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Protective and Curative Effect of Clove Isolates on Trichloroacetic Acid-Induced Sperm-Endocrine Deficit, Redox Imbalance and Histomorphology in Adult Rat

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Abstract

Environmental pollutants, such as Trichloroacetic Acid (TCA), have been implicated in compromising male reproductive health by inducing sperm-endocrine deficits, redox imbalances, and histomorphological alterations. This research investigates the protective and curative effects of clove isolates, particularly Eugenol, on TCA-induced reproductive toxicity in adult male rats. The study employs various parameters, including body and testes weight, reproductive hormone levels, oxidative stress markers, sperm analysis, and histological profiles. Sixty adult male Wistar rats were divided into ten groups, with treatments involving TCA, Eugenol Isolates from Clove (EIC), and combinations of both. The results indicate significant changes in body weight and testes weight, with TCA-treated groups showing pronounced effects. EIC supplementation, however, mitigated these changes, emphasizing the potential protective role of clove isolates. Reproductive hormone analysis revealed a decrease in testosterone levels induced by TCA, while EIC treatment demonstrated a capacity to elevate testosterone levels. The interaction of Eugenol with luteinizing hormone (LH) underscored the complexity of hormonal effects, with dose-independent impacts requiring further exploration. Oxidative stress marker analysis demonstrated TCA-induced alterations, while EIC treatment exhibited synergistic effects, particularly in reducing oxidative stress. Sperm analysis revealed TCA-induced adverse effects, which were mitigated by EIC, suggesting a potential role in preserving reproductive health. Histological examination delineated distinct profiles, with EIC showing potential therapeutic benefits. The study concludes that Eugenol from clove isolates may have protective and curative effects against TCA-induced reproductive deficits, redox imbalance, and histomorphological abnormalities. These findings contribute valuable insights into the potential applications of clove isolates in mitigating the detrimental effects of environmental toxins on male reproductive health. Further investigations are warranted to elucidate underlying mechanisms and optimize the therapeutic potential of Eugenol in safeguarding male reproductive function.

Keywords: environmental pollutants, Trichloroacetic Acid (TCA), male reproductive health, sperm-endocrine deficits, redox imbalances, histomorphological alterations, protective effects, curative effects, clove isolates, Eugenol, TCA-induced reproductive toxicity

1. Introduction

In recent years, there has been a growing concern over the deleterious impact of environmental pollutants on

reproductive health, particularly the intricate interplay between endocrine disruption, oxidative stress, and histomorphological alterations in the male reproductive system (Agarwal *et al.*, 2021). One such environmental toxin, Trichloroacetic Acid (TCA), a byproduct of water chlorination, has been implicated in impairing male reproductive function (Kumar & Singh, 2022). The adverse effects of TCA on sperm quality, endocrine regulation, and testicular morphology pose significant threats to fertility and overall reproductive well-being (Karavolos *et al*., 2020).

As researchers strive to develop effective therapeutic strategies against the reproductive toxicity induced by environmental pollutants, natural compounds derived from medicinal plants have emerged as promising candidates. Among these, clove (*Syzygium aromaticum*) and its isolates have gained attention for their diverse pharmacological properties, including antioxidant, anti-inflammatory, and anti-microbial effects (Checa *et al.*, 2016; Zhang *et al.*, 2020). However, the specific protective and curative effects of clove isolates on TCA-induced sperm-endocrine deficit, redox imbalance, and histomorphological alterations in the male reproductive system remain largely unexplored.

This research aims to bridge this gap by investigating the potential ameliorative effects of clove isolates on TCA-induced reproductive toxicity in adult rats. By assessing sperm parameters, endocrine profiles, oxidative stress markers, and histomorphological changes, this study seeks to elucidate the mechanisms underlying the protective and curative actions of clove isolates. Understanding these mechanisms not only holds implications for male reproductive health but also contributes to the broader field of environmental toxicology and natural product-based therapeutics.

Recent studies have highlighted the multifaceted benefits of medicinal plants in mitigating reproductive toxicity induced by environmental contaminants. For instance, research by Sharma *et al*. (2022) demonstrated the protective effects of curcumin against endocrine disruption and oxidative stress in male rats exposed to industrial pollutants. Similarly, the antioxidant properties of resveratrol have been shown to counteract reproductive impairments caused by various environmental toxins (Li *et al*., 2021). However, the specific efficacy of clove isolates in the context of TCA-induced reproductive toxicity remains an area requiring comprehensive investigation.

This research presents a novel contribution to the existing literature by exploring the protective and curative potential of clove isolates against TCA-induced sperm-endocrine deficit, redox imbalance, and histomorphological alterations. By shedding light on the molecular mechanisms involved, this research aims to provide valuable insights for the development of targeted therapeutic interventions to safeguard male reproductive health in the face of environmental challenges.

2. Materials and Methodology

Experimental Animals

A total of Sixty (60) adult male Wistar rats weighing an average of 110g were procured from the Animal House of the College of Health Science, Benue State University Makurdi and were allowed to acclimatize for fourteen (14) days in mesh net-covered plastic cages in ten (10) groups of six (6) and given *ad libitum* access to grower Vital feed pellet and water before the commencement of the experiment. The weights of rats were measured at acquisition, during acclimatization, before and after administration of extract and at the end of the experiment using electronic weighing balance.

Animal Cages

A total of ten (10) plastic cages measuring 30cm×20cm in size were obtained, in which the experimental animals were housed, acclimatized and fed throughout the duration of the experiment.

Trichloroacetic Acid (TCA)

Trichloroacetic acid and sodium hydroxide pellets, that were used to neutralize TCA solution (K_a=0.3) to required pH 7.0–7.5, was purchased from a chemical shop in Abuja. The purity of TCA and sodium hydroxide was ensured to be >99.0%. Trichloroacetic acid is stable in neutral solution and is classified as non-biodegradable.

Animal Feeds

The animal feed (UAC Vital feed Grower made in Nigeria) was purchased from feed store in Wurukum area of Makurdi and stored at optimum temperature in the animal house.

Experimental Design

The sixty (60) adult male Wistar rats were divided into ten (10) groups of six (6) rats each, and administered the research substances as follows:

Group 1 - Negative Control (Placebo): 2 ml/kg body weight of normal saline daily for 30 days through an

orogastric canula.

Group 2 - EIC Low Dose: 4 mg/kg of EIC via orogastric canula for 15 days.

Group 3 - EIC Standard Dose: 10 mg/kg of EIC for 15 days via orogastric canula.

Group 4 - TCA Low Dose: 200 mg/kg of TCA for 15 days through an orogastric canula.

Group 5 - TCA High Dose: 400 mg/kg of TCA for 15 days through an orogastric canula.

Group 6 - TCA Low Dose + EIC Low Dose: 200 mg/kg TCA for the first 15 days + 4 mg/kg of EIC for another 15 days through an orogastric canula.

Group 7 - TCA High Dose + EIC Standard Dose: 400 mg/kg TCA for the first 15 days + 10 mg/kg of EIC further 15 days through an orogastric canula.

Group 8 - EIC Low Dose + TCA Low Dose: 4 mg/kg of EIC for the first 15 days + 200 mg/kg of TCA for another 15 days via orogastric canula.

Group 9 - EIC Standard Dose + TCA High Dose: 10 mg/kg of EIC for first 15 days + 400 mg/kg of TCA for another 15 days through an orogastric canula.

Group 10 - EIC Standard Dose + TCA Low Dose (Extended): 4 mg/kg of EIC for 30 days in combination with 200 mg/kg of TCA for 30 days through an orogastric canula.

Animal Sacrifice

Upon sacrifice, the rats were weighed before decapitation. Following sacrifice, blood samples were promptly collected from the heart of each rat. A midline abdominal incision was made to expose the reproductive organs. The testes and epididymis were excised, and the weight of each animal's testes was assessed using an electronic analytical and precision balance.

Testis volume was determined using the water displacement method. Both testes of each rat were measured, and the average value for each parameter was considered as one observation. One of the testes from each animal was preserved in Bouin's fluid for subsequent histological examination. Serum and the remaining testis of each animal were stored at –25^{o}C for biochemical assays.

Serum and Testicular Testosterone Estimation

The enzyme immunoassay approach, previously reported (Tietz, 1995), was used to assess the amounts of testosterone in plasma. It was based on the idea of competitive binding between TT and TT-horseradish peroxidase conjugate for a constant quantity of rabbit anti-TT. In summary, TT standards, controls, samples (blood sera and testicular homogenate supernatants), TT-horseradish peroxidase conjugate reagent, and rabbit anti-TT reagent were incubated for 90 minutes at 37°C on goat anti-rabbit IgG-coated wells. After the unbound TT peroxidase conjugate was eliminated, the wells were cleaned. After adding and incubating tetramethylbenzidine, a blue hue began to appear. After adding 1N hydrochloric acid, the colour development was halted, and the absorbance was measured spectrophotometrically at 450 nm. Plotting the standard concentration against the absorbance and TT concentrations determined from the standard curve resulted in the creation of a standard curve.

Serum Hormonal Assay- Luteinizing Hormone and Follicle Stimulating Hormone (FSH)

The tests were conducted in accordance with the methodology that Amballi modified in 2007. The blood that was drawn and placed into simple containers was briefly left to coagulate. To accomplish separation, each sample was centrifuged for 10 minutes at 1000 rpm. Each time, the collected serum was divided into aliquots, labelled, and kept at –200^{0}C. The samples were analyzed for hormone estimation using enzyme immunoassay (EIA) in accordance with the World Health Organisation (WHO) matched reagent programme protocol (manual) for EIA kits (protocol/version of December 1998 for LH, FSH). One aliquot of each specimen was taken at a time to prevent repeated freezing and thawing.

Biochemical Analysis

Assay of Superoxide dismutase (SOD) Activity

Rukmini *et al.* (2004) reported that superoxide dismutase activity was tested using the Winterbourn *et al.* (1975) technique. The assay's basic idea was based on SOD's capacity to prevent nitro-blue tetrazolium (NBT) from being reduced. In summary, the reaction mixture included 0.1 ml of enzyme samples, 0.05 ml of 0.12 mM riboflavin, 0.1 ml of 1.5 mM NBT, 0.05 ml of 0.01M methionine, and 2.7 ml of 0.067M phosphate buffer at pH 7.8. To guarantee even lighting of the tubes, they were placed in a box with a 15W fluorescent bulb and covered with air foil for ten minutes. Control without the enzyme source was included. The absorbance was measured at 560nm. The quantity of enzyme needed to prevent the decrease of NBT by 50% under the given circumstances was defined as one unit of SOD. Units of the enzyme's activity were represented as mg of protein.

Estimation of Lipid Peroxidation (Malondialdehyde (MDA)

By using Buege and Aust's thiobarbituric acid reactive substances (TBARS) technique, colorimetric measurements of the tissue's lipid peroxidation were made (1978). Lipid peroxidation produces malondialdehyde (MDA), which is a major component of TBARS. In summary, 2 ml of the 1:1:1 ratio TBA-TCA-HCl reagent (thiobarbituric acid 0.37%, 0.25 N HCl, and 15% TCA) was added to 0.1 ml of tissue in Tris-HCl buffer, pH 7.5. The tissue was then put in a water bath for 15 minutes and allowed to cool. At 535 nm, the absorbance of the clear supernatant was measured in comparison to the reference blank. Malondialdehyde's molar absorptivity of 1.56 $x10^5$ M^{-1} cm^{-1}, which is represented as nmol/mg protein, was used to compute the concentration.

Assay of Catalase (CAT) Activity

The Aebi (1983) technique was used to test the catalase activity. 0.1 ml of tissue was pipetted into a cuvette that held 1.9 ml of pH 7.0, 50 mM phosphate buffer. The addition of 1.0 ml of recently made 30% (v/v) hydrogen peroxide (H_2O_2) initiated the reaction. Using spectrophotometry, the rate of H_2O_2 breakdown was determined by monitoring changes in absorbance at 240 nm. Units of the enzyme's activity were represented as mg of protein.

Haematoxylin and Eosin (H&E) Tissue Processing

The fixed specimens were processed overnight for dehydration, clearing, and impregnation using an automatic tissue processor (Sakura, Japan). The specimens were embedded in paraffin blocks using an embedding station (Sakura, Japan) and serial sections of 5um thickness were cut using a microtome (ModelRM2245, Leica Biosystems, Wetzlar, Germany). We used dan autostainer (Model 5020, Leica Biosystems, Wetzlar, Germany) for Hematoxylin & Eosin staining of the sections. The mounted specimens were observed and were scored under light microscopy at x40.

Statistical Analysis

For each number, the mean and standard error of the mean (S.E.M.) were determined. Duncan's multiple range tests were used in conjunction with one-way analysis of variance (ANOVA) to compare the treatment and control groups. At p~0.05, differences were deemed statistically significant.

Ethical Clearance

Ethical approval was sought and obtained from Human Research and Ethical Committee (HREC), College of Health Science, Benue state university, Makurdi with clearance number 08038619526. All experimental procedures carried out were in accordance with the guidelines on animal experiment as prescribed by the Ethics Committee.

3. Results

3.1 Gross Observations: Body Weight and Testes Weight

Figures 1 – 4 represents the changes in body weight and testes weight of the experimental animals across groups. There were significant ($p \leq 0.05$) changes in the body weight of all the experimental animals. However, the changes were more pronounced in the groups that were treated with either low or high dose of TCA. The groups that were treated with EIC and the high dose TCA has a significant increase in body weight when compared to negative control.

Also, a significant ($p \leq 0.05$) decrease in testes weight was observed in the groups treated with EIC or TCA alone when compared to the negative control group.

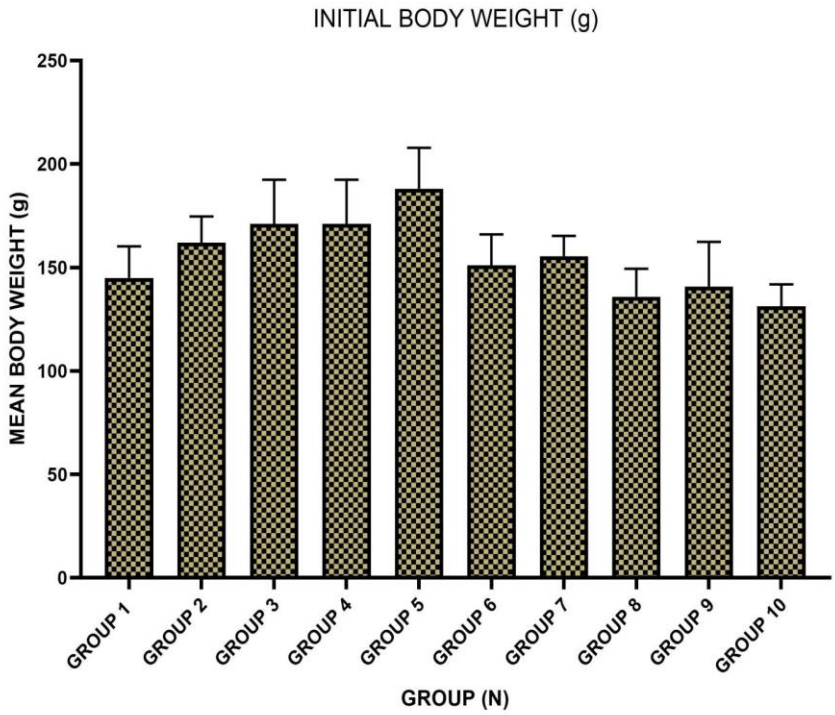

Figure 1. Simple Bar Chart Showing the Mean Initial Body Weight across groups treated with normal saline for Group 1, EIC, TCA and EIC, TCA combinations respectively for the rest of the groups

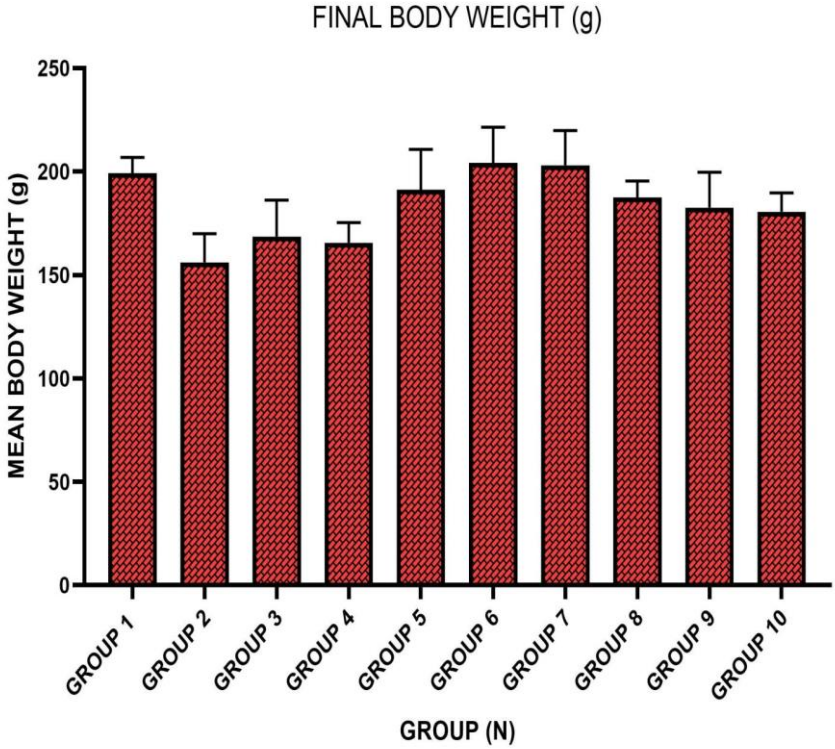

Figure 2. Simple Bar Chart Showing the Mean Final Body Weight across groups treated with normal saline for Group 1, EIC, TCA and EIC, TCA combinations respectively for the rest of the group

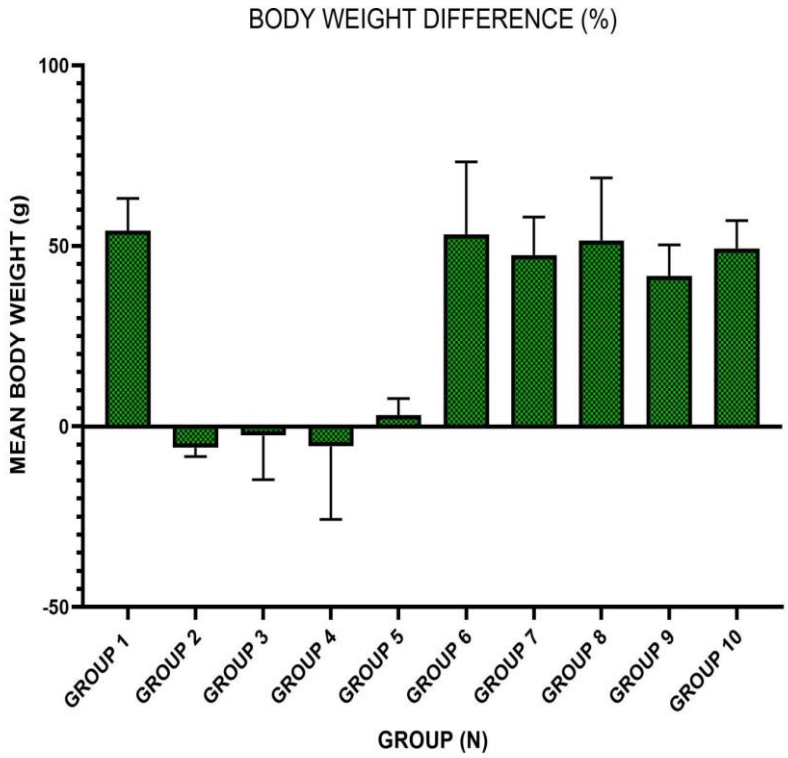

Figure 3. Simple Bar Chart Showing the Mean Body Weight Changes across groups treated with normal saline for Group 1, EIC, TCA and EIC, TCA combinations respectively for the rest of the group

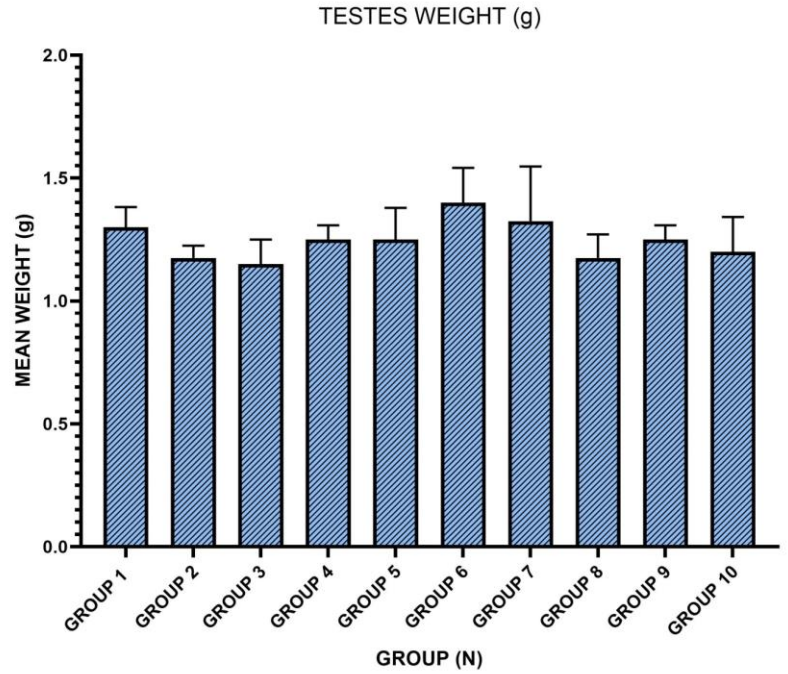

Figure 4. Simple Bar Chart Showing the Mean Testes Weight across groups treated with normal saline for Group 1, EIC, TCA and EIC, TCA combinations respectively for the rest of the group

3.2 Biochemical Analysis

3.2.1 Reproductive Hormones: Testosterone Levels, Follicle Stimulating Hormone and Luteinizing Hormone

The results reveal noteworthy findings in the context of hormonal changes:

Plasma and testicular testosterone levels experienced a statistically significant decrease ($p \leq 0.05$) in the positive control groups when compared to the negative control groups. Notably, groups treated with EIC isolates exhibited a significant ($p \leq 0.05$) increase in plasma and testicular testosterone levels when compared to groups treated with TCA alone. In the case of follicle-stimulating hormone (FSH), there were no significant ($p > 0.05$) differences between the FSH levels of rats treated with EIC and the positive control group. Regarding luteinizing hormone (LH), only groups 7 and 9 displayed a significant increase in LH levels compared to the positive control group. It's worth highlighting that the groups treated exclusively with EIC showed hormonal values similar to those of the negative control group. Surprisingly, the effect of TCA and EIC treatment did not exhibit a dose-dependent pattern, as there were no significant ($p > 0.05$) differences between the high-dose groups and their respective counterparts.

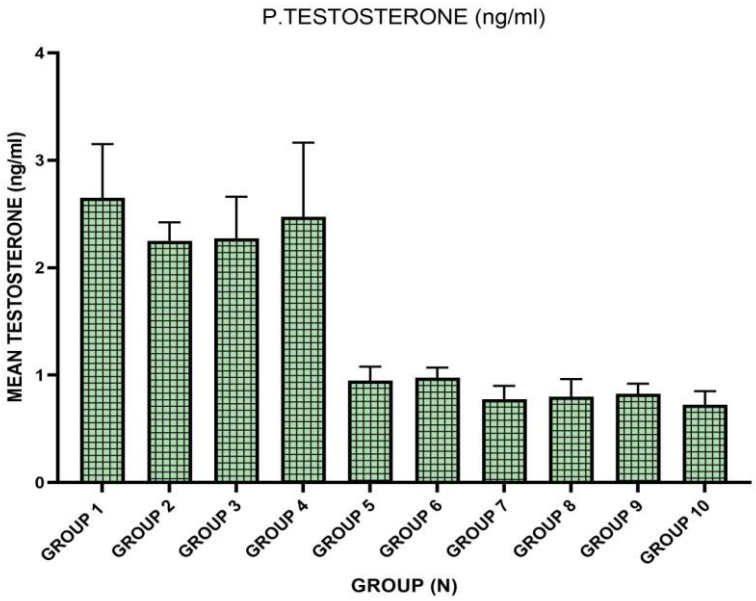

Figure 5. Simple Bar Chart Showing the Mean Plasma Testosterone Levels across groups treated with normal saline for Group 1, EIC, TCA and EIC, TCA combinations respectively for the rest of the group

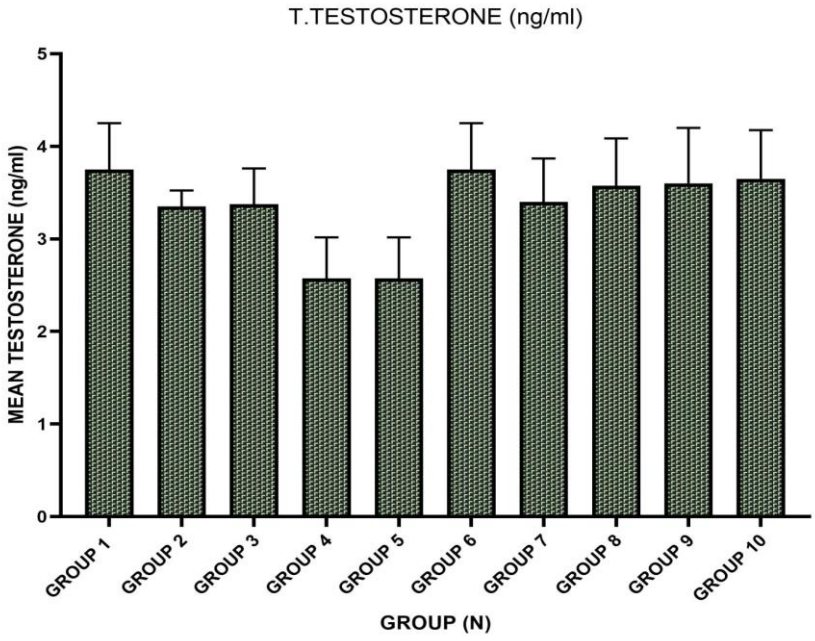

Figure 6. Simple Bar Chart Showing the Mean Testicular Testosterone Levels across groups treated with normal saline for Group 1, EIC, TCA and EIC, TCA combinations respectively for the rest of the group

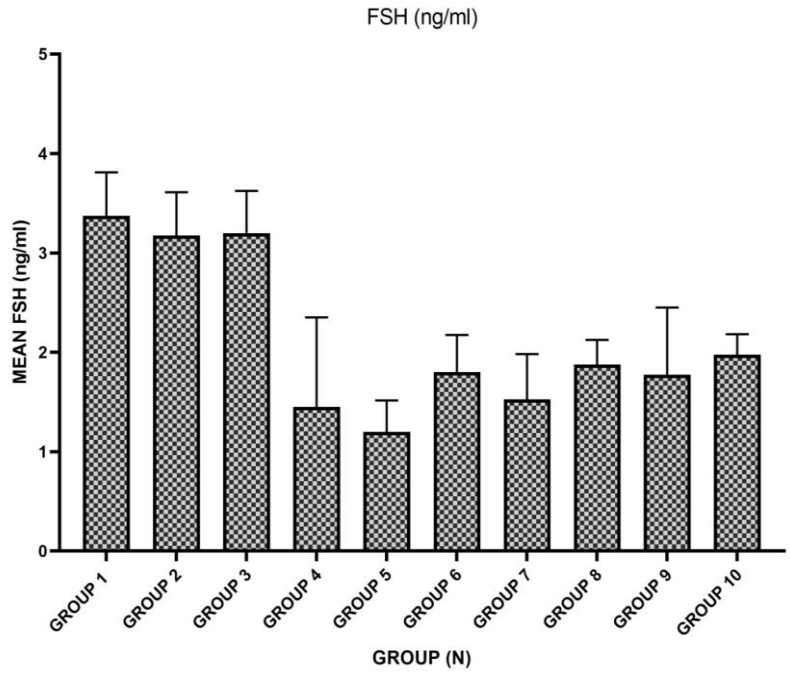

Figure 7. Simple Bar Chart Showing the Mean Follicle stimulating hormone across groups treated with normal saline for Group 1, EIC, TCA and EIC, TCA combinations respectively for the rest of the group

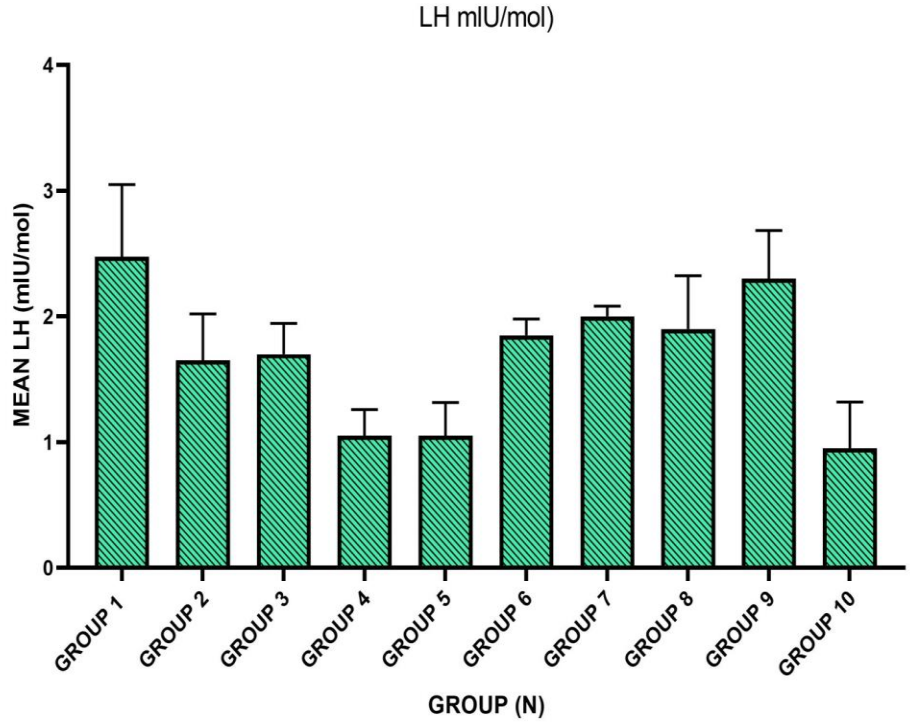

Figure 8. Simple Bar Chart Showing the Mean Luteinizing hormone across groups treated with normal saline for Group 1, EIC, TCA and EIC, TCA combinations respectively for the rest of the group

3.2.2 Oxidative Stress Markers: SOD, CAT and MDA

As depicted in Figures 9-11, the positive control group exhibited a noteworthy reduction in SOD and CAT levels, coupled with a substantial increase in MDA levels when compared to the negative control group. Moreover, the results revealed a significant elevation in SOD and CAT levels and a marked decrease in MDA levels among the

treatment groups (groups 6-8) in comparison to the positive control group. Notably, when comparing the higher doses of TCA (group 5) and EIC, it is evident that the higher dose of EIC, in conjunction with TCA, induced a more pronounced reduction in SOD levels and MDA levels, respectively, compared to their corresponding lower doses.

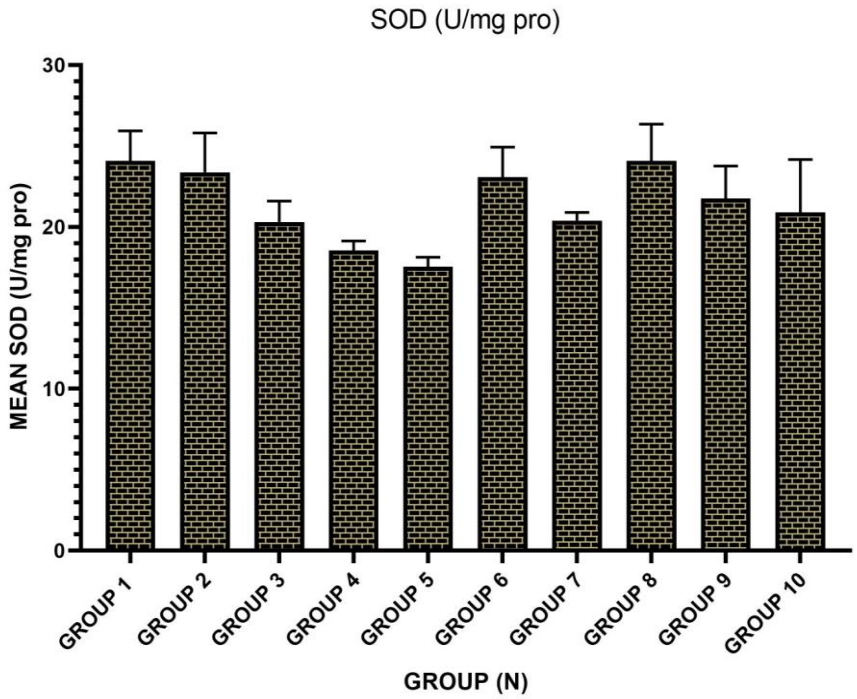

Figure 9. Simple Bar Chart Showing the Mean Superoxide dismutase across groups treated with normal saline for Group 1, EIC, TCA and EIC, TCA combinations respectively for the rest of the groups

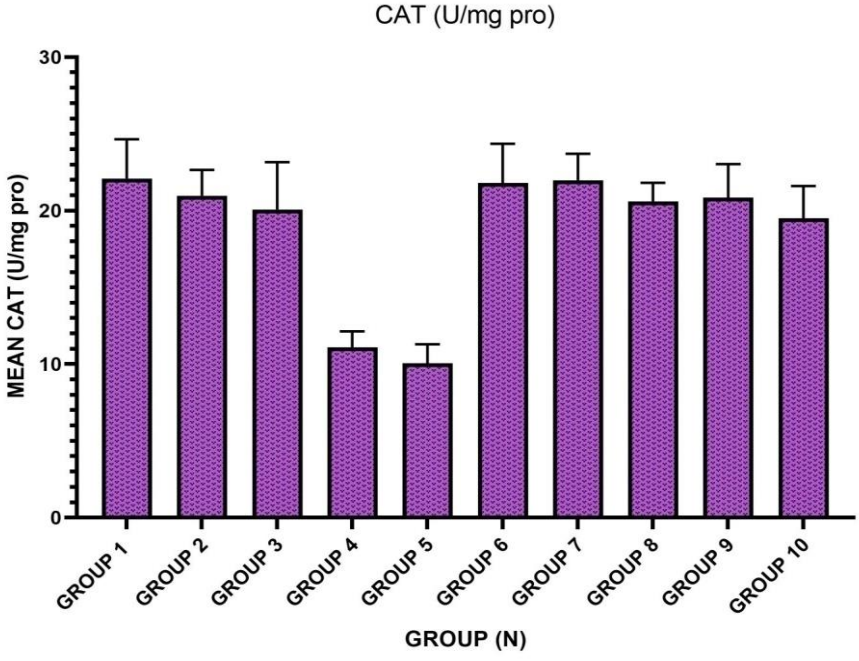

Figure 10. Simple Bar Chart Showing the Mean Catalase across groups treated with normal saline for Group 1, EIC, TCA and EIC, TCA combinations respectively for the rest of the group

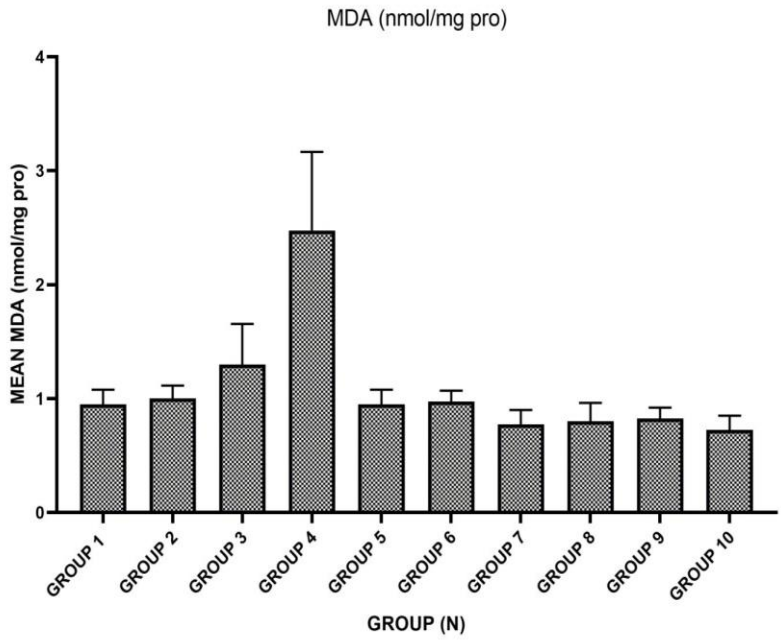

Figure 11. Simple Bar Chart Showing the Mean Malondialdehyde across groups treated with normal saline for Group 1, EIC, TCA and EIC, TCA combinations respectively for the rest of the group

3.3 Sperm Analysis

3.3.1 Assessment of Sperm Quality: Sperm Count and Morphology

The rat models subjected to exclusive TCA treatment exhibited noteworthy changes in various sperm parameters. There was a substantial and statistically significant ($p \leq 0.05$) decrease in sperm count and normal sperm morphology, accompanied by a significant ($p \leq 0.05$) increase in abnormal sperm morphology when contrasted with the negative control group (Group 1). These alterations in sperm characteristics highlight the detrimental effects of TCA treatment on reproductive parameters.

Conversely, the groups treated with EIC displayed a striking contrast in these parameters. There was a significant increase in sperm count and a decrease in abnormal sperm morphology, signifying a potentially beneficial impact when compared to the group treated solely with TCA. This suggests the potential of EIC to counteract some of the negative effects of TCA on sperm quality.

It's worth noting that Group 6 exhibited a non-significant ($p > 0.05$) increase in normal sperm morphology when compared to the positive control group. While this increase wasn't statistically significant, it does hint at a potential positive influence of the treatment in this specific context. In comparing the corresponding dose groups, only Group 7 and Group 9 demonstrated statistically significant ($p \leq 0.05$) changes. Group 7 showed a decrease in normal sperm morphology, while Group 9 exhibited an increase in normal sperm morphology along with changes in abnormal sperm morphology. These specific responses highlight the dose-dependent nature of the treatment effects on sperm morphology.

Additionally, the classification of sperm morphology revealed no statistically significant differences between the negative and positive control groups and the experimental groups. This implies that the morphological aspects of sperm were not significantly affected by the experimental treatments, as observed in the data. Overall, the findings shed light on the complex interplay between TCA, EIC, and sperm morphology, with both detrimental and potentially beneficial effects on various parameters.

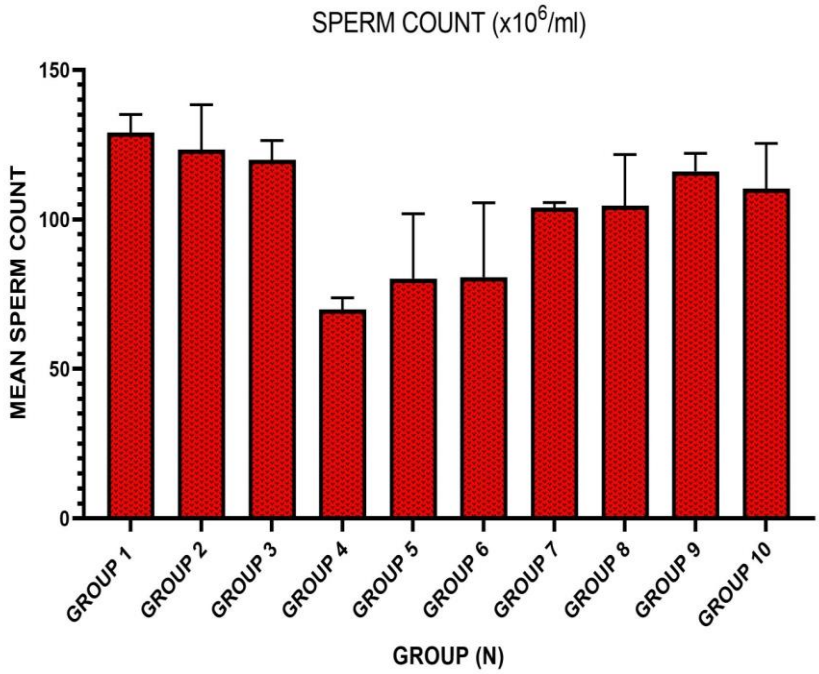

Figure 12. Simple Bar Chart Showing the Mean Sperm Count across groups with significant decreases or increases at P≤0.05 when compared to group 1 (negative control), groups 4&5 (positive controls), groups 2, 4,6 and 8 (low dose group). N = 4.

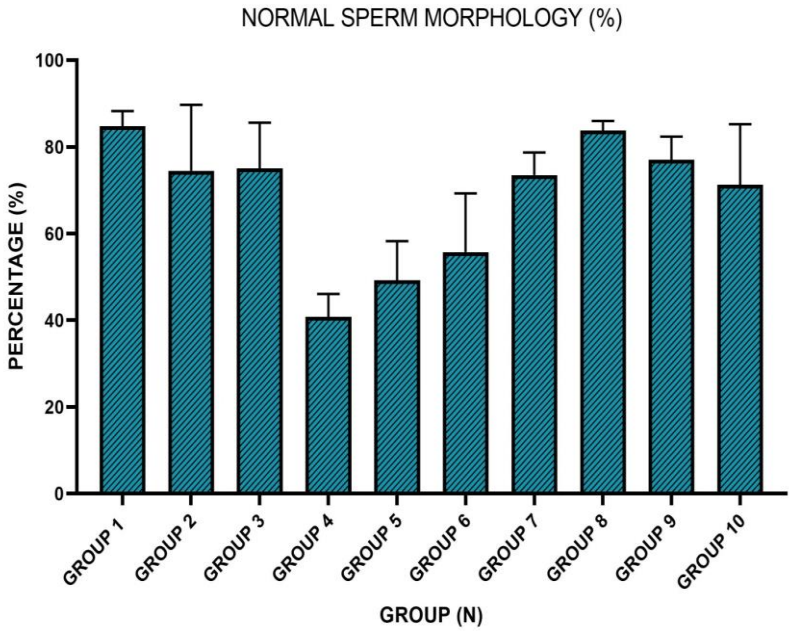

Figure 13. Simple Bar Chart Showing the Mean Normal Sperm Morphology across groups with significant decreases or increases at P≤0.05 when compared to group 1 (negative control), groups 4&5 (positive controls), groups 2, 4,6 and 8 (low dose group). N = 4.

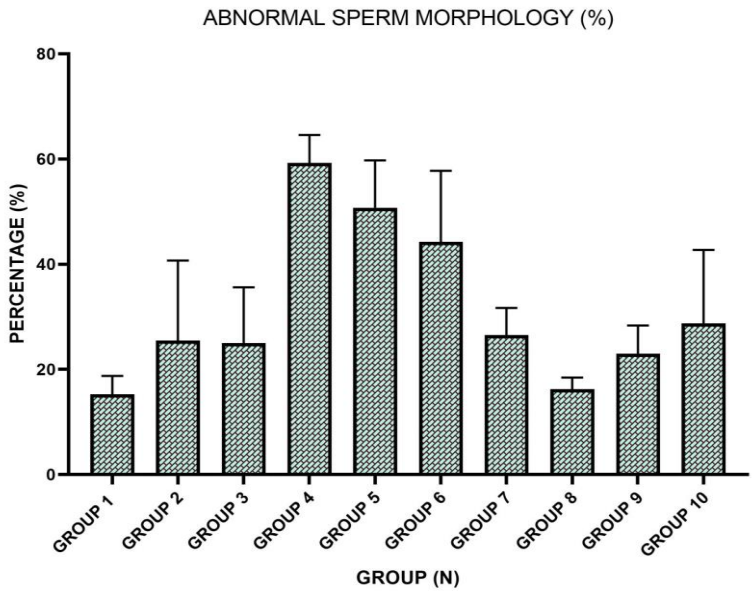

Figure 14. Simple Bar Chart Showing the Mean Abnormal Sperm Morphology across groups with significant decreases or increases at P≤0.05 when compared to group 1 (negative control), groups 4&5 (positive controls), groups 2, 4,6 and 8 (low dose group). N = 4.

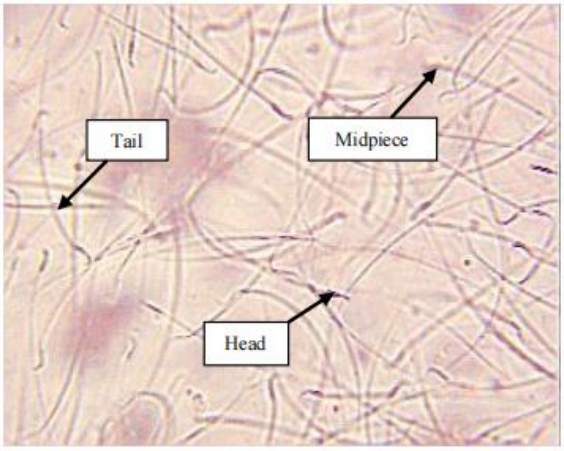

Plate 1: Photomicrograph of sperm from group 1 showing the Head, Tail, and Midpiece. Stain: Papanicolaou; Magnification: x100.

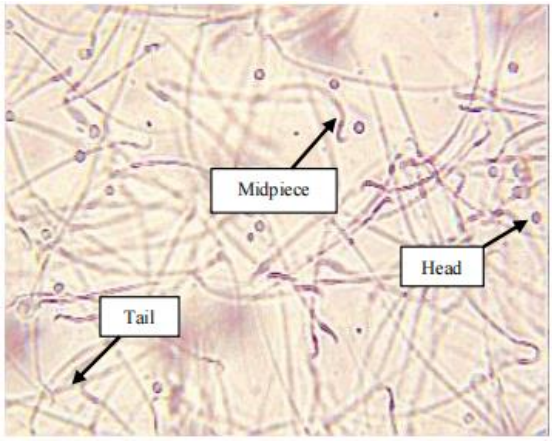

Plate 2: Photomicrograph of sperm from group 2 showing the Head, Tail, and Midpiece. Stain: Papanicolaou; Magnification: x100.

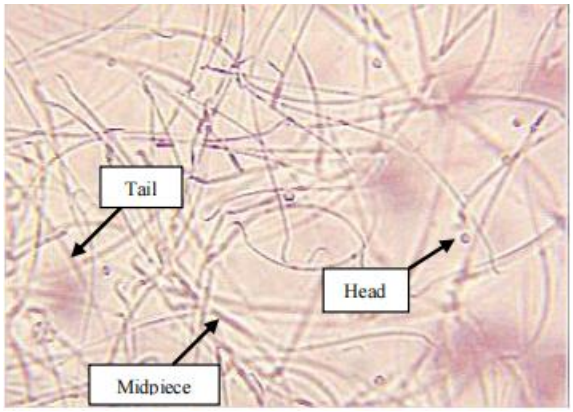

Plate 3: Photomicrograph of sperm from group 3 showing the Head, Tail, and Midpiece. Stain: Papanicolaou; Magnification: x100.

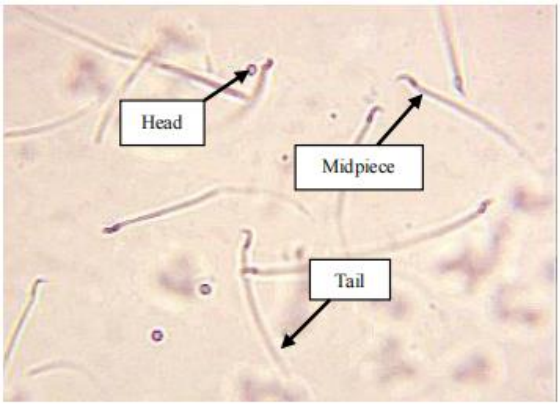

Plate 4: Photomicrograph of sperm from group 4 showing the Head, Tail, and Midpiece. Stain: Papanicolaou; Magnification: x100.

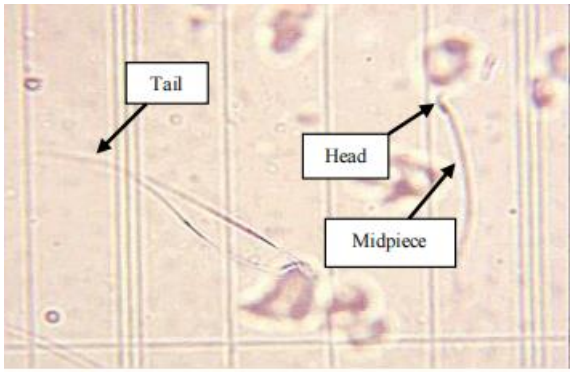

Plate 5: Photomicrograph of sperm from group 5 showing the Head, Tail, and Midpiece. Stain: Papanicolaou; Magnification: x100.

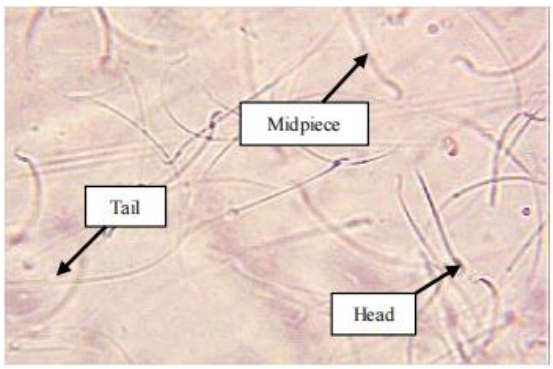

Plate 6: Photomicrograph of sperm from group 6 showing the Head, Tail, and Midpiece. Stain: Papanicolaou; Magnification: x100.

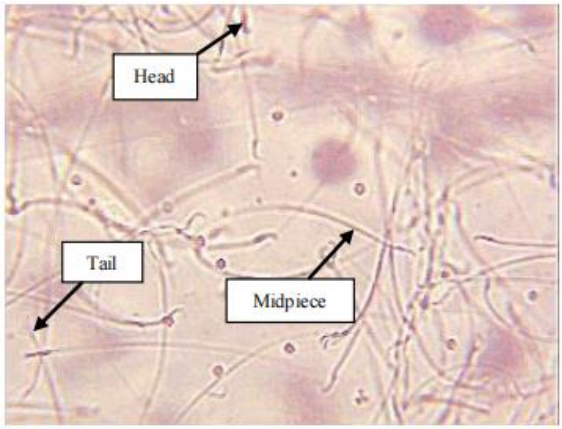

Plate 7: Photomicrograph of sperm from group 7 showing the Head, Tail, and Midpiece. Stain: Papanicolaou; Magnification: x100.

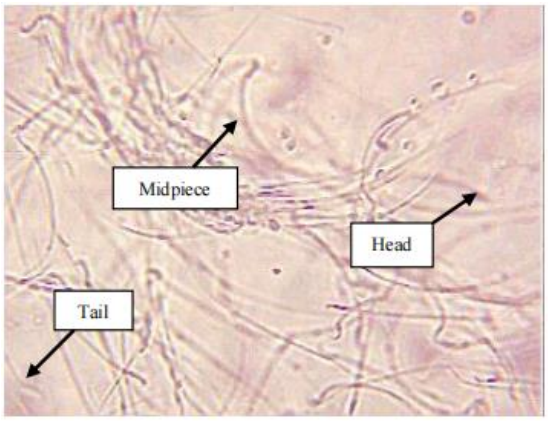

Plate 8: Photomicrograph of sperm from group 8 showing the Head, Tail, and Midpiece. Stain: Papanicolaou; Magnification: x100.

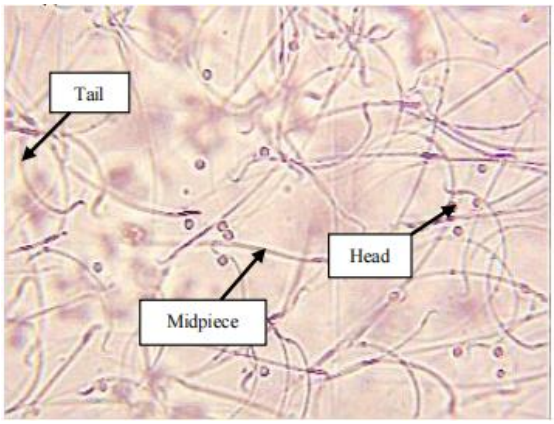

Plate 9: Photomicrograph of sperm from group 9 showing the Head, Tail, and Midpiece. Stain: Papanicolaou; Magnification: x100.

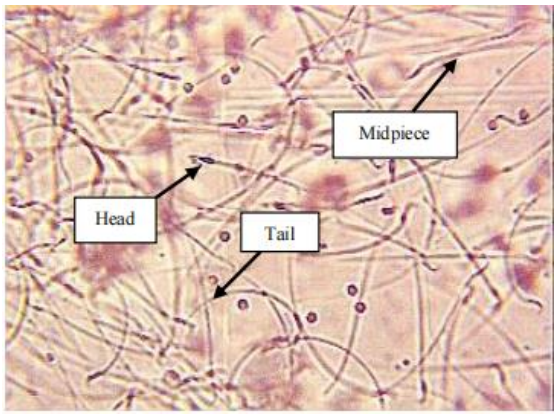

Plate 10: Photomicrograph of sperm from group 10 showing the Head, Tail, and Midpiece Stain: Papanicolaou; Magnification: x100.

3.3.2 Histological Observations

Histological observations of the testicular tissue in groups 1 - 3 revealed normal histological characteristics, with an abundance of spermatozoa extending towards the lumen. The Leydig cells remained intact, and spermatid retention was evident within the seminiferous tubules (Plate 11 - 13).

In contrast, groups 4 and 5 exhibited abnormal seminiferous tubule morphology, marked by spermatid retention, tubular atrophy, and widespread disorganization of germ cells. The testicular architecture showed signs of degeneration, with the absence of interstitial space and areas of necrosis. Additionally, several maturing

spermatogenic cells were observed within the seminiferous tubules. Some cells exhibited nuclear membrane rupture, accompanied by nucleus fragmentation (karyorrhexis) (Plate 14 - 15).

Conversely, groups 6 - 10 displayed distinct features, characterized by spermatogonia cells with deeply stained nuclei and damaged sperm cells. The majority of seminiferous tubules appeared shrunken with a wavy outline. Thickening and hyalinization of basement membranes were evident, and the lumens of the seminiferous tubules were primarily occupied by fragments of disintegrated cells. Although there were some similarities to groups 4 and 5, these observations set groups 6 -10 apart and preclude direct comparison to the histological features of groups 1-3.

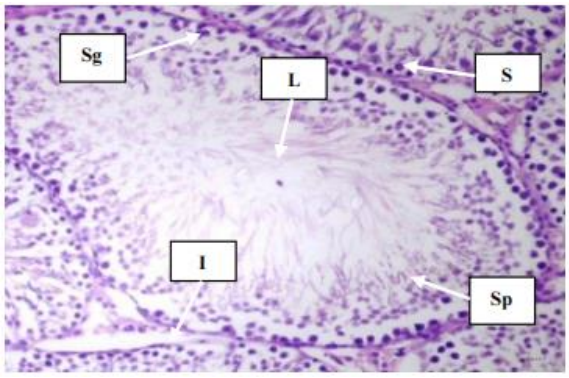

Plate 11: Photomicrograph of testes from group 1 **showing** the lumen (L), Spermatozoa (Sp); Sertoli Cells (S) and interstitum (I)**. Stain:** H&E; **Magnification**: x40

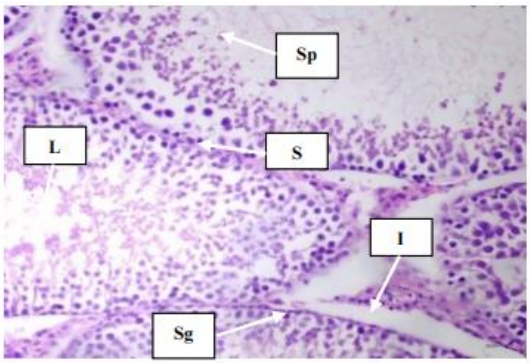

Plate 12: Photomicrograph of testes from group 2 showing the lumen (L), Spermatozoa (Sp); Sertoli Cells (S) and interstitum (I)**. Stain:** H&E; **Magnification**: x40

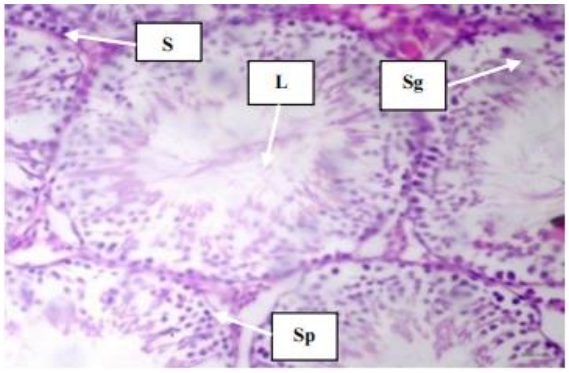

Plate 13: Photomicrograph of testes from group 3 showing the lumen (L), Spermatozoa (Sp); Sertoli Cells (S) **Stain:** H&E; **Magnification**: x40

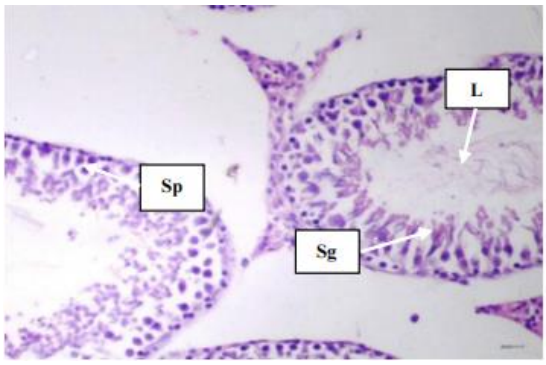

Plate 14: Photomicrograph of testes from group 4 showing the lumen (L), Spermatozoa (Sp); Sertoli Cells (S) and interstitum (I)**. Stain:** H&E; **Magnification**: x40

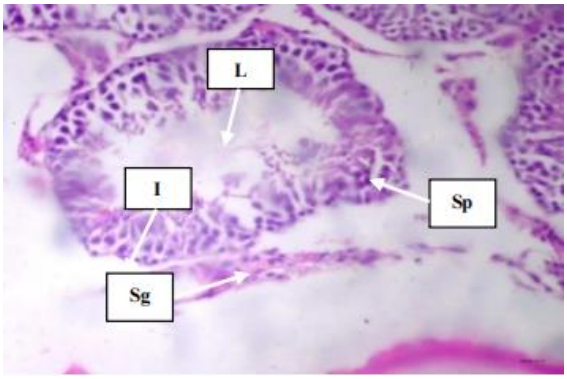

Plate 15: Photomicrograph of testes from group 5 showing the lumen (L), Spermatozoa (Sp); Sertoli Cells (S) and interstitum (I)**. Stain:** H&E; **Magnification**: x40

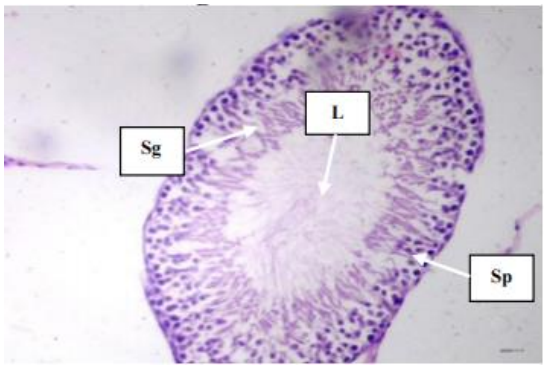

Plate 16: Photomicrograph of testes from group 6 showing the lumen (L), Spermatozoa (Sp); Sertoli Cells (S) and interstitum (I)**. Stain:** H&E; **Magnification**: x40

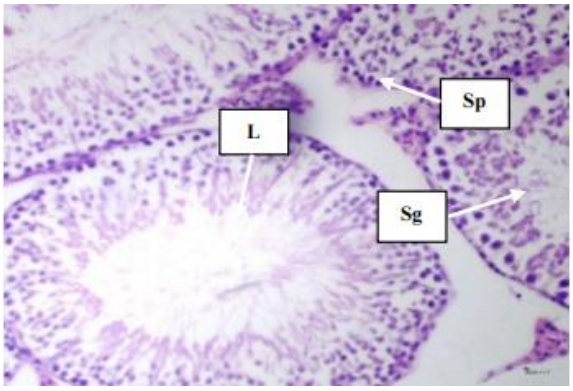

Plate 17: Photomicrograph of testes from group 7 showing the lumen (L), Spermatozoa (Sp); Sertoli Cells (S) and interstitum (I)**. Stain:** H&E; **Magnification**: x40

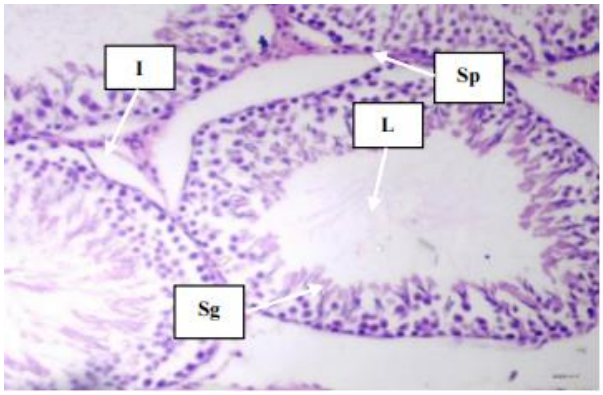

Plate 18: Photomicrograph of testes from group 8 showing the lumen (L), Spermatozoa (Sp); Sertoli Cells (S) and interstitum (I)**. Stain:** H&E; **Magnification**: x40

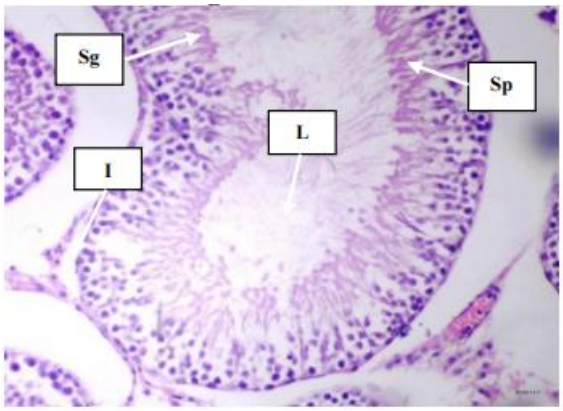

Plate 19: Photomicrograph of testes from group 9 showing the lumen (L), Spermatozoa (Sp); Sertoli Cells (S) and interstitum (I)**. Stain:** H&E; **Magnification**: x40

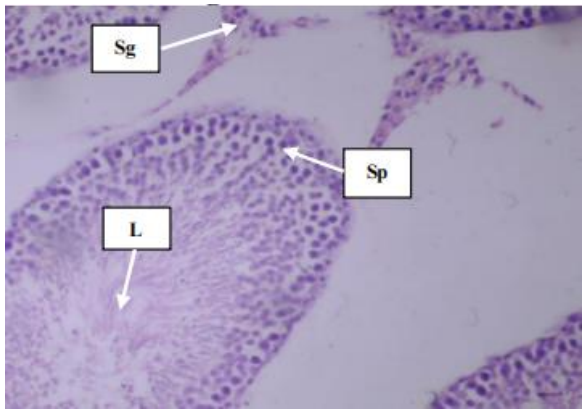

Plate 20: Photomicrograph of testes from group 10 showing the lumen (L), Spermatozoa (Sp); Sertoli Cells (S) and interstitum (I)**. Stain:** H&E; **Magnification**: x40

4. Discussion

4.1 Gross Parameters: Body Weight and Testes Weight

The evaluation of alterations in body weight among the different experimental groups, as depicted in figures 1 - 4, unveiled statistically significant differences ($p \leq 0.05$). Notably, the influence on body weight was particularly prominent, especially in groups subjected to varying doses of Trichloroacetic Acid (TCA). Specifically, the groups treated with Eugenol Isolates from Clove (EIC) and the high TCA dose exhibited a significant increase in body weight compared to the negative control group. This observation underscores the discernible impact of both EIC and high-dose TCA on physiological parameters related to body mass, highlighting their potential effects on the weight dynamics of the experimental subjects. A more in-depth exploration of these findings holds promise in yielding valuable insights into the metabolic and physiological implications associated with the administered treatments.

It is essential to draw attention to a prior investigation by Acharya *et al.* (1995), which reported diminished body weight gains in animals treated with Dichloroacetic Acid (DCA) at doses of 500 and 5000 ppm, accompanied by a concurrent reduction in total serum protein levels across all doses. Additionally, Mather et al. (1990) observed elevated organ to body weight ratios in the liver and kidney of rats administered either TCA (5000 ppm) or DCA (500 or 5000 ppm). Further studies by Wan & Grimble (1998) revealed adverse effects of Trichloroacetic Acid (TCA) injections in rats, leading to the loss of muscle protein and skin weight. Davis (1990) reported that exposure to TCA in drinking water resulted in reduced water and food consumption, along with a decline in body weight over a 14-day period. Gavage with Dichloro- and Trichloroacetic acids (DCA and TCA) induced a 15% weight loss in male rats (Davis, 1992). Notably, exposure to Trichloroethylene (TCE) for 240 hours in rats did not result in apparent weight loss, liver injury, or hematological changes (Koizumi *et al.*, 1984). The

interaction between TBARS+TCA was found to induce a significant loss of body weight in rats, as reported by Acharya *et al.* (1995).

Furthermore, in comparison to the negative control group, a significant ($p \leq 0.05$) reduction in testis weight was observed in groups treated with either EIC or TCA alone. Testis weight predominantly relies on the mass of developed spermatogenic cells, and a decrease may be attributed to a decline in the number of germ cells, suppression of spermatogenesis, and altered steroidogenic enzyme activity (Takahashi & Oishi, 2001; Yang *et al.*, 2006). Consistent with various investigations (Katz *et al.*, 1981; Toth *et al.*, 1992; Linder *et al.*, 1997), the present study unveiled testicular damage associated with spermatogenic impairment and degeneration of the germinal epithelium in a dose-dependent manner following repeated exposure to comparable chemicals.

The histopathological abnormalities observed in the current study align with prior research by Xie *et al.* (2014), suggesting that pathogenic alterations in the seminiferous epithelium may disrupt Sertoli and germ cells, impairing spermatogenesis and potentially leading to the loss of germ cells.

4.2 Biochemical Analysis

4.2.1 Reproductive Hormone Changes: Plasma & Serum Testosterone, Follicle Stimulating Hormone and Luteinizing Hormone

Pituitary gonadotropins, encompassing luteinizing hormone and follicle-stimulating hormone, are well-established for their role in testosterone secretion and promotion of proper spermatogenesis. While FSH directly affects seminiferous tubules, LH stimulates the generation and release of testosterone by Leydig cells (MacLachlan *et al.*, 2002; Spaliviero *et al*., 2004).

A notable observation is the substantial and statistically significant decrease ($p \leq 0.05$) in plasma and testicular testosterone levels within the positive control groups when compared to their counterparts in the negative control groups. This implies a clear impact on testosterone levels within the experimental context. The findings of this study diverge from those of Davies (1990), whose study reported that high doses of Trichloroacetic Acid (TCA) led to a decrease in plasma glucose and lactate concentrations but did not affect reproductive hormones in rats. Additionally, a considerable drop in testosterone levels was observed, contrary to the work by Linder *et al*. (1997), which found no effect on testosterone levels in rats administered a comparable drug for 14 consecutive days at 18 to 1440 mg/kg b.w. In contrast, this study suggests a different outcome. Subchronic exposure to Dibromoacetic Acid (DBAA) was found to potentially influence reproductive outcomes in rats, with DBAA exhibiting stronger testicular toxicity compared to Dichloroacetic Acid (DCA), as highlighted by Linder *et al*. (1994). Nonetheless, these outcomes align with those discovered in rats given bromochloroacetic acid treatment by Klinefelter *et al* (2002). The decrease in plasma levels of LH and FSH observed in rats exposed to DCA suggests that DCA interferes with the production and release of anterior pituitary hormones. Groups subjected to EIC displayed a remarkable increase in both plasma and testicular testosterone levels when contrasted with groups treated solely with TCA. This underscores the potent influence of EIC on testosterone levels, suggesting its potential as an effective intervention.

Concerning FSH levels, a unique pattern emerges. Rats treated with EIC did not exhibit any statistically significant differences in their FSH levels when compared to the positive control group. This finding suggests that EIC treatment might not significantly influence FSH levels, offering an intriguing area for further investigation.

The story takes a different turn when considering luteinizing hormone (LH). Only groups 7 and 9 demonstrated a notable and statistically significant increase in their LH levels compared to the positive control group. This selective response to EIC treatment with regard to LH underscores the complexity of its effects on different hormones. A notable observation is that groups exclusively treated with EIC exhibited hormonal values akin to those of the negative control group. This suggests that EIC treatment may restore hormonal balance, or at least maintain it at levels consistent with the control group unaffected by the experimental factors. Surprisingly, when considering the effect of TCA and EIC treatment, it becomes apparent that this impact is not dependent on the dosage administered. No statistically significant differences were observed between the high-dose treatment groups and their corresponding counterparts. This dose-independent effect raises questions about the mechanisms underlying the observed hormonal changes and warrants further investigation.

4.2.2 Oxidative Stress Markers: SOD, CAT and MDA

This study revealed significant disparities between the positive and negative control groups. The positive control group exhibited a noteworthy increase in malondialdehyde (MDA) levels and a marked decline in catalase (CAT) and superoxide dismutase (SOD) levels compared to the negative control group, indicating a substantial alteration in oxidative stress indicators. This outcome aligns with previous research on TCA, where oxidative stress indicators accumulated in response to dichloroacetate injection (Calcutt *et al.*, 2009), and TCA exposure led to lipid peroxidation and alterations in antioxidant systems in rats (Çelik, 2007).

Studies by Austin *et al*. (1996) demonstrated a surge in TCA-induced thiobarbituric acid-reactive substances (TBARS), indicative of lipid peroxidation, in mice livers nine hours after administration.

Numerous investigations have explored the potential of Trichloroacetic Acid (TCA) to induce oxidative stress and peroxisome proliferation. The detoxification of Reactive Oxygen Species (ROS) involves antioxidant enzymes, constituting a crucial aspect of the immune system. The primary defense against oxygen-related damage is the SOD–CAT system, where Superoxide Dismutase (SOD) plays a pivotal role in converting superoxide anions to hydrogen peroxide (H2O2) during oxidative stress (Halliwell & Gutteridge, 1989). H_2O_2 elimination is subsequently managed by either Catalase (CAT) or Glutathione Peroxidase (GPx), with GPx predominantly operating in the testes (Peltola *et al*., 1992). Increased SOD activity may result from heightened superoxide anion generation, indicating an adaptive response to combat free radicals, with a subsequent rise in CAT activity (Cheung *et al*., 2001; Braga *et al*., 2009).

Conversely, elevated levels of ROS pose a threat to testicular function. The testes possess a robust antioxidant system, including the glutathione family, superoxide dismutase, catalase, and non-enzymatic antioxidants, shielding against the harmful effects of ROS (Aitken & Roman, 2008). Nevertheless, excessive exposure to environmental toxins has been shown to disturb the pro-oxidant/antioxidant equilibrium in the testes, impairing their function (Saradha & Mathur, 2006).

It is noteworthy however, that various studies have explored TCA-induced oxidative stress responses in mice, including lipid peroxidation and oxidative DNA damage, with temporary increases observed in single-dose trials. Conversely, peroxisome proliferation-related reactions persisted for up to 10 weeks after TCA dosage. Findings of the current study indicate a consistent pattern among treatment groups, specifically groups 6 through 8, showing reduced MDA levels, indicative of lowered lipid peroxidation, and increased SOD and CAT levels, suggesting improved antioxidant defenses compared to the positive control group. Interestingly, the combination of higher doses of Eugenol Isolates from Clove (EIC) with TCA exhibited synergistic effects, leading to a more pronounced reduction in SOD levels and concurrent decrease in MDA levels, suggesting a potent mitigation of lipid peroxidation. This underscores the potential dose-dependent synergies between EIC and TCA in reducing oxidative stress.

4.3 Sperm Analysis

The examination of sperm parameters in this study, as depicted in Figures 12 – 14, offers valuable insights into the impact of Trichloroacetic Acid (TCA) treatment on adult male Wistar rats. Specifically, the exclusive administration of TCA resulted in significant alterations in sperm count and morphology, with a marked decrease in normal sperm morphology and a simultaneous increase in abnormal sperm morphology ($p \leq 0.05$). This observation aligns with previous research, such as Toth *et al.* (1992) study, which linked chronic dichloroacetate treatment to testicular degeneration and decreased sperm counts in rats. Similarly, brominated acetic acids, including dibromoacetic acid (DBAA) and bromochloroacetic acid (BCA), have been associated with defects in spermatogenesis and reduced fertility in adult rats.

Particularly, epididymal sperm counts were significantly impacted by dichloroacetate treatment in rats at specific dose levels (Toth *et al*., 1992). These findings underscore the adverse effects of TCA on crucial reproductive parameters, raising concerns about its potential implications for fertility and reproductive health. Histological alterations in rats treated with TCA further support these findings, indicating disrupted spermatogenesis due to Leydig cell degeneration and reduced testosterone production, as highlighted by Sanghamitra *et al*. (2008), and the negative impact of environmental pollutants on testicular function reported by Akingbemi *et al*. (2004) and Murugesan *et al*. (2007).

In contrast, the groups subjected to Eugenol Isolates from Clove (EIC) treatment exhibited noteworthy improvements in sperm parameters. A significant increase in sperm count and a reduction in abnormal sperm morphology suggest a potential favorable influence of EIC in mitigating the deleterious effects of TCA on sperm quality. These promising outcomes hint at EIC's capacity to partially restore the integrity of sperm parameters in the face of TCA-induced challenges, offering hope for maintaining reproductive health.

An intriguing observation arises from the analysis of Group 6, revealing a non-significant ($p > 0.05$) increase in normal sperm morphology compared to the positive control group. Although not statistically significant, this increase suggests a potential positive impact of the treatment within this specific context, warranting further exploration into the nuanced effects of the interventions.

Upon scrutinizing the dose-dependent responses, it becomes apparent that only Group 7 and Group 9 displayed statistically significant ($p \leq 0.05$) alterations. Group 7 witnessed a decline in normal sperm morphology, while Group 9 exhibited an increase in normal sperm morphology alongside changes in abnormal sperm morphology. These specific responses underscore the importance of dosage considerations in understanding the treatment effects on sperm morphology within this experimental context.

Additionally, the analysis of sperm morphology classification provided valuable insights, revealing no statistically significant differences between the negative and positive control groups and the experimental groups. This suggests that certain structural aspects of sperm, as assessed in this study, remained largely unaffected by the experimental treatments. This finding contributes to our understanding of the specific dimensions of sperm morphology influenced by the interventions, guiding further research in this area.

In summary, the collective results from Figures 5 and 6 shed light on the intricate interplay between TCA, EIC, and sperm morphology. These findings underscore the potential for both detrimental and beneficial effects on various sperm parameters, prompting critical questions about the complex dynamics at play and their broader implications for reproductive health.

4.4 Histological Profile

The histological examination of testicular tissues in groups 1-3 revealed a standard profile characterized by an abundance of spermatozoa extending towards the lumen, intact Leydig cells, and noticeable spermatid retention within the seminiferous tubules. In contrast, groups 4 and 5 exhibited abnormal seminiferous tubule morphology, marked by spermatid retention, tubular atrophy, and widespread disorganization of germ cells. Degeneration signs were evident in the absence of interstitial space and areas of necrosis, with maturing spermatogenic cells displaying nuclear membrane rupture and nucleus fragmentation (karyorrhexis).

Singh (2005) conducted a study involving dissection, weight measurement, and histological examination of testes in pups from various dose groups. Fetal testes exhibited a significant reduction in average weights, particularly noticeable at doses equal to or exceeding 1,200 mg/kg-day when compared to the control group. Histological examination of fetal rat testes within the 1,200 mg/kg-day dose group revealed a distinct pattern, including a reduction in seminiferous tubule diameter, predominantly in the peripheral region. This effect became more pronounced in groups receiving higher doses, accompanied by a reported reduction in the length of seminiferous tubules at elevated dose levels.

Closer inspection at higher magnification revealed an elevated occurrence of apoptosis in both gonocytes and Sertoli cells within the seminiferous tubules, particularly evident at doses equal to or exceeding 1,200 mg/kg-day. This detailed histological analysis highlights the nuanced impact of varying doses on testicular morphology, underscoring the importance of considering both dosage levels and specific histopathological alterations in understanding the observed effects.

Conversely, groups 6-10 presented distinctive characteristics, featuring spermatogonia cells with deeply stained nuclei and damaged sperm cells. The seminiferous tubules in these groups displayed a shrunken appearance with a wavy outline, accompanied by thickening and hyalinization of basement membranes. The lumens of the seminiferous tubules were predominantly occupied by fragments of disintegrated cells. While sharing some similarities with groups 4 and 5, the histological features of groups 6-10 stand apart, preventing a direct comparison to the observations in groups 1-3.

Eugenol, a key component in clove oil, exhibits therapeutic potential across various physiological domains, as evidenced by multiple studies. Damiani *et al.* (2003) found that Eugenol induces smooth muscle relaxation in rats by blocking voltage-sensitive and receptor-operated channels modulated by endothelial-generated nitric oxide. Additionally, Al-Okbi *et al.* (2014) demonstrated that clove essential oil and Eugenol microemulsions significantly improve fatty liver and dyslipidemia in rats, providing protection against cardiovascular diseases and complications associated with fatty liver and other tissue disruptions.

5. Conclusion

The study delved into the protective and curative effects of Clove Isolates, particularly Eugenol, on Trichloroacetic Acid (TCA)-induced sperm-endocrine deficit, redox imbalance, and histomorphology in adult male rats. The investigation spanned diverse parameters, including body and testes weight, reproductive hormone levels, oxidative stress markers, sperm analysis, and histological profiles.

The alterations in body weight observed, especially in groups subjected to TCA and Eugenol Isolates from Clove (EIC), emphasized the profound impact of these treatments on physiological parameters related to body mass. These findings align with prior research on the adverse effects of TCA on body weight and organ-to-body weight ratios. The significant reduction in testes weight in groups treated with EIC or TCA alone indicated potential spermatogenic impairment and alterations in steroidogenic enzyme activity.

Reproductive hormone analysis revealed a substantial decrease in testosterone levels within positive control groups, while EIC treatment demonstrated a capacity to elevate testosterone levels, suggesting a potential protective effect. The intricate interplay of Eugenol with luteinizing hormone (LH) highlighted the complexity of its hormonal effects, and dose-independent impacts raised questions for further exploration.

Oxidative stress marker analysis demonstrated TCA-induced alterations, while EIC treatment exhibited

synergistic effects, particularly in reducing oxidative stress. Sperm analysis showcased TCA-induced adverse effects, mitigated by EIC, offering hope for preserving reproductive health. The histological examination delineated distinct profiles, with EIC showing potential therapeutic benefits.

In conclusion, this study underscores the multifaceted interactions between TCA, Eugenol Isolates from Clove, and various physiological parameters. The results suggest potential protective and curative effects of Eugenol against TCA-induced reproductive deficits, redox imbalance, and histomorphological abnormalities. These findings contribute valuable insights into the potential applications of Clove Isolates in mitigating the detrimental effects of environmental toxins on male reproductive health. Further investigations are warranted to elucidate the underlying mechanisms and optimize the therapeutic potential of Eugenol in safeguarding male reproductive function.

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