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Investigation of the Effects of *Diplotaenia turcica* Root Extract on Sperm Parameters and Antioxidant Markers in Methotrexate-Administered Rats

Saadet BELHAN[1,*] Uğur ÖZDEK[2]

[1] *Van Yüzüncü Yıl University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, 65080, Van, Türkiye*
[2] *Van Yüzüncü Yıl University, Vocational School of Health Services, 65080, Van, Türkiye*

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ABSTRACT This experimental study was designed to evaluate the potential toxic effects of Methotrexate (MTX), an antineoplastic agent, on sperm parameters and antioxidant defense mechanisms in rats, as well as to assess the protective potential of *Diplotaenia turcica*, an endemic plant species from Turkey. A total of 21 male Wistar albino rats weighing 250–300 g was included in the study and randomly assigned to three groups of seven animals each. The control group received only physiological saline solution. The MTX group was administered a single intraperitoneal dose of 20 mg/kg MTX. In the MTX + *Diplotaenia turcica* root extract group, following MTX injection, the plant extract was given orally at 150 mg/kg daily throughout the study. At the end of the 7-day period, testicular tissues were collected under ketamine anesthesia. Sperm concentration and the percentage of abnormal sperm were assessed using samples obtained from the cauda epididymis, while portions of testicular tissue were used for biochemical analyses, including measurement of malondialdehyde (MDA) levels, glutathione (GSH) content, and catalase (CAT) activity. The results demonstrated that MTX administration significantly impaired sperm parameters, with a notable decrease in sperm concentration and an increase in the percentage of abnormal sperm. Additionally, MTX elevated MDA levels and reduced GSH content and CAT activity. Administration of *Diplotaenia turcica* root extract partially alleviated these adverse effects, contributing to the preservation of testicular tissue integrity. These findings underscore the biomedical potential of endemic plants in supporting reproductive health and protecting against toxic insults.

Keywords: *Diplotaenia turcica root extract, Catalase, MDA, Methotrexate, Sperm parameters.*

ÖZ

Metotreksat Uygulanan Sıçanlarda *Diplotaenia turcica* Kök Ekstraktının Sperm Parametreleri ve Antioksidan Parametreler Üzerindeki Etkisinin Araştırılması

Bu deneysel çalışma, antineoplastik bir ajan olan Metotreksat (MTX)'ın sıçanlarda sperm parametreleri ve antioksidan savunma mekanizmaları üzerindeki olası toksik etkilerini, ayrıca Türkiye'ye özgü endemik bir bitki olan *Diplotaenia turcica*'nın koruyucu potansiyelini araştırmak amacıyla gerçekleştirilmiştir. Çalışmada, 250–300 g ağırlığında toplam 21 erkek Wistar albino sıçan kullanılmış ve hayvanlar her biri 7 sıçandan oluşan üç gruba ayrılmıştır. Kontrol grubuna yalnızca serum fizyolojik solüsyonu verilmiştir. MTX grubunda hayvanlara tek doz 20 mg/kg intraperitoneal MTX uygulanmıştır. MTX + *Diplotaenia turcica* kök ekstraktı grubunda ise MTX enjeksiyonunu takiben, 150 mg/kg dozunda bitki ekstraktı oral yolla, deney süresince her gün verilmiştir. Yedi günün sonunda, tüm sıçanlardan ketamin anestezisi altında testis dokuları çıkarılmıştır. Kauda epididimisten elde edilen örnekler kullanılarak sperm yoğunluğu ve anormal sperm oranı değerlendirilmiş; diğer testis dokusu biyokimyasal analizler için ayrılarak malondialdehit (MDA) düzeyi, glutatyon (GSH) miktarı ve katalaz (CAT) aktivitesi ölçülmüştür. Bulgular, MTX uygulamasının sperm parametrelerinde belirgin bozulmalara yol açtığını, özellikle sperm yoğunluğunda anlamlı azalmalara, anormal sperm oranında ise anlamlı artışlara yol açtığını göstermiştir. Bunun yanı sıra MDA düzeyinde artışa ve GSH miktarı ile CAT aktivitesinde azalmaya neden olduğunu göstermiştir. Ancak *Diplotaenia turcica* kök ekstraktının bu zararlı etkileri kısmen sınırlayarak testiküler doku bütünlüğünün korunmasına katkı sağladığı belirlenmiştir. Bu sonuçlar, endemik bitkilerin üreme sağlığı ve toksisiteye karşı biyomedikal potansiyellerine ışık tutmaktadır.

Anahtar Kelimeler: *Diplotaenia turcica kök ekstraktı, Katalaz, MDA, Metotreksat, Sperm parametreleri.*

✉ *Corresponding author: saadetbelhan@yyu.edu.tr

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INTRODUCTION

Methotrexate (MTX), a folic acid antimetabolite, is widely used in the treatment of various conditions due to its antitumoral, anti-inflammatory, antimicrobial, and immunosuppressive properties (Cole et al. 2006). Despite its established clinical efficacy, the toxic side effects of MTX pose significant limitations for its therapeutic use. Literature reports indicate that MTX can cause adverse effects, particularly in the gastrointestinal system, as well as hepatotoxicity, nephrotoxicity, bone marrow suppression, pulmonary toxicity, and detrimental impacts on the reproductive system (Manna et al. 2023; Wani et al. 2023). Regarding reproductive toxicity, the testicular tissue is a major target organ in males, as the rapid proliferation and division of spermatogenic cells render them highly susceptible to environmental toxins and chemotherapeutic agents (Zhou et al. 2023). MTX-induced testicular toxicity has been shown to involve multiple mechanisms, including oxidative stress, inflammatory responses, and apoptotic cell death (Arafa et al. 2024; Tarmookh et al. 2024).

Diplotaenia turcica, an endemic plant species from Türkiye, was formerly identified as Diplotaenia cachrydifolia until it was reclassified as a distinct species in 2011 based on studies by Pimenov and colleagues in the Bitlis–Van–Hakkâri region (Pimenov et al. 2011). Locally referred to as "siyabo," the plant is recognized for its applications in traditional medicine. Beyond medicinal uses, *Diplotaenia turcica* is commonly incorporated into the production of "herbed cheese" (otlu peynir), a regional specialty. The plant's root has traditionally been employed to treat various conditions, including diabetes, hypertension, and rheumatic disorders, and it is also used as an antidote for snake and scorpion bites (Kaval et al. 2014; Uce and Tuncturk 2014; Ozdek et al. 2020). Belonging to the Umbelliferae family, *Diplotaenia turcica* shares a lineage with species that have demonstrated diverse biological activities in experimental studies, including antihyperglycemic, hypolipidemic, and antioxidant effects (Razavi et al. 2010; Ahmed et al. 2011; Farkhad et al. 2012; Duran et al. 2015). This taxonomic placement underscores the significance of exploring the potential biological properties of *Diplotaenia turcica*.

The root extract of *Diplotaenia turcica* has been shown to reduce blood glucose levels and promote beta-cell regeneration in streptozotocin-induced diabetic rats by reducing pancreatic oxidative stress and enhancing antioxidant enzyme activities. Its hydroalcoholic extract has exhibited strong antioxidant and anti-Alzheimer effects, which are attributed to its high phenolic and flavonoid content. In addition, the plant has demonstrated antimicrobial activity against various bacterial strains. Green-synthesized copper nanoparticles derived from *Diplotaenia turcica* have also shown significant antioxidant, antimicrobial, and DNA-protective properties (Ozdek 2020; Ozdek et al. 2020; Seckin 2021; Seckin and Meydan 2021).

Investigating natural products that may prevent the adverse effects of MTX on testicular tissue and sperm parameters is of great importance. Evaluating the potential protective effects of the root extract of *Diplotaenia turcica* against MTX-induced oxidative stress and sperm damage may contribute to the development of novel therapeutic approaches aimed at preserving reproductive health and reducing toxicity. Accordingly, this study aims to elucidate the effects of MTX-induced reproductive toxicity on sperm quality and the antioxidant defense system, and to determine the potential mitigating role of *Diplotaenia turcica* against this damage.

MATERIAL AND METHODS

The study was conducted following the approval of the Local Ethics Committee for Animal Experiments at Van Yuzuncu Yil University (Date: 26/06/2025; Ethics Approval No: 2025/07-12).

Extraction of Plant Material

The roots of *Diplotaenia turcica*, collected during May–June following taxonomic identification, were thoroughly washed with distilled water. Subsequently, the roots were cut into small pieces, shade-dried, and ground into a fine powder using a mechanical grinder. A 100 g portion of the powdered material was macerated in 1000 mL of 96% ethanol for 24 h. Following maceration, the mixture was filtered, and the filtrate was concentrated using a rotary evaporator at 50 °C and 70 rpm. The resulting extract was further dried in a water bath at 40 °C until complete evaporation was achieved (Farkhad et al. 2012).

Animals and Experimental Design

This study was carried out using 21 adult male Wistar albino rats, weighing 250–300 g each. During the experimental period, the rats were maintained in standard cages under standardized laboratory conditions, with ambient temperature regulated at 22±2 °C and a 12-hour light/12-hour dark photoperiod. Cage maintenance was performed on a daily basis, and the animals were provided unrestricted access to standard feed and drinking water throughout the study.

For the experimental setup, the rats were randomly assigned to three groups, each comprising seven animals:

Control group: Received only physiological saline solution (0.9% NaCl) throughout the study.

MTX group: Administered a single intraperitoneal dose of 20 mg/kg MTX (Hassanein et al. 2023; Waly et al. 2023; Abbas et al. 2024; Taleahmad et al. 2024; El-Shitany et al. 2025).

MTX + *Diplotaenia turcica* root extract group: Following a single intraperitoneal injection of 20 mg/kg MTX, the animals were given *Diplotaenia turcica* root extract orally at a dose of 150 mg/kg daily for 7 days.

At the end of the experimental period, all animals were fasted for 12 h. Anesthesia was then induced by intraperitoneal administration of ketamine at 75 mg/kg. Under anesthesia, one testis from each rat was excised for biochemical analyses and stored in a deep freezer at -80°C. These samples were used to assess oxidative stress and antioxidant parameters, including MDA levels, GSH content, and CAT activity. The cauda epididymis from the opposite testis was collected for spermatological evaluations.

Semen Collection and Evaluation of Spermatological Parameters

Sperm samples were obtained from the cauda epididymis to evaluate spermatological parameters, following the method described by Aksu et al. (2015). The cauda epididymis was carefully dissected and removed, then placed in 2 mL of sterile physiological saline solution preheated to 37 °C in a sterile Petri dish. Several small incisions were made in the tissue using a sterile scalpel to facilitate the release of spermatozoa into the medium. The preparation was incubated at 37 °C for approximately 15 min to allow sperm to disperse into the solution. The resulting suspension was homogenized and used for

spermatological analyses, including sperm count, motility, and morphology.

Sperm concentration was determined using a slightly modified version of the method described by Sonmez et al. (2005), substituting an Eppendorf tube for the conventional hemocytometer. Briefly, 10 µL of the sperm suspension, prepared by mincing the cauda epididymis in physiological saline, was aspirated with an automatic pipette and transferred into an Eppendorf tube containing 990 µL of Hayem's solution. The tube was vortexed for 15 s, and approximately 10 µL of the diluted suspension was then loaded into the counting chambers of a Thoma hemocytometer (HHH, Germany). The sample was allowed to settle for 5 min to enable sperm sedimentation, after which sperm counting was performed under phase-contrast microscopy at 400× magnification.

The method described by Turk et al. (2008) was used to assess abnormal sperm cells. A drop of the homogenized sperm suspension was placed on a clean, dry slide and spread to form thin smears. After air-drying, the smears were stained with 1% eosin–nigrosin dye. The slides were then examined under a microscope at 400× magnification. For each sample, at least 200 sperm cells were evaluated, and morphological abnormalities in the head, neck, and tail regions were recorded. The proportion of sperm with normal morphology was calculated and compared among the groups.

Evaluation of Biochemical Parameters

In this study, commercial sandwich ELISA kits were used for all biochemical analyses, in accordance with the manufacturers' instructions. The GSH ELISA kit (Cat. No: ELK8577) was used to determine GSH levels, the MDA kit (Cat. No: YLA0029RA) was used to measure MDA levels, and the CAT kit (Cat. No: YLA0123RA) was used to assess CAT activity.

Statistical Analysis

The normality of data distribution was assessed using the Kolmogorov–Smirnov test. The results were expressed as mean±standard deviation (SD) of the number of observations. Post-hoc power analysis was performed using the observed effect size of the main outcome parameters, demonstrating that the study achieved a statistical power of over 80% at α=0.05. Comparisons of group means were performed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test for post hoc comparisons between groups. The lettering system (a, b, c) used in the tables represents the results of Duncan's test. The level of statistical significance was set at $p<0.05$. All statistical analyses were performed using SPSS Statistics (Version 20.0; SPSS Inc., Chicago, IL, USA).

RESULTS

Spermatological Findings

As shown in Table 1, sperm concentration was significantly reduced in the MTX administered group compared with the control group ($p<0.001$). In contrast, the group receiving *Diplotaenia turcica* root extract alongside MTX exhibited a significant increase in sperm concentration relative to the MTX only group ($p<0.001$). Examination of sperm morphology indicated that MTX administration impaired sperm structural integrity, leading to a significantly higher proportion of abnormal sperm compared with controls ($p<0.001$). Conversely, co-administration of *Diplotaenia turcica* root extract with MTX resulted in a significant reduction in the percentage of abnormal sperm ($p<0.001$).

Biochemical Findings

Analysis of the biochemical data presented in Table 2 revealed a significant increase in MDA levels in the MTX administered group compared with the control group ($p<0.001$). In contrast, the group receiving both MTX and *Diplotaenia turcica* root extract exhibited a significant reduction in MDA levels relative to the MTX only group ($p<0.001$). Evaluation of CAT enzyme activity showed a significant decrease in the MTX group compared with controls ($p<0.001$), whereas co-administration of *Diplotaenia turcica* root extract with MTX led to a significant increase in CAT activity ($p<0.001$). Similarly, GSH levels were significantly lower in the MTX group compared with the control group ($p<0.001$), while GSH levels in the MTX+*Diplotaenia turcica* root extract group were significantly higher than in the MTX only group ($p<0.001$).

Table 1: Effects of *Diplotaenia turcica* root extract on sperm parameters in methotrexate-administered rats.

Groups	Sperm Concentration ($x10^6$)	Abnormal Sperm Rate (%)	p
Control group	96.71 ± 1.49^a	10.00 ± 0.81^c	0.001
MTX group	58.85 ± 2.91^c	45.85 ± 1.57^a	0.001
MTX + *Diplotaenia turcica* root extract group	67.28 ± 1.11^b	25.57 ± 1.71^b	0.001

Each value is expressed as mean±SD (n=7). $p<0.05$ was considered statistically significant. Different lowercase letters (a, b, c) within the same column indicate statistically significant differences between groups according to Duncan's multiple range post hoc test.

Table 2: Effects of *Diplotaenia turcica* root extract on testicular tissue MDA and GSH levels and CAT enzyme activity in methotrexate administrated rats.

Groups	MDA (nmol/g tissue)	CAT (µg/g tissue)	GSH (µg/g tissue)	p
Control group	4.11 ± 0.04^c	1.37 ± 0.05^a	18.19 ± 0.52^a	0.001
MTX group	10.01 ± 0.68^a	0.59 ± 0.05^c	7.82 ± 0.20^c	0.001
MTX + *Diplotaenia turcica* root extract group	6.18 ± 0.59^b	0.90 ± 0.06^b	12.29 ± 0.32^b	0.001

Each value is expressed as mean±SD (n=7). $p<0.05$ was considered statistically significant. Different lowercase letters (a, b, c) within the same column indicate statistically significant differences between groups according to Duncan's multiple range post hoc test.

DISCUSSION AND CONCLUSION

Chemotherapeutic agents are recognized not only for their therapeutic benefits but also for their potential to induce toxic side effects across multiple organ systems. Antimetabolite drugs such as MTX, which are widely used, can adversely affect the reproductive system, leading to impaired fertility. This underscores the importance of protective and adjunctive treatment strategies and underscores the need to explore the potential benefits of biologically active compounds derived from natural sources in mitigating such side effects. In this context, *Diplotaenia turcica*, an endemic plant whose pharmacological properties remain incompletely

understood, is believed to offer protective effects against chemotherapy-induced oxidative stress and reproductive dysfunction through its bioactive constituents. The present study experimentally investigated the effects of this plant extract on MTX-induced reproductive damage.

The data obtained in our study indicate that MTX administration has several adverse effects on male reproductive health. Specifically, sperm concentration was significantly reduced in the MTX group compared with the control group ($p<0.001$), suggesting that MTX exerts toxic effects on testicular tissue, thereby inhibiting spermatogenesis and reducing sperm production. In addition, analysis of sperm morphology revealed a significant increase in the percentage of morphologically abnormal sperm in the MTX administered group ($p<0.001$), indicating that MTX may cause structural damage to germ cells and disrupt normal sperm development. Previous studies have reported that MTX disrupts cellular architecture in the testes by inducing apoptosis in spermatocytes and spermatids, which compromises the structural integrity of seminiferous tubules and leads to increased production of abnormal sperm (Taleahmad et al. 2024). The deterioration in sperm parameters may also be linked to the susceptibility of polyunsaturated fatty acids in sperm plasma membranes to reactive oxygen species (Rashid and Sil 2015). MTX-induced testicular damage is understood to involve oxidative stress, inflammation, and apoptosis, collectively causing substantial disruption of spermatogenesis (Mansour et al. 2021; Arafa et al. 2024). Our findings regarding sperm parameters are in agreement with previous reports in the literature (Meng et al. 2022; Delkhosh et al. 2024; Kabartan et al. 2025).

The co-administration of *Diplotaenia turcica* root extract with MTX resulted in a significant increase in sperm concentration and a significant reduction in the percentage of morphologically abnormal sperm ($p<0.001$). These results align with previous findings reported by Belhan et al. (2020) and suggest that *Diplotaenia turcica* root extract has protective effects against MTX induced testicular toxicity. This protective effect is likely mediated by the antioxidant constituents of the extract, which mitigate oxidative damage caused by reactive oxygen species, thereby preserving germ cells and supporting spermatogenesis.

In this study, CAT enzyme activity, as well as GSH and MDA levels, were measured in the testicular tissues of rats from the experimental groups to evaluate oxidative damage and tissue deterioration induced by MTX. The results revealed a significant decrease in CAT activity and GSH levels ($p<0.001$), along with a pronounced increase in MDA levels ($p<0.001$) in the MTX administered group. These findings are in agreement with previous studies showing that MTX impairs antioxidant defense systems, leading to the accumulation of reactive oxygen species and increased lipid peroxidation (Waly et al. 2023; Abbas et al. 2024; Taleahmad et al. 2024; El-Shitany et al. 2025; Kabartan et al. 2025). The elevation in MDA levels indicates enhanced oxidative damage to cellular membranes, which may contribute to functional impairments in testicular tissue (Taleahmad et al. 2024).

Moreover, co-administration of *Diplotaenia turcica* root extract with MTX resulted in a significant restoration of CAT activity and GSH levels to near-control values, accompanied by a significant reduction in MDA levels. These findings indicate that *Diplotaenia turcica* enhances endogenous antioxidant defense mechanisms through its potent antioxidant constituents, thereby markedly attenuating MTX induced oxidative stress. The decrease in lipid peroxidation suggests preservation of cellular membrane integrity and functional improvement in testicular tissue. Overall, the biochemical results of this study demonstrate a protective effect of *Diplotaenia turcica* root extract against MTX induced testicular toxicity, in line with previous biochemical findings reported by Ozdek et al. (2020) in diabetic rats.

In conclusion, this study demonstrated that MTX administration induces significant impairments in sperm parameters and elevates oxidative stress in the testicular tissue of male rats. *Diplotaenia turcica* root extract, due to its antioxidant properties, partially mitigated these adverse effects by preserving sperm concentration, reducing the proportion of abnormal sperm, and supporting testicular antioxidant defense mechanisms. These findings suggest that *Diplotaenia turcica* root extract may serve as a potential protective agent against MTX induced reproductive toxicity. Future studies are warranted to perform detailed chemical analyses of the plant's active components, evaluate different dosage regimens and long-term effects, and assess the feasibility of clinical application in humans. Additionally, investigating the protective effects of *Diplotaenia turcica* root extract against other chemotherapeutic agents beyond MTX will provide further insight into its potential role in therapeutic contexts.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

AUTHOR CONTRIBUTIONS

Idea/Concept: SB, UÖ
Supervision/Consultancy: SB
Data Collection and/or Processing: SB
Analysis and/or Interpretation: SB, UÖ
Writing the Article: SB
Critical Review: SB, UÖ

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