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Propolis Protects Against Reproductive Toxicity Induced by Di(2-ethylhexyl) Phthalate (DEHP) in Male Rabbits

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ABSTRACT

Propolis, a natural resinous substance produced by bees, is well-documented for its antioxidant and protective properties, whereas diethylhexylphthalate (DEHP) is a known endocrine disruptor with potential reproductive toxicity. This study aimed to evaluate the effects of propolis, DEHP, and their combination on semen quality parameters and oxidative stress biomarkers in male rabbits. Twenty adult male rabbits were divided into four groups: control, propolis-treated, DEHP-exposed, and a combination of propolis and DEHP. The results demonstrated that DEHP significantly reduced semen quality, as evidenced by decreased ejaculate volume, sperm concentration, motility, and total sperm count ($p < 0.05$). Additionally, DEHP exposure led to an increase in dead and abnormal sperm percentages, along with a reduction in initial fructose concentration and packed sperm volume. Conversely, propolis supplementation significantly improved semen quality parameters compared to the control, mitigating DEHP-induced reproductive toxicity. Notably, rabbits treated with both propolis and DEHP showed partial improvement in semen quality parameters compared to the DEHP group, suggesting a protective effect of propolis. Furthermore, oxidative stress markers revealed a significant decrease in glutathione (GSH), glutathione peroxidase (GPx), glutathione S-transferase (GST), catalase (CAT), and superoxide dismutase (SOD) levels in the DEHP-exposed group ($p < 0.05$), alongside an increase in thiobarbituric acid-reactive substances (TBARS), indicating elevated lipid peroxidation. Propolis treatment significantly enhanced antioxidant enzyme activities and reduced TBARS levels, highlighting its potent antioxidative properties. The combination group exhibited improved antioxidant defense mechanisms compared to the DEHP group, reinforcing the role of propolis in counteracting DEHP-induced oxidative damage..

Keywords: Propolis, Diethylhexylphthalate (DEHP), Semen Quality, Oxidative Stress, Male Rabbits.

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1. INTRODUCTION

Di(2-ethylhexyl) phthalate "DEHP" is a widely used plasticizer in the production of polyvinyl chloride "PVC" materials, found in various consumer products, including food packaging, medical devices, and cosmetics[1]. Due to its high environmental prevalence, "DEHP" exposure is a significant public health concern, particularly for its endocrine-disrupting properties[2]. Studies have demonstrated that "DEHP" interferes with male reproductive health by inducing oxidative stress, apoptosis, and hormonal imbalances, leading to reduced fertility and testicular dysfunction[3]. Several animal studies have highlighted the detrimental effects of "DEHP" on male reproductive function [4]. Research on male rats demonstrated that "DEHP" exposure significantly decreased sperm count, motility, and testosterone levels while increasing lipid peroxidation and oxidative stress markers in the testes[5]. Similarly, a study on male rabbits reported histopathological alterations in testicular tissue and a decline in reproductive hormone levels following "DEHP" administration[6]. These findings confirm that "DEHP" disrupts spermatogenesis and reproductive endocrinology through oxidative stress-mediated mechanisms [7]. Propolis, a natural resinous substance collected by honeybees from plant exudates, has been extensively

studied for its antioxidant, anti-inflammatory, and antimicrobial properties [8]. The bioactive compounds in propolis, such as flavonoids, phenolic acids, and terpenoids, contribute to its ability to mitigate oxidative damage and modulate immune responses[9]. In reproductive toxicology, propolis has been investigated as a protective agent against various environmental toxicants, showing promising effects in restoring sperm quality, testicular histology, and hormonal balance[10]. In contrast, propolis has shown protective effects against chemically induced reproductive toxicity in animal models [11]. A study on male rats treated with cadmium-induced testicular damage revealed that propolis supplementation significantly improved sperm parameters, reduced oxidative stress, and restored normal testicular histology[12]. Another investigation in rabbits demonstrated that propolis administration counteracted bisphenol A-induced reproductive toxicity by enhancing antioxidant enzyme activity and reducing apoptosis in testicular tissue[13]. These studies collectively suggest that propolis exerts a protective role against environmental toxicants affecting male reproductive health [14]. This study aims to evaluate the protective effects of propolis against reproductive toxicity induced by "DEHP" in male rabbits by assessing sperm quality, oxidative stress markers, and reproductive hormone levels

2. MATERIALS AND METHODS

Tested Compounds and Experimental Animals

This study utilized di-(2-ethylhexyl) phthalate (DEHP) and propolis as the primary tested compounds. DEHP (purity 99.0%) was procured from Sigma–Aldrich (USA), while propolis was obtained from California Health Products, Inc. (Los Angeles, CA, USA).

Experimental Animals

The study was conducted on mature male New Zealand White rabbits aged seven months, with an initial body weight of **2.917 ± 28.9 g.** The animals were individually housed in cages under controlled environmental conditions and were monitored weekly for body weight throughout the three-month experimental period. A total of twenty rabbits were randomly assigned into four experimental groups (n = 5 per group): **Group I (Control):** Rabbits received an equivalent volume of the vehicle (corn oil) by oral gavage daily for 12 consecutive weeks. **Group II (Propolis-treated):** Rabbits were administered propolis orally at a dose of **50 mg/kg body weight (B.W.),** dissolved in corn oil, for 12 weeks, following the method of [15]. **Group III (DEHP-treated):** Rabbits received **500 mg/kg B.W. of DEHP** daily via gavage, corresponding to **1/50th of its lethal dose** as described by [4]. **Group IV (DEHP + Propolis):** Rabbits were co-administered **500 mg/kg B.W. of DEHP** and **50 mg/kg B.W. of propolis** daily by gavage for 12 weeks. The administered doses of DEHP and propolis were adjusted weekly based on the animals' body weight measurements obtained during the preceding week.

Specimen Collection

Blood Sampling and Biochemical Analysis

Blood samples were collected biweekly from the ear vein of all rabbits in the morning before access to food and water. The samples were immediately placed on ice and divided into two tubes: One tube contained **heparin** to obtain **plasma**, which was separated via centrifugation at **860 × g for 20 minutes** and stored at **−80°C** until analysis. The second tube lacked anticoagulants to obtain **serum** for subsequent biochemical assessments. The stored plasma samples were analyzed for oxidative stress markers and antioxidant enzyme activities: **Glutathione S-transferase (GST; EC 2.5.1.18):** Determined using the method of [16]. **Catalase (CAT; EC 1.11.1.6):** Measured by the Luck method, which assesses the decomposition of hydrogen peroxide[17]. **Superoxide dismutase (SOD; EC 1.15.1.1):** Evaluated following the method of [18]. **Thiobarbituric acid-reactive substances (TBARS):** Quantified according to [19] .

Semen Analysis

Semen samples were collected weekly throughout the 12-week experimental period, resulting in a total of **60 ejaculates per treatment group.** Collection was performed using an **artificial vagina and a teaser doe.** The following semen characteristics were assessed: **Ejaculate Volume:** Measured in a graduated collection tube after gel mass removal. **Sperm Concentration:** Determined using a **Neubauer hemocytometer** slide (GmbH + Co., Hamburg, Germany) with a weak eosin staining method [20]. **Total Sperm Output:** Calculated as the product of ejaculate volume and sperm concentration. **Seminal Fructose Concentration:** Measured immediately post-collection following [21]. **Sperm Viability and Morphology:** Evaluated using **eosin-nigrosine blue staining** [22]. **Sperm Motility:** Assessed via visual inspection under **10× magnification** using a light microscope. Total motile sperm count was determined by multiplying the percentage of motile sperm with the total sperm output. **Reaction Time:** Measured in seconds, from exposure to a doe until the completion of erection. **Seminal pH:** Measured immediately post-collection using **pH indicator paper** (Universalindikator pH 0-14, Merck, Darmstadt, Germany). **Packed Sperm Volume (PSV):** Recorded. **Total Functional Sperm Fraction (TFSF):** Computed as the product of **total sperm output, motility (%), and normal morphology (%)**[23].

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 8 and Minitab software (version 17). Data normality was assessed before statistical analysis. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to evaluate significant differences among groups. A *P*-value of <0.05 was considered statistically significant

3. RESULTS

The results presented in Table 1 indicate significant variations in semen quality parameters among the different experimental groups. Propolis supplementation led to a notable enhancement in semen quality, whereas DEHP exposure resulted in marked deterioration. The combination of propolis and DEHP partially mitigated the negative effects of DEHP, demonstrating the potential protective role of propolis. Ejaculate volume was significantly higher in the propolis-treated group (0.92 ± 0.018 ml) compared to the control (0.64 ± 0.017 ml), indicating that propolis positively influences semen production. Conversely, DEHP exposure significantly reduced ejaculate volume (0.48 ± 0.018 ml), which suggests a detrimental impact on semen secretion. The combination group showed an intermediate value (0.80 ± 0.017 ml), indicating a partial protective effect of propolis. Similarly, sperm concentration followed the same trend, with the highest concentration observed in the propolis group (319 ± 7.2 x10^6/ml) and the lowest in the DEHP group (229 ± 4.7 x10^6/ml), suggesting that DEHP significantly impairs spermatogenesis. The TSO was significantly improved in the propolis group (299 ± 10.3 x10^6) compared to the control (172 ± 5.9 x10^6). DEHP exposure resulted in a substantial decrease (114 ± 5.6 x10^6), whereas co-treatment with propolis partially restored sperm output (201 ± 5.6 x10^6). Sperm motility followed a similar pattern, where propolis treatment significantly enhanced motility (73.5 ± 0.9%) compared to control (67.8 ± 0.7%). DEHP exposure severely reduced sperm motility (60.9 ± 0.9%), reinforcing its negative impact on sperm function. The combination treatment resulted in a sperm motility value (67.9 ± 0.7%) close to that of the control. TFSF was significantly increased in the propolis-treated group (184 ± 8.3 x10^6) while it was markedly reduced in the DEHP group (54 ± 3.3 x10^6). This finding suggests that propolis improves the proportion of functionally viable sperm. Similarly, the percentage of normal sperm was highest in the propolis group (86 ± 0.4%) and lowest in the DEHP group (77 ± 0.7%). DEHP exposure resulted in a significant increase in dead sperm percentage (37.5 ± 1.14%), which was significantly higher than the control (26.3 ± 0.82%). Propolis treatment significantly reduced dead sperm count (17.9 ± 1.07%), while co-administration with DEHP maintained an intermediate level (28.9 ± 0.69%). A similar trend was observed for abnormal sperm, with the DEHP group showing the highest percentage (71.7 ± 3.83%), whereas propolis-treated rabbits exhibited a significantly lower abnormal sperm percentage (41.3 ± 3.61%). Fructose levels were significantly higher in the propolis group (275 ± 4.0 mg/100 ml) compared to the control (258 ± 3.9 mg/100 ml). DEHP exposure significantly decreased initial fructose (199 ± 5.9 mg/100 ml), indicating an impaired energy supply for spermatozoa. Propolis co-administration with DEHP partially restored fructose levels (222 ± 3.4 mg/100 ml). Similarly, the initial semen pH was significantly elevated in the DEHP group (8.26 ± 0.034), whereas propolis reduced pH (7.44 ± 0.038). The combination group (7.67 ± 0.036) had a value closer to the control (7.58 ± 0.022), indicating a moderating effect of propolis. DEHP exposure significantly increased reaction time (5.65 ± 0.201 sec), suggesting a negative impact on libido. In contrast, propolis significantly reduced reaction time (2.98 ± 0.145 sec), indicating a potential enhancement in sexual behavior. The combination treatment (4.49 ± 0.107 sec) showed an intermediate value, implying partial protection by propolis against DEHP-induced sexual dysfunction.

Table 1. The overall means (±SEM) of semen quality of as affect by propolis, diethylhexylphthalate (DEHP) and/or their combination (means ± SE)

Items	Control	Propolis	DEHP	Propolis + DEHP
Ejaculates volume (EV; ml)	0.64± 0.017^{c}	0.92 ± 0.018^{a}	0.48 ± 0.018^{d}	0.80 ± 0.017^{b}
Sperm concentration (SC; x10^{6}/ml)	264 ± 6.5^{b}	319 ± 7.2^{a}	229 ± 4.7^{c}	255 ± 4.1^{b}
Total sperm count (TSO; x10^{6})	172 ± 5.9^{c}	299 ± 10.3^{a}	114 ± 5.6^{d}	201 ± 5.6^{b}

Sperm motility (SM ;%)	67.8± 0.7[b]	73.5± 0.9[a]	60.9± 0.9[c]	67.9± 0.7[b]
Total motile sperm per ejaculate (TMS; x10^6)	118 ± 4.3[c]	220 ± 8.8[a]	71 ± 3.9[d]	139 ± 4.4[b]
Total function sperm fraction (TFSF; x10^6)	94± 3.6[b]	184 ± 8.3[a]	54 ± 3.3[c]	109± 3.6[b]
Normal sperm (NS; %)	82± 0.3[b]	86± 0.4[a]	77± 0.7[d]	80± 0.3[c]
Dead sperm (DS; %)	26.3± 0.82[b]	17.9± 1.07[c]	37.5± 1.14[a]	28.9± 0.69[b]
Semen initial fructose (IF; mg/100 ml)	258 ± 3.9[b]	275± 4.0[a]	199 ± 5.9[d]	222 ± 3.4[c]
Live sperm (LS; %)	13.0±1.09[bc]	18.1±0.44[a]	10.1±0.69[c]	14.0±1.48[b]
Abnormal sperm (AbS; %)	60.05±3.12[b]	41.3±3.61[c]	71.7±3.83[a]	58.7±1.51[b]
Packed sperm volume (PSV; %)	15.1 ± 0.16[b]	18.1 ± 0.38[a]	12.65 ± 0.28[c]	14.3 ± 0.16b
Initial hydrogen ion concentration (pH)	7.58 ± 0.022[b]	7.44 ± 0.038[c]	8.26 ± 0.034[a]	7.67 ± 0.036[b]
Reaction time (RT; sec.)	3.55 ± 0.099[c]	2.98 ± 0.145[d]	5.65 ± 0.201[a]	4.49 ± 0.107[b]

Data are expressed as mean ± SE of 5 rabbit. Within each row, means with different superscript (a, b, c or d) were significantly different at $p<0.05$. Where means superscripts with the same letters mean that there is no significant difference ($p>0.05$).

Table 2. Presents key oxidative stress markers, including glutathione-related enzymes and antioxidant defense parameters. The results demonstrate that DEHP exposure disrupts redox homeostasis, while propolis supplementation enhances antioxidant capacity and mitigates oxidative damage. GSH levels were significantly lower in the propolis-treated group (4.55 ± 0.065 U/ml) compared to the control (4.98 ± 0.104 U/ml), suggesting increased utilization of GSH due to enhanced detoxification activity. DEHP exposure significantly elevated GSH (5.22 ± 0.086 U/ml), likely due to compensatory upregulation. Co-administration with propolis maintained an elevated GSH level (5.08 ± 0.087 U/ml), indicating partial restoration of redox balance. GPx activity was highest in the propolis-treated group (10.82 ± 0.230 U/ml), reflecting enhanced antioxidant defense. DEHP exposure significantly reduced GPx (9.03 ± 0.243 U/ml), suggesting oxidative stress-induced depletion. Co-administration with propolis improved GPx activity (10.39 ± 0.185 U/ml), supporting the protective effect of propolis. Similarly, GST activity followed the same trend, with propolis treatment enhancing GST levels (1.147 ± 0.036 µmol/hr), while DEHP significantly reduced GST activity (0.908 ± 0.019 µmol/hr). CAT activity was highest in the propolis group (1.120 ± 0.020 U/min/ml), whereas DEHP significantly suppressed CAT (0.598 ± 0.033 U/min/ml). Co-administration partially restored CAT levels (0.916 ± 0.013 U/min/ml). SOD activity followed a similar pattern, with propolis increasing SOD (1.054 ± 0.025 U/ml) and DEHP reducing it (0.853 ± 0.026 U/ml). Lipid peroxidation was significantly elevated in the DEHP-treated group (1.483 ± 0.061), indicating increased oxidative stress. Propolis significantly reduced TBARS levels (1.139 ± 0.016), while the combination treatment resulted in values close to the control (1.219 ± 0.037), confirming the protective role of propolis.

Table 2. Average of plasma glutathione (GSH; U/ml), glutathione peroxidase (GPx; U/ml) and glutathione S-transferase (GST; µmol/hr), glutathione, catalase (CAT; U/min/ml), superoxide dismutase (SOD; U/ml) and thiobarbituric acid-reactive substances (TBARS) of male rabbits treated with propolis, di-ethylhexylphthalate (DEHP) and/or their combination (means ± SE).

[abc] Means with different superscript letters within rows are significantly different (P<0.05).

Items	Control	Propolis	DEHP	Propolis + DEHP
Glutathione (GSH; U/ml)	4.98± 0.104[a]	4.55± 0.065[b]	5.22± 0.086[a]	5.08± 0.087[a]
Glutathione peroxidase (GPx; U/ml)	9.95± 0.171[b]	10.82± 0.230[a]	9.03± 0.243[c]	10.39± 0.185[ab]
Glutathione S-transferase (GST; µmol/hr)	1.010± 0.017[b]	1.147± 0.036[a]	0.908± 0.019[c]	1.032± 0.019[b]
Catalase (CAT; U/min/ml)	0.990 ± 0.018[b]	1.120 ± 0.020[a]	0.598 ± 0.033[c]	0.916± 0.013[b]
Superoxide dismutase (SOD; U/ml)	0.902 ± 0.022[b]	1.054 ± 0.025[a]	0.853 ± 0.026[b]	0.931± 0.021[b]
Thiobarbituric acid-reactive substances (TBARS)	1.223 ± 0.036[b]	1.139 ± 0.016[b]	1.483 ± 0.061[a]	1.219 ± 0.037[b]

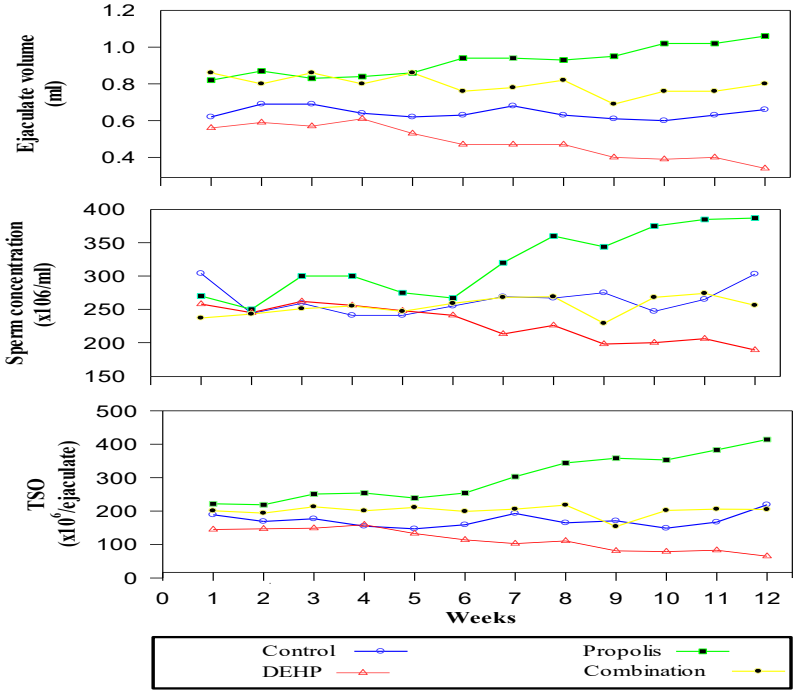

Figure .1. Changes in ejaculate volume, sperm concentration and total sperm output (STO) during treatment of male rabbits with propolis, di-ethylhexylphthalate (DEHP) and/or their combination.

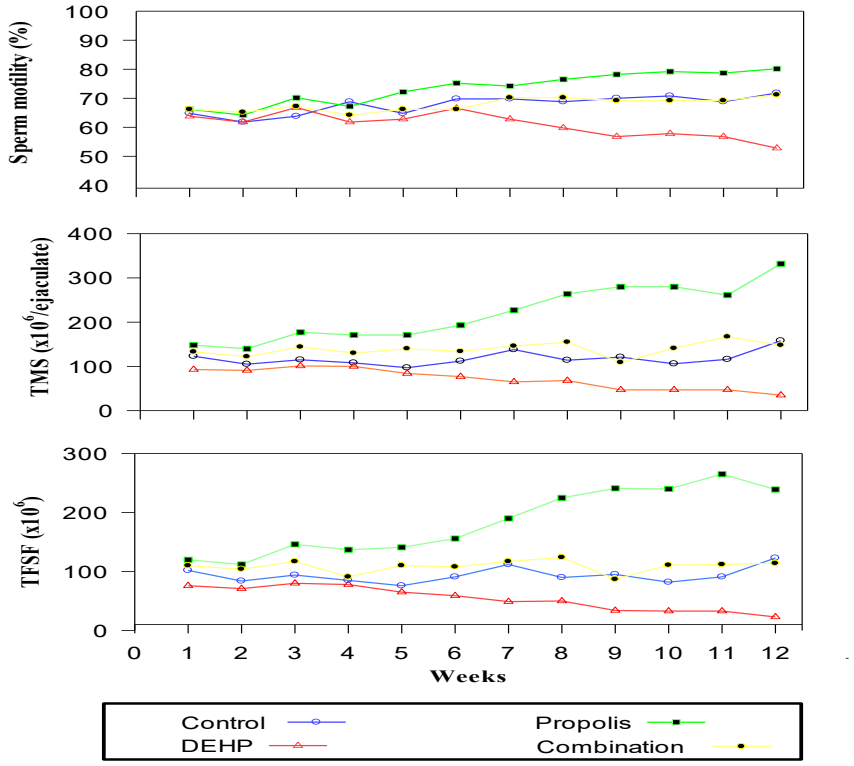

Figure .2. Changes in sperm motility, total motile (TMS) and total function sperm fracation (TFSF) during treatment of male rabbits with propolis, di-ethylhexylphthalate(DEHP) and/or their combination.

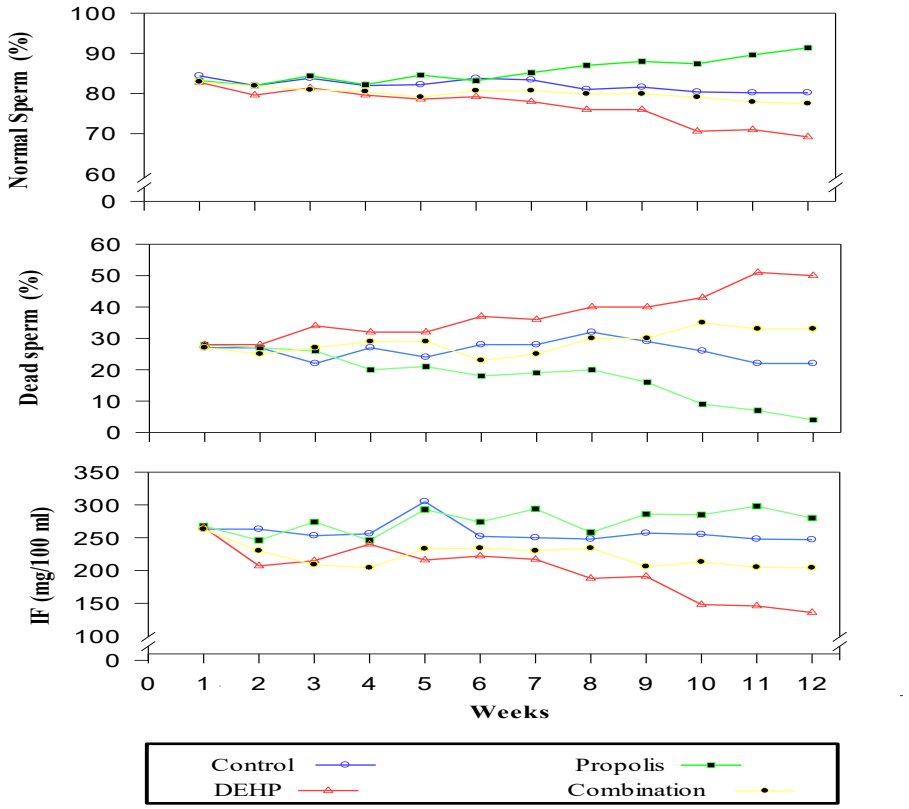

Figure 3. Changes in normal sperm, dead sperm and initial fructosen (IF) during treatment of male rabbits with propolis, di-ethylhexylphthalate(DEHP) and/or their combination.

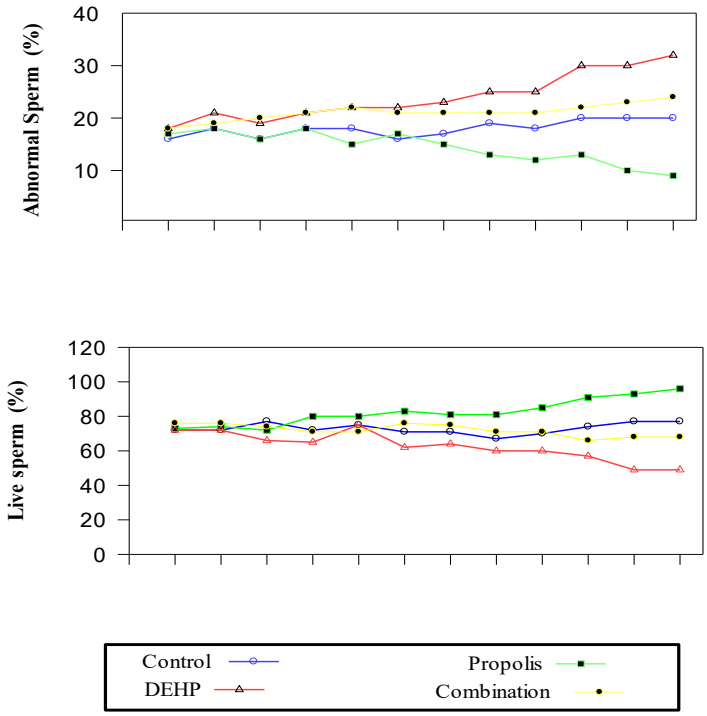

Figure 4. Changes in Abnormal sperm, Live sperm during treatment of male rabbits with propolis, di-ethylhexylphthalate(DEHP) and/or their combination.

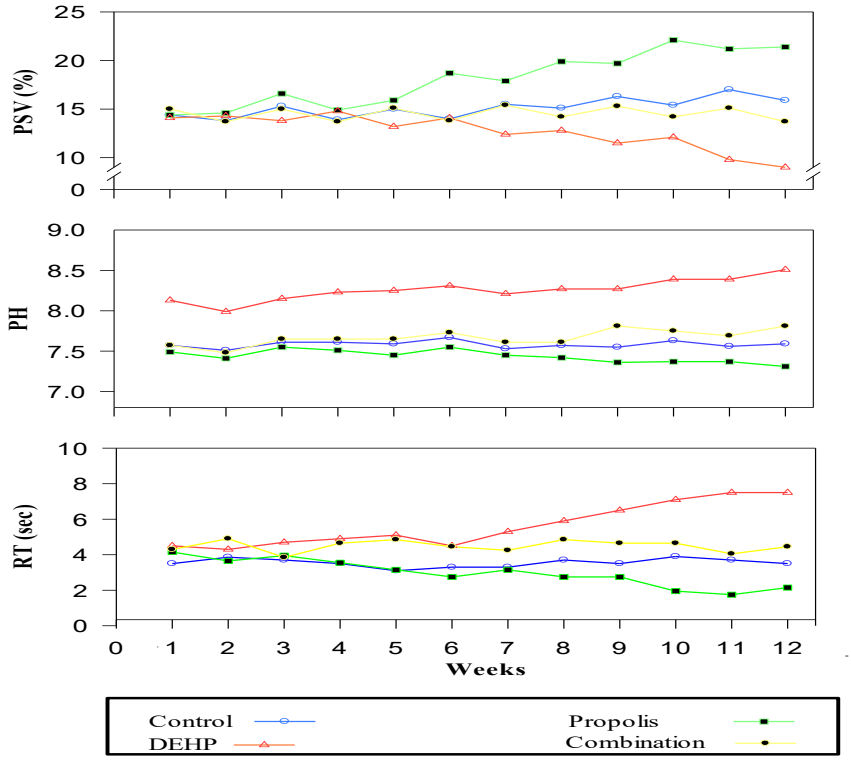

Figure 5. Changes in packed sperm volume, initial hydrogen ion concentration and reaction time during treatment of male rabbits with propolis, di-ethylhexylethylphthalate (DEHP) or combination.

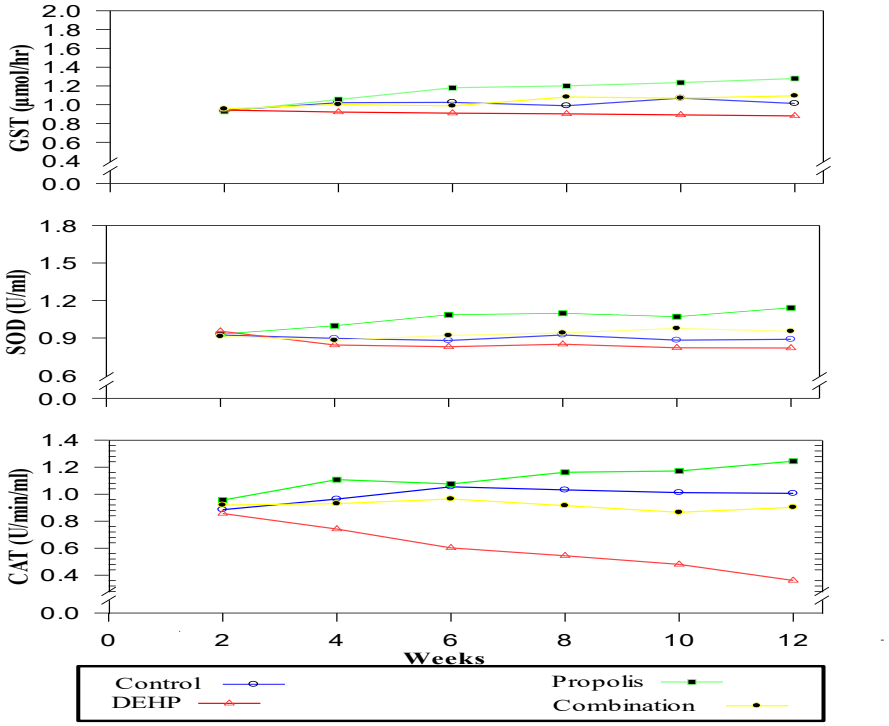

Figure 6. Changes in the activity of plasma glutathione S-transferase, superoxide dismutase and catalase during treatment of male rabbits with propolis, di-ethylhexylphthalate (DEHP) and/or their combination

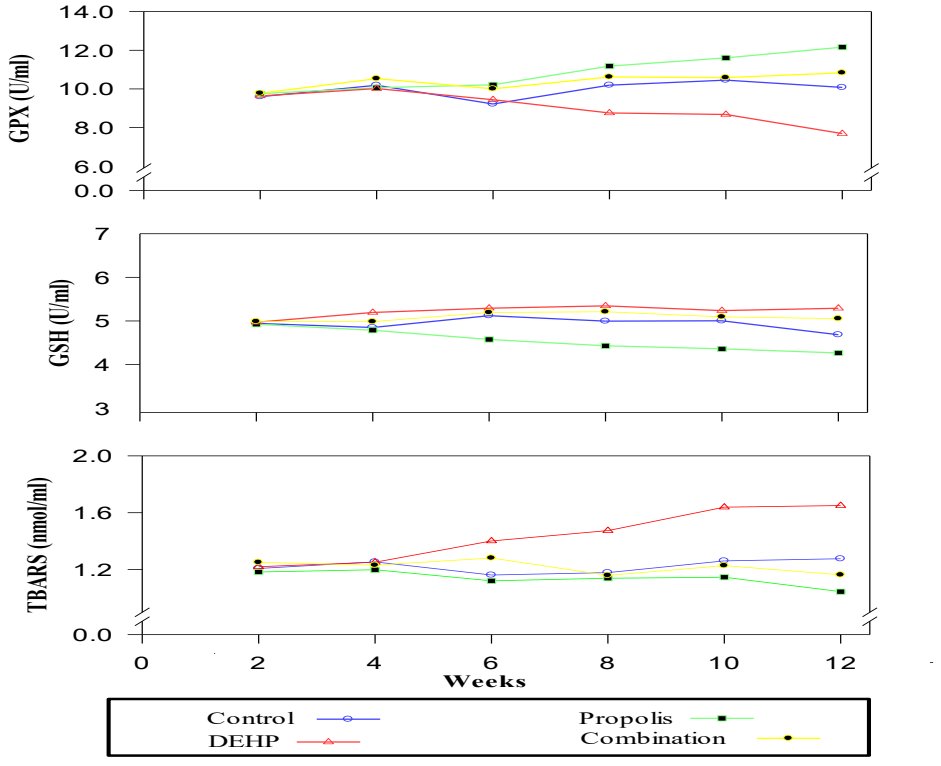

Figure7. Changes in the activity of plasma glutathione peroxidase (GPx), glutathione (GSH) and TBARS during treatment of male rabbits with propolis, Di-ethylhexylphthalate (DEHP) and/or their combination.

4. DISCUSSION

The results in Table 1 demonstrate that exposure to diethylhexyl phthalate (DEHP) significantly reduced semen quality, as

reflected in the decrease in ejaculate volume (EV), sperm concentration (SC), total sperm count (TSO), sperm motility (SM), and total functional sperm fraction (TFSF). The decline in these parameters suggests that DEHP exerts deleterious effects on spermatogenesis and sperm function. This finding aligns with previous studies indicating that DEHP disrupts endocrine function and impairs testicular steroidogenesis, leading to reduced sperm production and quality [24]. Conversely, propolis supplementation significantly improved semen parameters compared to the control group [25]. Propolis increased sperm concentration, motility, total sperm count, and functional sperm fraction, suggesting its protective role in enhancing male fertility. These improvements may be attributed to the antioxidant properties of propolis, which mitigate oxidative stress and lipid peroxidation in spermatozoa, thus preserving sperm integrity[26]. The combined treatment of propolis and DEHP showed partial restoration of sperm quality, suggesting that propolis counteracts some of DEHP's toxic effects but does not fully prevent its detrimental impact [27]. Sperm morphology and viability were significantly affected by DEHP exposure, as evidenced by increased abnormal sperm (AbS) and dead sperm (DS) percentages, coupled with a significant decrease in normal sperm (NS) and live sperm (LS) percentages. These observations support previous findings that phthalates induce testicular toxicity by disrupting the Sertoli cell function and impairing sperm maturation[28]. Propolis treatment significantly reduced the percentage of abnormal sperm and dead sperm while increasing the proportion of normal and live sperm, indicating its protective role against sperm damage. This aligns with previous studies showing that propolis exerts a cytoprotective effect on sperm cells by enhancing antioxidant defense mechanisms [29]. The combined group (propolis + DEHP) exhibited intermediate values, suggesting that while propolis alleviates DEHP-induced toxicity, it does not completely reverse its adverse effects. Seminal fructose, an indicator of seminal vesicle function, was significantly reduced in the DEHP group, suggesting impaired seminal fluid production. This reduction in seminal fructose may be linked to DEHP's endocrine-disrupting effects, which compromise androgen-dependent accessory gland function [30]. Propolis supplementation significantly increased seminal fructose levels, indicating improved seminal vesicle function, likely due to its androgen-enhancing and antioxidant properties[31]. The propolis + DEHP group showed an intermediate response, reinforcing the partial protective effect of propolis against DEHP toxicity. Similarly, semen pH was significantly elevated in the DEHP group, while propolis supplementation maintained a physiological pH. Elevated pH in the DEHP group suggests an imbalance in the seminal plasma environment, potentially contributing to sperm dysfunction[32]. The restoration of normal pH values by propolis further supports its role in maintaining sperm viability and function [33]. The findings in Table 2 reveal that DEHP exposure significantly disrupted the antioxidant defense system in male rabbits, as evidenced by reduced levels of glutathione peroxidase (GPx), glutathione S-transferase (GST), catalase (CAT), and superoxide dismutase (SOD), along with elevated thiobarbituric acid-reactive substances (TBARS), an indicator of lipid peroxidation. These results are consistent with previous studies reporting that DEHP induces oxidative stress by generating reactive oxygen species (ROS), which damage cellular membranes and impair antioxidant enzyme activity[34]. Propolis treatment significantly enhanced antioxidant enzyme activity and reduced TBARS levels, demonstrating its potent antioxidant properties. The improvement in antioxidant status aligns with prior research indicating that propolis contains flavonoids and polyphenols, which scavenge free radicals and upregulate antioxidant enzyme expression[11]. The propolis + DEHP group exhibited partial restoration of antioxidant defense, suggesting that propolis mitigates, but does not fully counteract, DEHP-induced oxidative damage.

5. CONCLUSION

DEHP exposure negatively impacted semen quality and oxidative stress parameters, whereas propolis supplementation significantly improved reproductive function and antioxidant status. The partial recovery observed in the combination group suggests that propolis may mitigate DEHP-induced reproductive toxicity through its antioxidant properties. These findings provide valuable insights into the protective role of propolis against environmental toxins affecting male fertility

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