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Determination of the Effects of Helichrysum Plicatum DC. Subspecies Plicatum Ethanol Extract on Thallium Sulfate Induced Testicular Toxicity in Rat

Helichrysum Plicatum DC Subsp Plicatum Etanol Ekstraktının Sıçanlarda Talyum Sülfatın Neden Olduğu Testis Toksisitesi Üzerine Etkilerinin Belirlenmesi

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ABSTRACT

Objective: : In this study, we aimed to determine the effects of the ethanol extract of *Helichrysum plicatum* DC (HPE) plant on thallium sulfate (TS)-induced testicular toxicity in rats.

Material and Method: For this purpose, a total of twenty four Sprague Dawley rats, six rats in each group, were used in the study. Rats were administered a single dose of TS via IP and HPE via oral gavage for 8 days. After the administrations, the rats were sacrificed and blood and testicular tissues were collected. Testicular tissues were preserved for use in biochemical, histopathological, immunohistochemistry and in situ hybridization analyzes. The cauda epididymis was trimmed by separating from the testis and the resulting liquid was used for semen analysis.

Results: The sperm motility decreased and the rate of dead and abnormal spermatozoa increased in parallel to the increase ($P<0.001$) in oxidative damage in the TS group. Histopathological examination revealed degenerative and necrotic changes in the TS group. Cleaved caspase 3 gene expression decreased in the control group and TS group ($P<0.001$). The mean testicular biopsy score (MTBS) was the lowest in the TS group ($P<0.001$). There was a statistical difference between the groups in terms of immunohistochemical (IHC) and in situ hybridization (IST) parameters.

Conclusion: In this process, it was observed that HPE could protect against TS-induced damage in testicular tissues of rats

Keywords: *Helichrysum plicatum, oxidative stress, rat, sperm, thallium sulphate*

ÖZET

Giriş: Bu çalışmada, *Helichrysum plicatum* DC (HPE) bitkisinin ratlarda talyum sülfat (TS) ile indüklenen testis toksisitesi üzerindeki etkilerinin belirlenmesi amaçlanmıştır.

Materyal ve Metot: Bu amaçla çalışmada her grupta 6 adet olmak üzere toplam 24 adet Sprague Dawley cinsi rat kullanıldı. Uygulamaların ardından ratlar sakrifiye edilerek kan ve testis dokuları alındı. Testis dokuları, biyokimyasal ve histopatolojik analizlerde kullanılmak üzere saklandı. Kauda epididimis testisten ayrılarak trimlendi ve elde edilen sıvı sperma analizi için kullanıldı.

Bulgular: Çalışma bulgularımıza göre TS grubunda sperm motilitesinin azaldığı, ölü ve anormal spermatozoa oranının arttığı; buna karşılık oksidatif hasarın arttığı ($P<0.001$) saptanmıştır. Histopatolojik incelemede, TS grubunda nekrotik ve dejeneratif değişiklikler saptandı. Klivaj kaspaz 3 gen ekspresyonu, kontrol grubu ve TS grubunda azaldı ($P<0.001$). Ortalama testis biyopsi skoru (OTBS) TS grubunda en düşüktü ($P<0.001$). Gruplar arasında immünohistokimyasal (IHK) ve *in situ hibridizasyon* (ISH) parametreleri açısından istatistiksel fark vardı.

Sonuç: HPE'nin sıçanların testis dokularında TS kaynaklı hasara karşı koruma sağlayabildiği görülmüştür.

Anahtar kelimeler: *Helichrysum plicatum, oksidatif stres, sıçan, sperm, talyum sülfat*

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INTRODUCTION

Heavy metals are environmental toxicants that enter the food chain and threaten the life of living things (Khushboo et al., 2018). Heavy metals enter the body and cause dysfunction by accumulating in vital organs such as liver, kidney, heart, and brain (Singh and Kalamdhad, 2011). They also accumulate in the testis and epididymis, causing hormonal dysfunction and thus infertility (Akarsu et al., 2022; Bhardwajet al., 2021). Thallium (Tl) is a heavy metal that is generally found in nature in the form of Tl (I) and Tl (III) and is toxic to living things even at very low concentrations (Bramanti et al.,2018). Tl crosses the placenta and testis barrier in animals of different species and causes reproductive dysfunction (Varão et al., 2021). Thallium sulfate (TS) causes histological disruptions in germ cells (Genchi et al., 2021) and seminiferous epithelium (Sindhi et al., 2022) in animals. Similarly, Tl toxicity impairs functions by increasing the level of reactive oxygen species (ROS) in tissues (Chang et al., 2023).

Today, the interest in herbal products in the field of health continues to increase (Belhan et al., 2017). There are about 185 genera and more than 1240 species within the *Helichrysum* species of the *Asteraceae* family (Bayer et al., 2007). *Helichrysum* species are widely used in the treatment of many conditions (Kulevanova, Stefova, and Stafilov, 2000). *Helichrysum plicatum DC. subsp. plicatum* extract (HPE) has antimicrobial, antidiabetic, spasmolytic, nephroprotective, and antimutagenic activities (Bigović et al., 2017). In addition, *Helichrysum* species display antioxidant properties (Bigović, 2010). The main components of *Helichrysum* plicatum are flavonoid substances such as helichrysin A and B, apigenin, naringenin, isoastragalin, and isosalipurposide (Aslan et al., 2007)

In this study, the possible effects of HPE against testicular damage caused by TS were investigated using oxidative stress, cleaved caspase 3 gene expression, in situ hybridization, and immunohistochemistry as well as spermatological analyses.

MATERIAL and METHOD

Preparation of Plant Extract

Plant material

The aeriel fragment of *Helichrysum plicatum* was collected from Kop Mountain (Bayburt, Turkey) at an altitude of 2100 meters and identified by Meryem ŞENGÜL (Atatürk University, Erzurum, Turkey).

Extraction procedure

HPE was prepared by the method used by Bayir et al. (2011). The plants were first dried at room temperature (24 °C) for 10 days, then pulverized, and extracted with ethanol (1:5) for 48 hours. After the extract was filtered with filter paper, the ethanol was evaporated under low temperature and pressure using a rotary evaporator (RV 05 Basic 1B IKA Group, Wilmington, NC, U.S.A.). The extract was dissolved in 5% dimethyl sulfoxide (DMSO) and was stored at 4 °C until the experiment.

Chemical, Dosage and Design of Experimental Animals

Unless stated otherwise, all chemicals used in the study and thallium sulfate (Tl_2SO_4) were purchased from Sigma-Aldrich (St Louis, MO, USA). Thallium sulphate was prepared by dissolving in 0.9% saline. Rats were recruited and adapted one week before the study. A total of 24 Sprague Dawley rats were used in the study. Rats were housed under standard laboratory conditions throughout the experiment. Water and food were given ad libitum.

Experimental Design

The animals were divided into four groups of six rats each and the following experimental procedure was applied:

Control: The rats were given 5% DMSO solution by intraperitoneal (IP) route as a single dose (Apaydin Yildirim et al., 2017).

Group I (TS): The rats were given thallium sulphate (10 mg/kg) via IP route as a single dose (Gibson and Becker, 1970).

Group II (TS + HPE): The rats were given thallium (I) sulphate (10 mg/kg, IP) as a single dose + HPE for 8 days (100 mg/kg, IP) (Apaydin Yildirim et al., 2017).

Group III (HPE): The rats were given HPE (100 mg/kg) via IP route for 8 days.

At the end of the administration period, animals were sacrificed under anesthesia with 10 mg/kg xylazine (Basilazin, Bavet, Turkey) and 60 mg/kg ketamine (Ketalar, Eczacıbaşı, Turkey). Cauda epididymis and testis tissues were excised. The right cauda epididymis was used for semen analysis. For lipid peroxidation and antioxidant status measurements, the left testis was stored in a deep freezer at -20 °C. For pathological examination, right testis tissues were stored in Bouin's solution until analysis. Blood from the heart of each rat was transferred into heparinized tubes and centrifuged. The obtained plasma was stored in a deep freezer at -20 °C until biochemical analysis.

Semen Evaluation

Right cauda epididymis was used to obtain sperm. First, right cauda epididymis tissue was trimmed into a petri dish containing 5 mL of physiological saline. The semen samples were incubated for 5 minutes at 35 °C for tissue-to-fluid migration. After the incubation period, the remaining pieces of cauda epididymis tissue were removed and the remaining liquid was used for sperm analysis. Semen motility, and abnormal sperm ratio analyzes were performed with an optical microscope (Primo Star; Carl Zeiss, Oberkochen, Germany) with a heated plate placed on it. A slide was placed on the heating plate at 35 °C. Approximately 20 µL of semen sample was dropped

on the slide and the estimated score average was calculated from three different areas (Akarsu et al., 2023).

The proportion of abnormal spermatozoa was measured on slides stained with a mixture of eosin and nigrosine (1.67% eosin, 10% nigrosine, and 0.1 M sodium citrate). The stained slides were examined with the aid of a light microscope (Primo Star) under X400 magnification. Two hundred spermatozoa were examined on each slide, and the head, tail, and total abnormality rates of spermatozoa were expressed as a percentage (Aksu et al., 2021) .

Measurements of Plasma Oxidative Stress Indices

Plasma malondialdehyde (MDA) level was calculated by the method described by Yoshika et al. (1979). Plasma glutathione (GSH) level was measured by the method described by Tietze (1969). Plasma catalase (CAT) enzyme activity was calculated by the method described by Goth (1991). Plasma superoxide dismutase (SOD) enzyme activity was measured by the method described by Sun et al. (1988). Plasma glutathione peroxidase (GPx) enzyme activity was analyzed by the method described by Matkovics (1988). Lipid peroxidation and oxidative stress parameters were calculated spectrophotometrically (Biotechepocha UV-visible EIA spectrophotometer).

Measurements of Testicular Tissue Oxidative Stress Indices

Tissue CAT enzyme activity was measured by the method described by Goth (1991). Tissue MDA level was determined as described by Placer et al. (1966). SOD activity was determined by the method applied by Sun et al. (1988). Tissue GPx activity was determined as described by Matkovics (1988). Tissue GSH level were was measured with the method applied by Fernandez and Videla (1981). Lipid peroxidation and oxidative stress parameters were calculated spectrophotometrically (Biotechepocha UV-visible EIA spectrophotometer).

Histopathological Examination

The left testes were immediately removed, fixed in 10% neutral formalin, and processed to paraffin block. Then, 5 µm sections were stained with hematoxylin-eosin and 10 randomly selected tubules were examined with a light microscope at 20X magnification. Mean testicular biopsy score (MTBS) was calculated according to Johnsen (1970). Each tubule was scored from 0 to 10 based on epithelial maturation.

Immunohistochemical Examination

Immunohistochemistry was performed with the streptavidin-biotin-peroxidase method. In short, 4 µm sections were deparaffinized and rehydrated. The slides were immersed in an antigen retrieval solution and heated in microwave for 15 minutes. Endogenous peroxidase activity was blocked in 3% H_2O_2 in methanol for 10 minutes and the slides were then incubated for 30 minutes at 24 °C in phosphate-buffered saline (PBS) containing 10% normal goat serum to prevent non-specific binding. These sections were then incubated with polyclonal rabbit cleaved caspase 3 antibody (Novus Biological, USA) at room temperature. The slides were counterstained with hematoxylin and 10 randomly selected tubules were examined with a light microscope (Primo Star) under X200 magnification and the values were written (Ileriturk et al., 2021).

In situ hybridization

In situ hybridization was done as recommended by the producer using Detection System Biotinylated Probes kit (Cat no: K0601, Carpinteria, USA). Paraffinization process were incubated at 57 °C for 1 hour, followed by a deparaffinization procedure by passing through the xylol alcohol series. Samples were incubated in heated pepsin-HCL solution for 5 minutes and then washed with PBS. AGATCATCACTGCTTCGTAATT/3Bio/oligonucleotide (Exiqon) was used in the hybridization step. Appropriate dilution ratio (1:50) was applied, sections were covered with coverslips, and the slides were incubated at 90°C for 45 minutes. Nuclear fast red was used as a chromogen. Afterwards, alcohol and xylene series were applied to the sections. In total 10 randomly selected tubules were viewed with a light microscope (Primo Star) under X200 magnification and values were determined (Sevim et al., 2019).

Statistical Analysis

All statistical analyzes were performed using the IBM SPSS (Version, 26.0) program. Statistical analysis of semen and biochemical data was performed using one-way analysis of variance (ANOVA) test and Duncan's multiple comparison test. Immunohistochemical and in situ hybridization parameters were measured using the non-parametric Kruskal-Wallis test. Mann-Whitney U test was used for pairwise comparisons. Results were expressed as mean ± standard error of the mean (SEM).

RESULTS

Spermatological Findings

Spermatological analysis results are presented in Table 1.Total motility in the TS group was lower than the control and other experimental groups ($P<0.001$). Head, tail abnormalities and dead spermatozoon rate in the TS and TS+HPE groups were statistically higher than in the control and HPE groups ($P<0.001$).

Oxidative Stress Findings

Oxidative stress analysis results are presented in Table 2 and Table 3.While testicular and plasma MDA levels were found to be the highest in the TS group, the levels were lower in the TS+HPE group ($P<0.001$). Lowest testicular and plasma GSH level, GPx and CAT enzyme activities were found in the TS group ($P<0.001$). The lowest plasma SOD activity was seen in the TS group ($P<0.001$). On the other hand, there was no difference between the groups in terms of testicular SOD activity.

Histopathological Findings

Histopathological findings are shown in Table 4 and Figures 1, 2 and 3. Statistically difference was observed between the TS and HPE groups (P<0.001). Seminiferous tubules were normal in the control and HPE groups. (Fig. 1 and 2). The regular structure of the spermatogenic cells deteriorated and the seminiferous tubules were observed as degenerated and necrotic in group 1 (Fig. 3). The degenerative and necrotic changes in 2 group were decreased compared to group 1 (Fig. 1) .

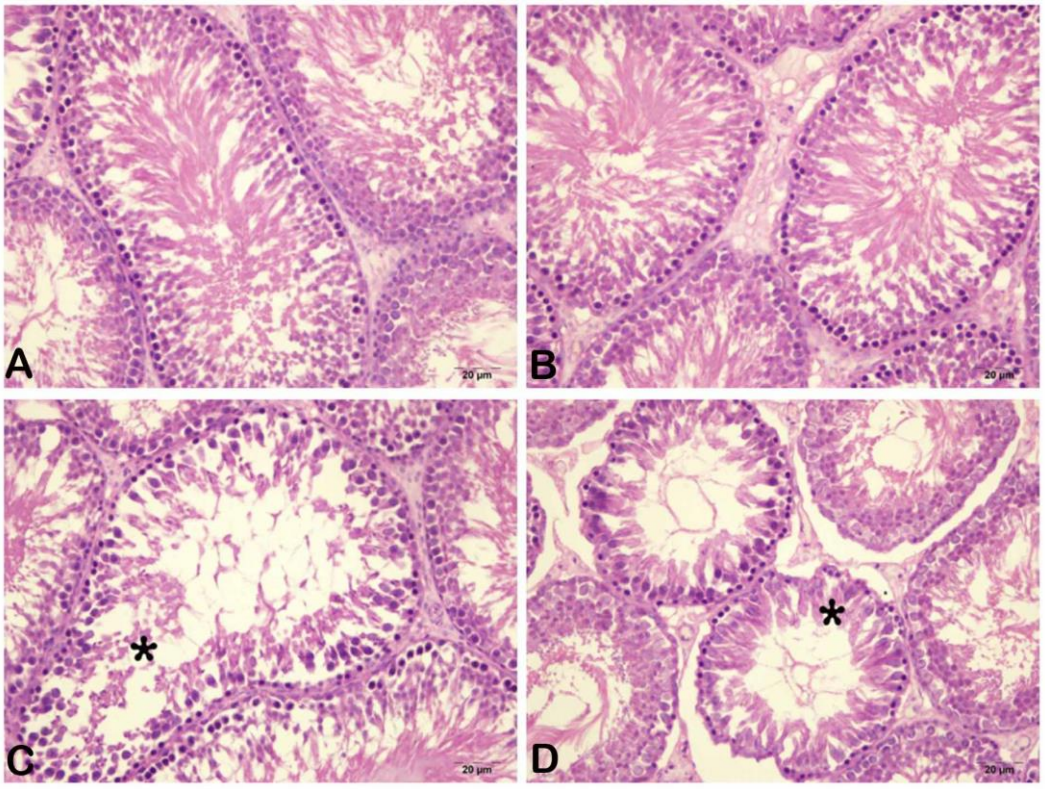

Figure 1. A- Control group [Normal structure]. B- Groups 3 [Normal structure]. C- Group 1 [Severe necrotic and degenerative changes in seminiferous tubules (*)]. D- Group 2 [Mild necrotic and degenerative changes in seminiferous tubules (*)].

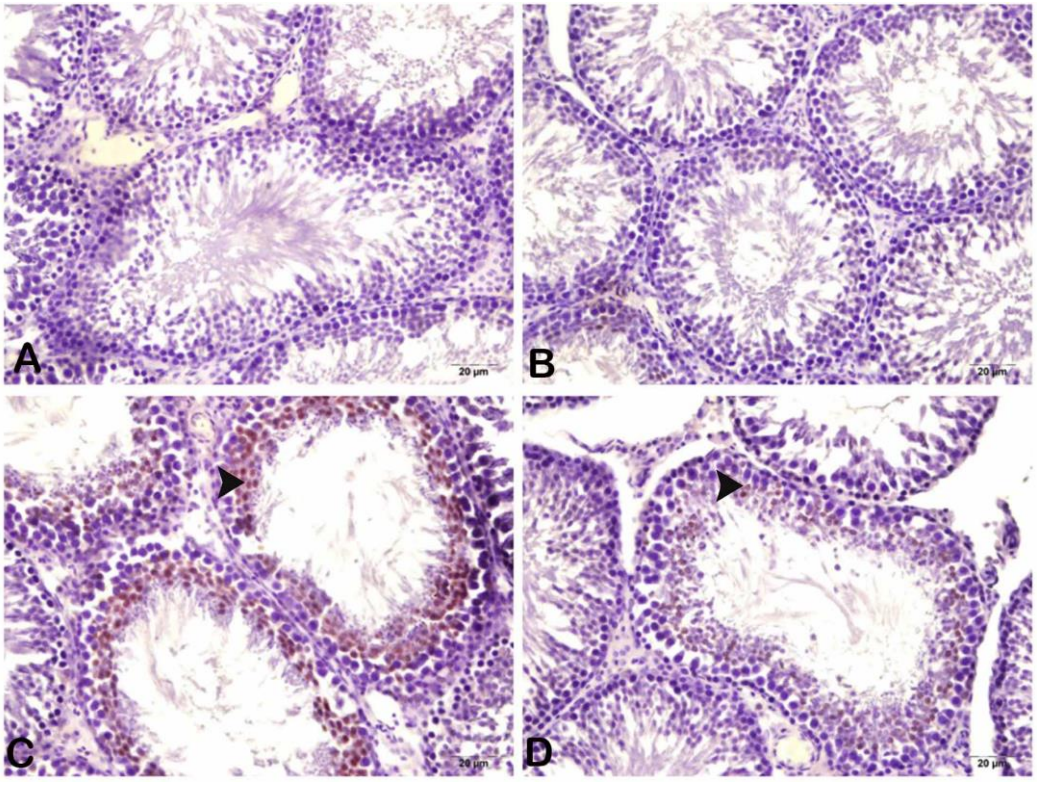

Figure 2. A- Control group [Cleaved caspase 3 immun negativity]. B- Groups 3 [Cleaved caspase 3 immun negativity]. C- Group 1 [Severe Cleaved Caspase 3 expression in seminiferous tubules (arrowhead)]. D- Group 2. [Moderate expression of Cleaved Caspase 3 in seminiferous tubules (arrowhead)].

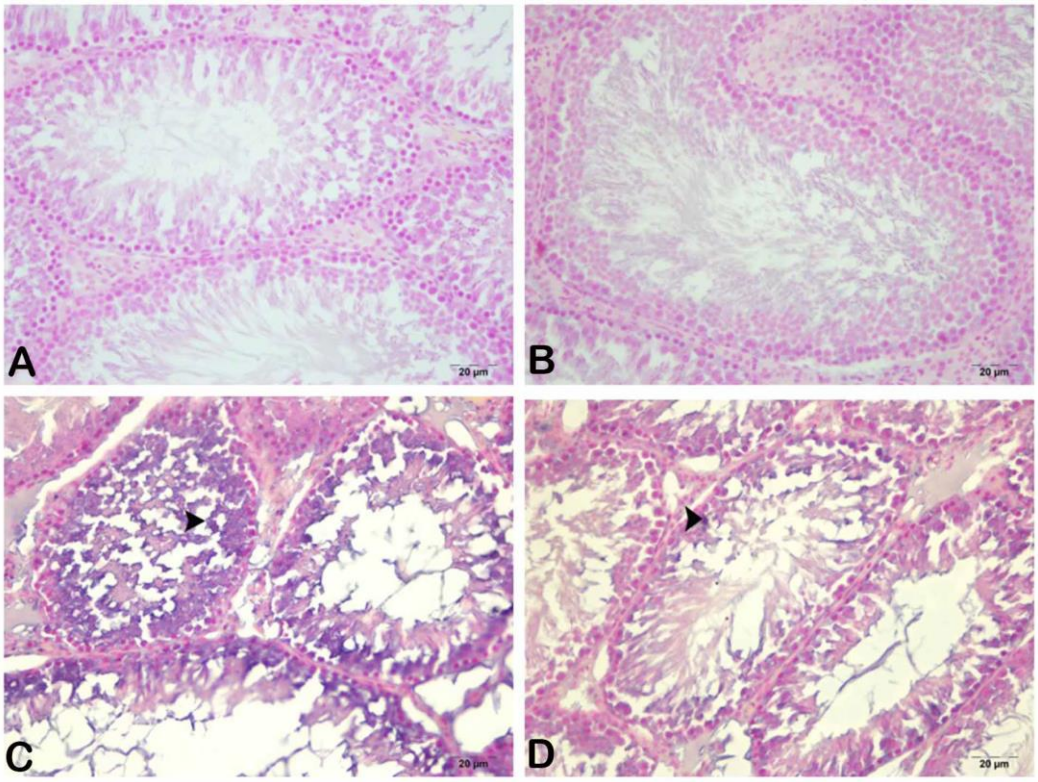

Figure 3. A- Control group [Cleaved caspase 3 immun negativity]. B- Groups 3 [Cleaved caspase 3 immun negativity]. C- Group 1 [Severe Cleaved Caspase 3 expression in seminiferous tubules (arrowhead)]. D- Group 2 [Moderate expression of Cleaved Caspase 3 in seminiferous tubules (arrowhead)].

Immunohistochemical and In situ Hybridization Findings

Immunohistochemical findings and in situ hybridization findings are shown in Table 4. Statistically significant difference was detected between the TS and HPE groups. It is noteworthy that the level of cleaved caspase 3 gene expression decreased in control and group 3. In the group 1, cleaved caspase 3 was expressed as severe in the seminiferous tubules, but in the group 2, the expression of cleaved caspase 3 was decreased ($P<0.001$).

DISCUSSION

The increase in researches on the effectiveness of plant-based active ingredients on biological structures draws attention. In line with this approach, it is thought that the research team's determination of the effect of HPE in terms of reproduction will contribute to the literature. Our findings show that Helichrysum plicatum extract has positive effects on spermatological, plasma/testicular tissue biochemical and histopathological parameters in rats. The protective effect of HPE against TS toxicity was tried to be revealed by apoptosis in the present study.

Apoptosis is a cell death event that is observed in pathological conditions as well as physiological conditions (Ileriturk et al., 2022). Caspases are cysteine proteases that break the peptide bond after aspartic acid and are one of the genes controlling apoptosis, known as programmed cell death (Metzstein et al., 1998). Bcl-2 family proteins activate procaspase-9 by affecting the mitochondrial membrane, but procaspase-8 is activated via cell receptors in the receptor-dependent exogenous pathway. Caspase-9 in the intrinsic pathway of apoptosis and caspase-8 in the exogenous pathway activate caspase-3 (cleaved), inducing cell apoptosis (Vermeulen et al.,, 2005). In the current study, the protective effect of HPE against TS-induced testicular damage was observed by determining that when HPE was applied with TS, the expression of cleaved caspase 3 in the testis tissue decreased by immunohistochemistry and in situ hybridization.

Testicular damage caused by TS is mostly due to increased oxidative stress. Oxidative stress causes testicular damage by increasing lipid peroxidation and mitochondrial lesions in germ cells, leading to dysfunction male reproductive system (Gur et al., 2022), further DNA damage and abnormality in germ cells (Kumar et al., 2002). The present results demonstrated that TS-induced testicular damage significantly decreased plasma and testis tissue antioxidant activities and increased significantly plasma and testis tissue MDA levels. The increased MDA level in group 1 was significantly decreased in treatment group. GSH levels, CAT, GPx and SOD activities were also significantly increased in treatment group compared to the group 1. GSH levels, CAT, GPx and SOD activities were suppressed, while the lipid peroxidation biomarker MDA, was significantly increased in the testis of group 1. Moreover, the present data clearly confirmed the marked increase in ROS in TS rat plasma and testis.

Furthermore, many of these changes suggest that HPE exhibits an antioxidant property in TS treated rats, possibly protecting the testes against the adverse effects of TS. It is known that oxidative stress has an important place in the pathogenesis of induced male reproductive disorders (Gur et al., 2023). The protective effect of HPE against lipid peroxidation and oxidative stress caused by TS was found to be significant in this sense.

The harmful effects of thallium and thallium sulfate on living tissues have been determined by following studies: placental transfer, embryotoxicity and teratogenicity efficiency (Sánchez-Chapul et al., 2023) , testicular toxicity in the rat in terms of reduced motility and immature germ cells, disarrangement of the tubular epithelium and ultrastructural changes in the Sertoli cells with cytoplasmic vacuolation (Álvarez-Barrera et al., 2019), oxidative stress in mice liver (Li et al., 2022). In our study, total motility was lower in the TS group than in the control and other experimental groups ($P<0.001$). Head, tail abnormalities and dead spermatozoa rate were statistically higher in the TS and TS+HPE groups than in the control and HPE groups ($P<0.001$). This situation suggested that TS caused a decrease in semen quality, while HPE had curative properties.

On the other hand, studies on Helichrysum plicatum show that, similar to our study, reduces the effectiveness of factors harmful to biological systems, because of its properties such as: antidiabetic and antioxidant potential (Miloglu et al., 2021).

Conclusion

In this study, it was tried to explain the effects of HPE (100 mg/kg) administration against lipid peroxidation and oxidative stress, histopathological changes and poor semen quality caused by thallium (I) sulfate (10 mg/kg) in male rats. Further scientific analysis and research will contribute to a better understanding of HPE-related efficacy.

Conflict of interest

There is no conflict of interest between the authors.

Ethical Standards

In this study, the animal experiments were performed according to the EU Guidelines for the Use of Laboratory Animals after the approval (Approval No. 2018/11-195) of Atatürk University Animal Experiments Ethics Committee.

Authors Contributions

ADÖ and BAY designed the study. SK, BM, ÖK, MÖ performed the experiments. SAA wrote and edited

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