

The use of 54 *Sprague-Dawley* rats (36 males; 18 females) were approved by Parul Institute of Pharmacy under the Institutional Animal Ethics Committee Approval (PIPH 42/24).

5. Collection, Identification, and Authentication of Plant

Bark samples of *M. esculanta* were obtained from the Department of Ayurveda Plant Store at Parul University, Vadodara. The Department of Botany, Parul University, Vadodara, identified and authenticated the plant with authentication number PU/PIA/DG-certi-625.

6. References

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Author Contribution

KR1 and VS conceptualised and designed the methodology. KR1 performed the experiments under the supervision of VS. KR2 conducted data analysis and prepared the manuscript draft. PT reviewed the research and manuscript. All authors have read and agreed to the published version of the manuscript.