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ORIGINAL RESEARCH

Protecting Spermatogenesis from Doxorubicin-Induced Damage: The Effects of *Prunus laurocerasus* on Oxidative Stress in an Animal Model

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Abstract

Objective: A side effect of chemotherapy is its tendency to increase oxidative stress, which can lead to infertility. This study examines the potential of *Prunus laurocerasus* fruit extract (PLFE) to reduce Doxorubicin (DOX)-induced oxidative stress and preserve sperm production.

Material-Method: Rats received daily doses of PLFE for 15 days, with DOX administered on day 13. Control groups had no treatment, while damage groups received only DOX. The protective effects of high (HD) and low (LD) doses of PLFE against DOX-induced damage were assessed by evaluating sperm count, motility, dead sperm ratio, and oxidative stress markers.

Results: PLFE significantly improved sperm concentration and reduced dead sperm percentage in both LD and HD groups compared to the DOX group ($p<0.05$), with no significant difference between LD and HD ($p>0.05$). Superoxide dismutase (SOD) activities and glutathione increased while malondialdehyde (MDA) decreased in PLFE groups compared to DOX ($p<0.05$) though these markers remained and couldn't reach the control levels ($p<0.05$). Catalase (CAT) activities did not differ between the HD+DOX and DOX groups ($p>0.05$), but the LD+DOX group showed an increase ($p < 0.05$ vs DOX), though lower than controls ($p<0.05$).

Conclusion: *P. laurocerasus* and its active compounds could be targets for developing treatments to reduce oxidative stress, preserve fertility, and improve reproductive success post-chemotherapy. Further studies are needed to adjust the application duration and dose, understand the mechanism of action, and clarify the limited impact on CAT activity, which suggests selective pathway activation in antioxidant defense.

Keywords: Doxorobucin, Phytotherapy, Spermatogenesis, Oxidative Stress, Chemotherapy, Infertility

INTRODUCTION

Male infertility has many physiological, genetic, and environmental causes[1,2]. Oxidative stress is a key factor in most cases of idiopathic male infertility. It plays a vital role in redox metabolism, which underpins essential processes from bioenergetics to metabolism, with mild oxidative stress being crucial for sperm cell capacitation[3–6]. Various environmental toxins and pathological processes can accumulate reactive oxygen species (ROS) at levels detrimental to general health and fertility[2,6]. Subfertility, defined as reduced fertility or subsequent secondary infertility, is also largely associated with oxidative stress and living conditions[3,7]. Sperm cell membranes contain a high concentration of unsaturated fatty acids[8], making them particularly susceptible to the harmful effects of ROS. Chemotherapeutic agents can negatively affect spermatogenesis by causing increased oxidative stress, impacting patients' quality of life post-treatment[9,10].

Doxorubicin (DOX) has been extensively used for the treatment of various tumors over the past 40 years. Despite its effectiveness in combating tumors, DOX induces significant oxidative stress in non-

target tissues, including the testes, making them susceptible to damage and highlighting the need to consider potential complications when administering this medication[11–15]. DOX can dramatically suppress spermatogenesis, though its precise impact on testicular tissue remains unclear[16–19]. Several studies have suggested that lipid peroxidation and apoptosis may play crucial roles in this toxicity[20,21]. High doses of DOX are used in experiments to replicate oxidative stress-induced damage in tissues[22,23]. The impact of various antioxidants in preventing this damage has been extensively researched[23–26].

Prunus laurocerasus (PL), also known as *Laurocerasus* officinalis, is a perennial evergreen tree from the Rosaceae family[27]. Found in the Black Sea region of Turkiye, this fruit is commonly referred to as "Taflan" and "Karayemiş". Although it is commonly eaten as a fresh fruit, it is also dried or boiled and consumed as jam or molasses. Its fruits, seeds, and leaves are used in traditional medicine, especially for diabetes[28]. Various extracts of Prunus are rich in phenolic and polyphenolic compounds, such as chlorogenic, coumaric, gallic, benzoic, and caffeic acids, and demonstrate significant antioxidant capacity, enhancing their pharmaceutical value[27,29–34].

Previous studies have shown the effects of different Prunus species on testicular tissue[35,36] and cancer cells[37,38]. Limited research exists on the protective effects of PL against chemotherapy side effects and its specific influence on spermatogenesis. This study addresses the existing research gap by examining the potential protective effects of PLFE on DOX-induced testicular toxicity.

MATERIALS AND METHODS

Extracts

Fruits harvested from the Central Black Sea region of Turkiye were extracted in ethanol, and the antioxidant capacity and reducing power were determined, as described in our previous study[12]. The PLFE was diluted to two different concentrations suitable for low-dose (LD) and high-dose (HD) applications and administered to the experimental animals as described below.

Animal model and experimental procedures

The Animal Ethics Committee at Ordu University approved all experimental protocols and animal procedures (decision dated 30th March 2020, reference number 14). The study strictly adhered to the established guidelines for the Care and Use of Laboratory Animals. Twenty-eight male Sprague-Dawley rats weighing 280±30 grams were acquired from OMÜ DEHAM. The animals were acclimatized for one week at the Ordu University Experimental Animal Research and Application Laboratory under controlled conditions (25±1°C temperature, 55% relative humidity, and a 12-hour light/dark cycle) to ensure their health and adaptation to the environment and randomly assigned to four distinct groups. Each group, comprising seven rats (n=7), received a specific pharmacological treatment. We provided the rats with routine care and feeding for 15 days with ad libitum access to food and water. Application doses of DOX and PLFE were selected based on previous studies[22,28,31,39], with the high dose (HD) confirmed as non-toxic in our earlier paper[12].

Group-1 (CTR): A healthy control group was given intragastric (I.G.) water for 15 days.

Group-2 (DOX): The damaged group was given water (I.G.) for 15 days and administered 15 mg/kg DOX intraperitoneally (I.P.) on the 13th day of the experiment.

Group-3 (LD+DOX): The treatment group was given a low dose (500 mg/kg) PLFE (I.G.) for 15 days and administered 15 mg/kg DOX (I.P.) on the 13th day of the experiment.

Group-4 (HD+DOX): The treatment group was given a high dose (1000 mg/kg) PLFE (I.G.) for 15 days and administered 15 mg/kg DOX (I.P.) on the 13th day of the experiment.

We administered gavage applications of 2-3 ml per dose to the animals at the same time every morning. On the 15th day of the experiment, the animals were euthanized under general anesthesia with intraperitoneal injections of 80 mg/kg Ketamine and 10 mg/kg Xylazine. Tissue samples were then collected for analysis.

Tissue collection and preparation

Following a thorough rinse with phosphate-buffered saline (PBS) to remove contaminants. Tissue samples were weighed using a precision balance, and the measurements were carefully documented. The cauda epididymis and testes were collected in a separate dish for sperm analysis (Figure 1). Testes were stored at -80°C in a deep freezer to evaluate oxidative stress and molecular studies. Relative testis weights (RTW) were calculated as a ratio to the final body weights.

Sperm analysis

Sperm count in the epididymis was determined using a modified version of the method developed by Kenjale et al.[40]. Briefly, the unilateral cauda epididymis was minced in 10 ml PBS in glass Petri

dishes using anatomical scissors and incubated at 37°C for 10 minutes with gentle shaking. The supernatant was diluted 10 times with PBS at room temperature. A volume of 10 microliters of the diluted sperm sample was carefully pipetted onto a hemocytometer. After allowing it to settle for 5 minutes, the sperm cells were counted using a light microscope. During the total count, the motile and stationary sperm were recorded. The percentage of motile sperm was then calculated by dividing the number of motile sperm by the total number of sperm and multiplying by 100 to obtain the percentage.

The Eosin-Nigrosin method [41] was employed to perform the dead sperm ratios. Accordingly, 20 µL of sperm suspension was treated with a standard solution containing 1% eosin Y and 5% nigrosin (GBL, Turkiye) and then dropped onto microscope slides and covered with a coverslip. The prepared slides were subsequently examined using light microscopy. In total, at least 200 sperm were counted on each slide, and the ratios of live (transparent) and dead (purple-stained) spermatozoa were calculated as percentages (%).

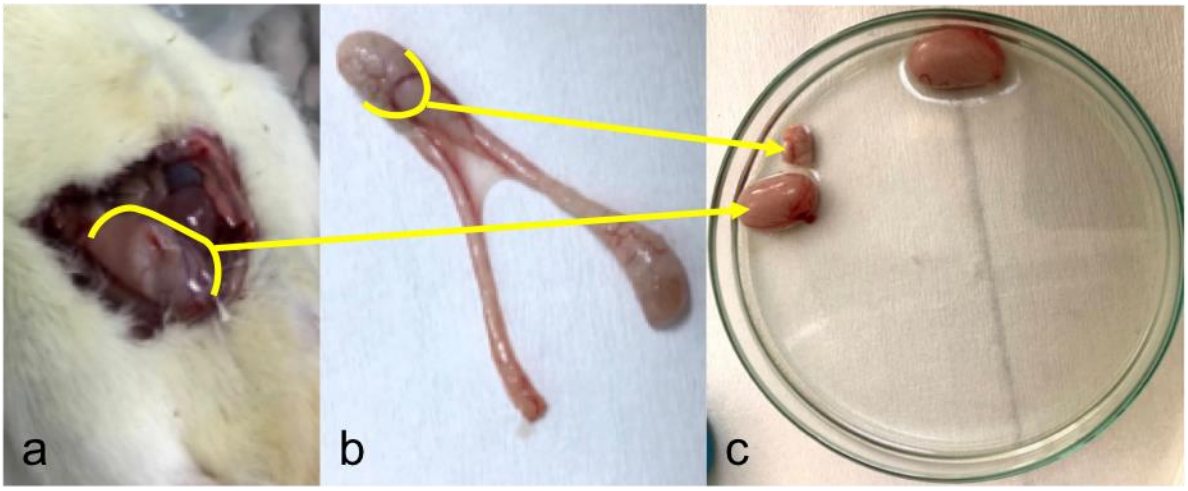

Figure 1. (a) Removal of tissues from a rat under anesthesia, (b) caput, corpus, and cauda epididymis with vas deferens, (c) tissue preparation on a 10 mm petri dish.

Assessment of oxidative stress in tissues

To quantify the extent of oxidative stress in the tissue, we conducted analyses for malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) activities using the prescribed methodologies.

MDA assay

The quantification of malondialdehyde (MDA) in the tissue was conducted using the thiobarbituric acid reactive substances (TBARS) method, a widely utilized technique for assessing lipid peroxidation and oxidative stress[42]. The tissue samples were weighed and homogenized in trichloroacetic acid (TCA) using a homogenizer. The supernatant was collected, followed by adding thiobarbituric acid (TBA) and butylated hydroxytoluene (BHT). The samples' optical density was measured at 535 nm using a spectrophotometer against a blank.

GSH assay

GSH in the tissue was measured using the modified Ellman method[43]. Tissue samples were efficiently homogenized and centrifuged. The supernatant was then combined with a solution of NaH2PO4 and 5,5′-dithiobis-(2-nitrobenzoic acid), followed by an incubation at room temperature for 5-10 minutes. The absorbance of the solution was quantified at a specific wavelength of 412 nm using a spectrophotometer, while a blank solution was used as the reference for calibration[44].

SOD activity

The method outlined by Sun et al. was employed to measure the superoxide dismutase (SOD) activity in the tissue[45]. The process entails generating the superoxide radical by utilizing xanthine and xanthine oxidase. This radical then transforms nitroblue tetrazolium into a red-colored formazan chromogen. The analysis was conducted using a spectrophotometer. The activity level was determined by measuring the intensity of the red color produced in the solution at a wavelength of 505 nm[45].

CAT activity

The classical method developed by Aebi[46] was used to determine CAT activity in this study. CAT is a

vital enzyme that catalyzes the breakdown of hydrogen peroxide (H2O2) into water and molecular oxygen, which is crucial in protecting cells from oxidative damage. The principle is based on monitoring the decrease in H2O2 concentration over time at 240 nm spectrophotometrically [46].

Statistical analysis

We conducted several statistical analyses to evaluate the data. Initially, the sample size in each group was determined through power analysis to ensure adequate statistical power for detecting significant effects. Data normality was assessed using the Kolmogorov-Smirnov test. Subsequently, a one-way analysis of variance (ANOVA) was employed to compare the means across the groups. Post-hoc comparisons between treatment groups were performed using Tukey's test to identify specific group differences. Pearson's correlation coefficient was calculated to examine the linear relationships between oxidative stress and sperm parameters, with corresponding p-values indicating statistical significance. A threshold of $p < 0.05$ was set to denote significance. All data are expressed as mean ± standard deviation. Statistical analyses were carried out using SPSS software (version 25), and results were visualized with Python (version 13.12.4).

RESULTS

Body and reproductive organs weights

The body weights of the experimental animals showed no significant differences between the groups ($p>0.05$) at the beginning and end of the experiment. The DOX group had a significantly lower relative testis weight (RTW) of 0.84 ± 0.58 compared to the control group's Relative testis weight (RTW)of 1.01 ± 0.05 ($p<0.05$). The groups receiving extracts (LD+DOX and HD+DOX) had RTWs of 1.05 ± 0.16 and 0.99 ± 0.11, respectively, both significantly higher than the DOX group ($p<0.05$). There was no significant difference between the RTWs of the extract and the control groups ($p>0.05$) (Table 1).

Table1. Animal's initial body weight (IBW), final body weight (FBW), and relative testis weight (RTW)

Group	IBW	FBW	RTW (%)
Control	259.67 ± 7.39	278.17 ± 14.19	1.01 ± 0.05 #
DOX	283.29 ± 16.79	273.00 ± 18.31	0.84 ± 0.58*
LD+DOX	283.33 ± 6.05	271.67 ± 16.52	1.05 ± 0.16#
HD+DOX	284.17 ± 23.96	280.67 ± 29.1	0.99 ± 0.11#

(n=7 for all groups; one-way ANOVA followed by Tukey's post-hoc test, values are means ± SDs; statistically significant differences *p<0.05 vs. the control; # p<0.05 vs. the DOX). Formula of RTW= [(Left testis+ Right testis weigts)/FBW]X100)

Sperm parameters

The sperm parameters for the control, DOX, LD+DOX, and HD+DOX groups were assessed. The control group exhibited the highest sperm concentration, motility, and the lowest percentage of dead sperm. In contrast, the DOX group showed significantly reduced sperm concentration and motility, along with a significantly higher percentage of dead sperm ($p<0.05$ for both parameters compared to the control group).

The groups receiving extracts (LD+DOX and HD+DOX) showed varied improvements in sperm parameters compared to the DOX group. Both extract groups had higher sperm concentration and motility ($p<0.001$ for both) and a lower percentage of dead sperm ($p<0.05$) than the DOX group. However, these values were still significantly different from the control group ($p<0.05$). The detailed values and statistical significance are presented in Table 2.

Table 2. Sperm parameters. Epididymal sperm concentration, motility, and dead sperm rates among the groups.

Group	Epidydimal Sperm Concentration (mil/ml)	Motility (%)	Dead Sperm (%)
Control	65.30 ± 2.9#	68.92 ± 12.12#	29.58 ± 4.22 #
DOX	23.98 ± 2.17*	41.08 ± 8.75*	58.17 ± 8.06 *
LD+DOX	41.85 ± 3.3*#	49.25 ± 4.17*	39.5 ± 1.64*#
HD+DOX	43.00 ± 4.3*#	51.58 ± 4.04*	39.08 ± 2.08*#

(n=7 for all groups; one-way ANOVA with Tukey's post-hoc test, values are means ± SDs; statistically significant differences *p<0.05, **p<0.01 vs. the control; and # p<0.01 vs. the DOX).

Oxidative stress parameters

As shown in Figure 2, the parameters assessed for evaluating oxidative stress in testicular tissue indicated that GSH, SOD, and CAT activities were significantly lower in the DOX group compared to the control group ($p<0.05$). At the same time, MDA levels were significantly higher ($p<0.05$), highlighting the testicular damage induced by our

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experimental model.

MDA levels were lowest in the control group (249.30 nmol/g) and highest in the DOX group (471.6 nmol/g) ($p < 0.01$). The MDA levels in the LD+DOX and HD+DOX groups were 376.87 nmol/g and 365.05 nmol/g, respectively. These levels were significantly lower than the DOX group ($p<0.05$) but higher than the control group ($p<0.05$ for both) (Figure 2a).

The control group had the highest average GSH level at 6.58 µmol/g, while the DOX group had the lowest at 1.58 µmol/g ($p<0.05$). The LD+DOX and HD+DOX groups exhibited GSH levels of 3.53 µmol/g and 3.73 µmol/g, respectively. The GSH level in the extract groups was significantly different from the control and DOX groups ($p<0.05$ for both). However, there was no significant difference between the two extract groups ($p>0.05$) (Figure 2b).

Similar to GSH, SOD activities were highest in the control group at 4.87 U/mg and lowest in the DOX group at 1.49 U/mg ($p<0.05$ control vs DOX). For LD+DOX, this value was 2.35 U/mg, while for HD+DOX, it was 2.57 U/mg. The LD+DOX group showed a significantly higher outcome than the DOX group ($p<0.05$). Additionally, the HD+DOX group also exhibited a significant increase relative to the DOX group ($p<0.05$) (Figure 2c).

The DOX group also had significantly lower CAT activity than the control group ($p<0.05$). The HD+DOX group did not show a significant difference compared to the DOX group ($p>0.05$) but remained lower than the control group ($p<0.05$). The LD+DOX resulted in a significant increase compared to the DOX group ($p<0.05$). The highest CAT activity was observed in the control group at 5.21 µmol H2O2/min/mg, decreasing progressively in the LD+DOX, HD+DOX, and DOX groups (3.67, 3.47, and 2.90 µmol H2O2/min/mg, respectively). No dose-dependent differences were observed between the extract groups ($p > 0.05$; Figure 2d).

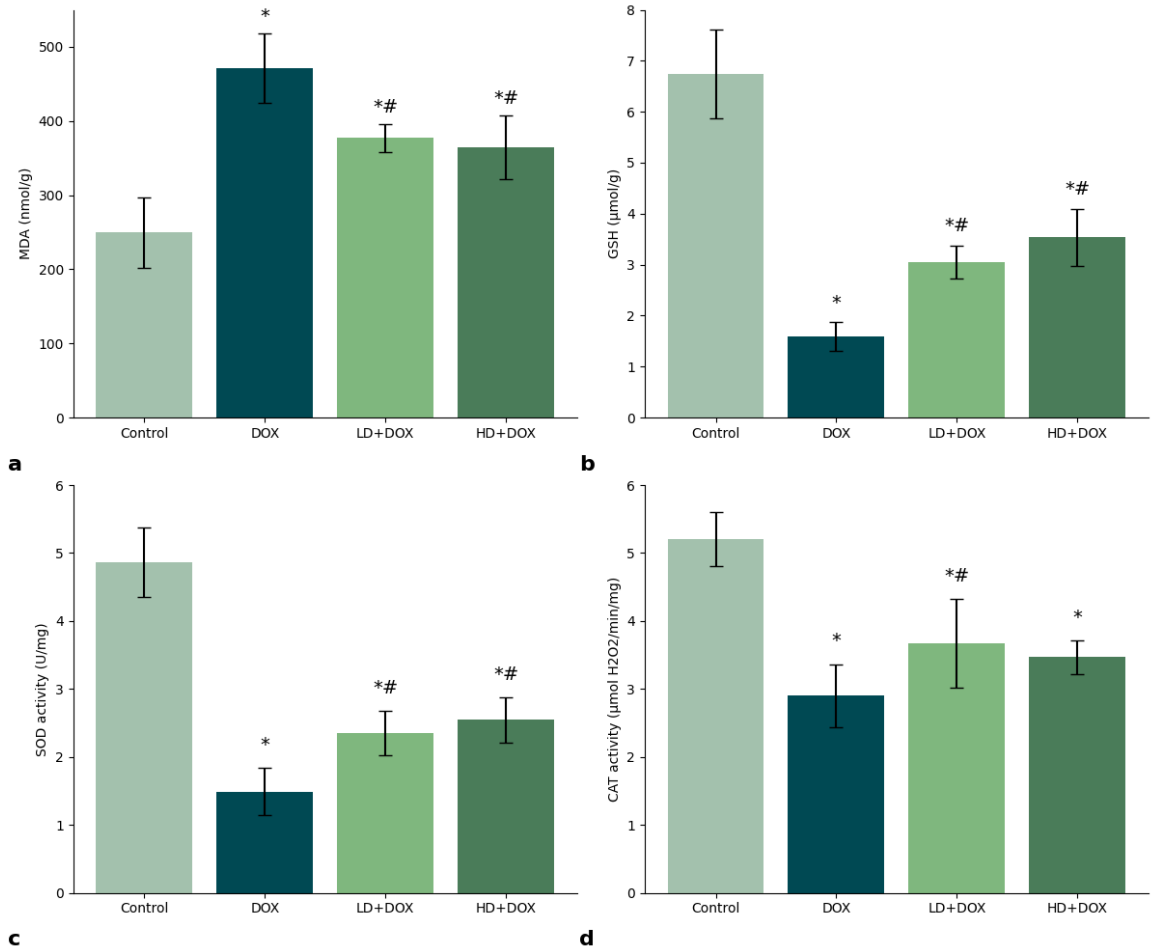

Figure 2. Testicular tissue levels of MDA (a), GSH (b), SOD activity (c), and CAT activity (d) in Control, DOX, LD+DOX, and HD+DOX groups. Data are mean ± SD (n=7). Statistical significance was determined using one-way ANOVA, followed by Tukey's post-hoc test (* $p<0.05$ vs. Control; # $p<0.05$ vs. DOX).

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Correlations with oxidative stress markers and sperm parameters

The correlation analysis revealed significant relationships between oxidative stress markers and sperm parameters. MDA showed a strong negative correlation with sperm count ($r = -0.93$, $p < 0.001$), suggesting that higher MDA levels are linked to lower sperm counts. GSH showed a positive correlation with sperm motility ($r = 0.71$, $p < 0.001$), while SOD was positively correlated with sperm vitality ($r = 0.73$, $p < 0.001$). Additionally, CAT demonstrated a slight positive correlation with sperm count ($r = 0.84$, $p < 0.001$) (Figure 3).

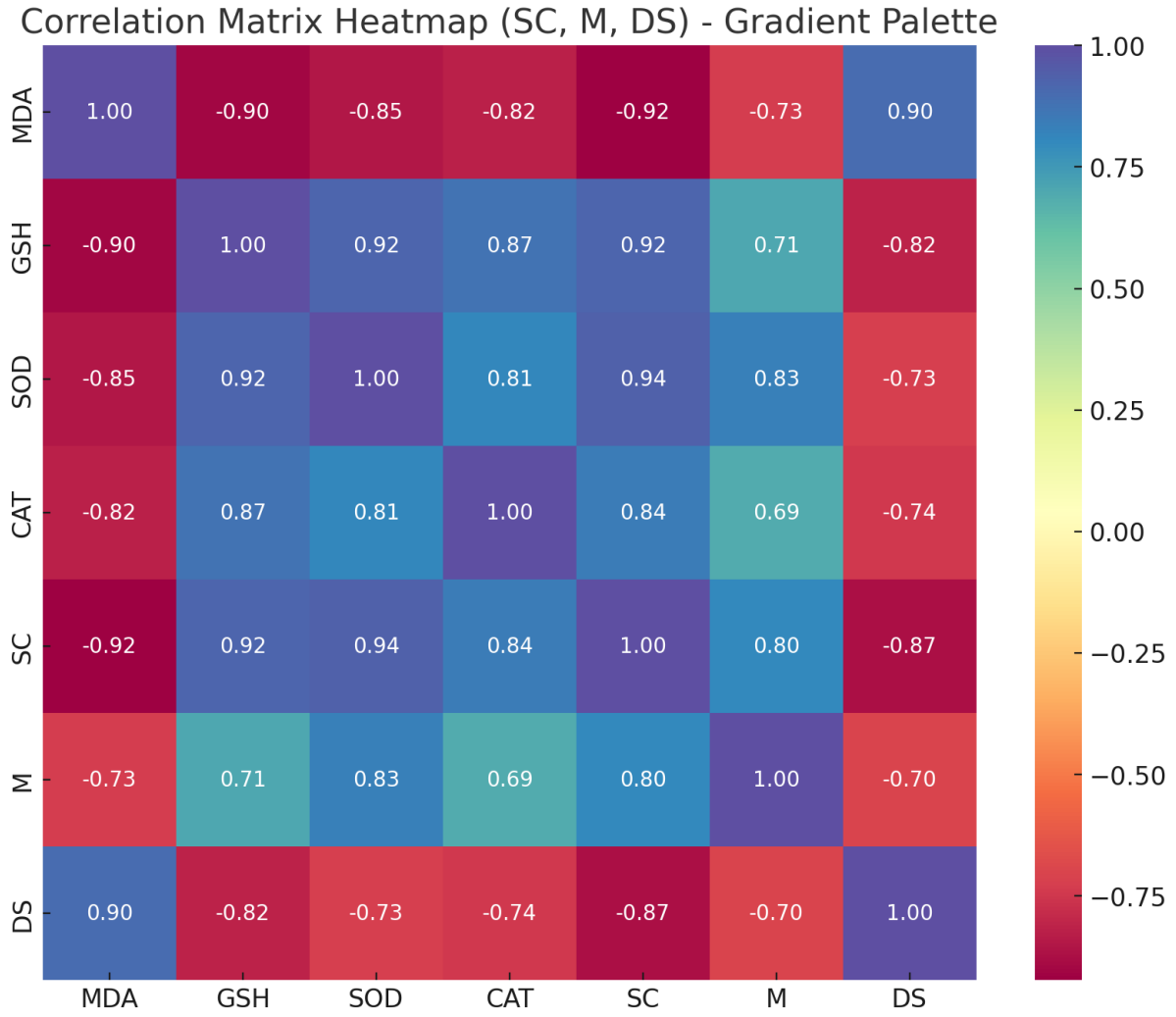

Figure 3. Heatmap showing the correlation matrix between oxidative stress markers (MDA, GSH, SOD activity, CAT activity) and sperm parameters (SC: Epididymal Sperm Count, M: Motility, DS: Dead sperm ratio). The color gradient ranges from cooler tones (blue) to warmer tones (burgundy), providing a clear visual representation of positive and negative correlations (Pearson's correlation coefficient).

DISCUSSION

Chemotherapeutic agents, such as doxorubicin (DOX), are known to induce oxidative stress, which disrupts spermatogenesis and can lead to secondary infertility, a significant concern for young men undergoing cancer treatment[9]. This study investigates the potential therapeutic impact of PLFE on DOX-induced reproductive toxicity, specifically focusing on oxidative stress and spermatogenesis.

This study underscores the significant reproductive toxicity induced by the chemotherapeutic agent DOX, emphasizing its potential to disrupt spermatogenesis, reduce sperm quality, and induce secondary infertility. Specifically, DOX exposure at 15 mg/kg was shown to cause testicular weight reduction, decreased sperm parameters, and increased oxidative stress markers within just two days post-administration, consistent with prior

research findings[17,24,25]. These detrimental effects highlight an urgent need to identify supportive therapies to protect reproductive function during chemotherapy, especially for younger male cancer patients at risk of infertility. The experimental outcomes demonstrated a promising protective effect of PLFE against DOX-induced oxidative stress.

Flavonoids, phenolic acids, and chlorogenic acids are powerful plant-based antioxidants that counteract oxidative stress in infertility[47,48]. By scavenging free radicals and reducing lipid peroxidation, phytochemicals help preserve sperm DNA integrity and cell membrane stability, offering therapeutic benefits against DOX toxicity[14,15,22,25,49].

Various PL extracts, well-recognized for their polyphenolic compounds, have demonstrated organ-protective effects across multiple studies, including against hepatic[12,50], renal[12,51], cardiac[13], and gastric[52] damages. Additionally, only one study specifically investigated Prunus laurocerasus fruit extract (PLFE) in relation to spermatogenesis[53]. They reported that a 7 mg/kg dose of PLFE administered over 8 weeks reduced pesticide-induced reproductive damage, improving sperm parameters by mitigating DNA damage and apoptosis. Nevertheless, full recovery in sperm concentration and motility was not achieved, which is similar to our findings. In our study, higher PLFE doses showed significant improvements in DOX-induced damage but did not normalize MDA levels, unlike Bakır et al.'s results with dimethoate[53].

The results of our study reveal that PLFE positively impacted sperm parameters by improving sperm concentration, motility, and vitality (Table 2). The administration of PLFE led to significant improvements in oxidative stress markers (Figure 2), with increases in glutathione (GSH) and superoxide dismutase (SOD) activities and reductions in malondialdehyde (MDA) levels. As indicated by MDA, the notable reduction in lipid peroxidation and a boost in key antioxidant enzymes for spermatogenesis[3–5] suggests PLFE's potential in counteracting ROS-induced cellular damage in reproductive tissues.

Interestingly, while PLFE was effective in enhancing GSH and SOD levels ($p<0.05$ LD+DOX and HD+DOX vs DOX for both parameters), it did not produce a dose-dependent increase in CAT activity ($p>0.05$ between LD+DOX and HD+DOX; Figure 2). This suggests that PLFE may exert selective antioxidant effects, primarily supporting the GSH and SOD pathways while having a more limited impact on the CAT pathway. This raises the question of whether PLFE's active compounds reach a functional threshold for CAT activity. Aitken and Drevet[6] emphasized catalase's essential role in preserving sperm motility by scavenging hydrogen peroxide, which supports our findings. However, in our study, the lower CAT activity in the PLFE-treated groups may have contributed to motility not reaching the control level.

The study's correlation analysis reinforces the role of oxidative stress in male fertility. A strong negative correlation between lipid peroxidation and sperm parameters—specifically, sperm count and vitality—highlights the detrimental impact of ROS on sperm cell membrane integrity. In contrast, the positive correlations of GSH and SOD with sperm count and motility reflect their protective roles against oxidative stress (Figure 3). These findings emphasize the importance of a balanced antioxidant environment in protecting spermatogenesis and overall sperm quality. Elevated ROS levels can impair the fluidity and permeability of sperm cell membranes[6], leading to decreased functionality. Additionally, these ROS levels disrupt energetic and mitochondrial processes[7,15] through genetic[15,18] and epigenetic[19] mechanisms, contributing to increased cell death[21]. DOX and natural compounds are known to modulate numerous molecular mechanisms[39,54,55], including oxidative stress response pathways such as NF-κB[56] and Nrf2[57–59], thereby enhancing antioxidant defenses and regulating inflammatory and apoptotic signals related to reproduction. In this context, it can be said that PLFE exerts its protective effects through these molecular pathways. However, the application of PLFE for a specific time interval (matching the duration of spermiogenesis) and the absence of molecular or histological analysis in our study limits our ability to elucidate the protective mechanisms of PLFE on spermatogenesis fully. Future studies should incorporate molecular-level investigations to identify the specific pathways targeted by PLFE's active compounds, allowing for a more comprehensive understanding of its protective effects.

Despite these encouraging results, several key points warrant further research. Notably, while PLFE improved oxidative stress markers and sperm parameters, it did not fully restore them to control levels. This partial improvement suggests that while PLFE has protective properties, it may not be a standalone therapeutic approach for mitigating chemotherapy-induced damage. Instead, optimizing

the dose and duration of PLFE treatment may be critical in maximizing its therapeutic benefits. Hormesis, wherein moderate stress levels induce protective effects but higher doses yield diminishing returns[60], might explain the observed plateau in PLFE efficacy at higher doses, particularly concerning CAT activity. Lower doses could activate endogenous antioxidant responses more effectively, while higher doses might lead to enzyme saturation or limited additional benefits. It should also be noted that certain flavonoids may inhibit CAT activity[61], which could explain the reduction in CAT activity observed at higher doses of PLFE.

Our findings indicate that higher doses of PLFE can significantly improve oxidative markers and sperm quality. This study is the first to explore PLFE's protective effects against chemotherapy-induced sperm damage, highlighting the varying impacts of different doses on testicular damage caused by DOX. Such experimental studies in rat models offer valuable insights into potential therapeutic approaches for human health. One of the strengths of our study is the systematic examination of different doses of PLFE, allowing us to assess its dose-response relationship.

In summary, this study is the first to systematically examine the protective effects of PLFE against chemotherapy-induced testicular toxicity. PLFE is a promising candidate for preserving male fertility by enhancing antioxidant defenses and reducing DOX-induced reproductive damage. However, further research is needed to optimize its therapeutic use, investigate its biochemical mechanisms, and determine its long-term effects on spermatogenesis. This research provides a foundation for developing phytotherapeutic interventions aimed at reducing the reproductive side effects of chemotherapy and underscores the importance of strengthening antioxidant defenses in male reproductive health. Future studies should examine the roles of PLFE's active compounds, particularly flavonoids and phenolic acids, in spermatogenesis at molecular and histological levels.

CONCLUSION

This study underscores the potential of PLFE as a supportive therapy for reducing DOX-induced oxidative damage in testicular tissue, suggesting its value in fertility preservation during chemotherapy. PLFE showed encouraging effects by lowering oxidative stress and enhancing sperm parameters. However, complete restoration to control levels was not achieved, highlighting the need for further research to refine the composition of extracts, dosage, and treatment duration. While PLFE activated several antioxidant pathways, its limited impact on catalase activity and the partial restoration of oxidative markers indicate that dosage optimization and further studies are essential.

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Author contributions: Conceptualization: [EK, SC]; Study Design: [EK]; Methodology: [EK, SC, GH, EGGP]; Data collection: [EK, SC, EGGP]; Statistics: [EK]; Writing: [EK]; All authors have read and approved the final version of the manuscript.

Conflict of interest: There is no potential conflict of interest relevant to this article.

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