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Effect of *Boswellia serrata* extract on Methotrexate induced testicular damage

Efecto del extracto de *Boswellia serrata* sobre el daño testicular inducido por Metotrexato

Gözde Arkalı*[1], Tutku Can Acısu[2], Nida Badıllı[2], Edanur Güler-Ekmen[1], Osman Sedat Tanyeri[1], Abdullah Toz[1], Mehmet Çay[1], Abdurrauf Yüce[1], Mesut Aksakal[1]

[1]*Fırat University, Faculty of Veterinary Medicine, Department of Physiology. Elazığ, Türkiye.*
[2]*Fırat University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemınation. Elazığ, Türkiye.*
**Corresponding Author: garkali@firat.edu.tr*

ABSTRACT

This study aimed to determine the effect of *Boswellia serrata* extract on Methotrexate- induced testicular damage by evaluating antioxidant system, reproductive organ weights, some spermatological parametres and serum Testesterone levels. For this purpose, 40 Sprague Dawley rats were divided into 4 groups. 1. Control Group (n=10): No treatment was given for 10 days. 2. *B. Serrata* Group (n=10): *B. Serrata* was given by gavage at a dose of 500 mg·kg^{-1} for 10 days. 3. Methotrexate Group (n=10): Methotrexate was given intraperitoneally as a single dose of 20 mg·kg^{-1}. 4. Methotrexate + *B. Serrata* Group (n=10): After methotrexate was given intraperitoneally as a single dose of 20 mg·kg^{-1}, 500 mg·kg^{-1} *B. Serrata* was given by gavage for 10 days. It was determined that *B. Serrata* significantly increased serum Testosterone levels (P<0.001), testicular GSH levels (P<0.001), motility of sperm (P<0.001), concentration of sperm (P<0.001), absolute ventral prostate (P<0.001) and absolute seminal vesicles (P<0.05) organ weight in Methotrexate + *B. Serrata* group. The decrease in testicular MDA levels (P>0.05) and the increase in GSH-Px enzyme activity of testes (P>0.05) and final body weight (P>0.05) were not significant in Methotrexate + *B. Serrata* group compared to the Methotrexate group. In conclusion, the negative effects of Methotrexate on the male reproductive system can be reduced by administering *B. Serrata* extract.

Key words: *Boswellia serrata* extract; Methotrexate; testicular injury; sperm; oxidative stress

RESUMEN

El objetivo fue determinar el efecto del extracto de *Boswellia Serrata* sobre el daño testicular inducido por metotrexato mediante la evaluación del sistema antioxidante, el peso de los órganos reproductores, algunos parámetros espermatológico y los niveles de testesterona sérica en este estudio. Para ello, 40 ratas Sprague Dawley se dividieron en 4 grupos. 1. Grupo Control (n=10): No se aplicó ningún tratamiento durante 10 días. 2. Grupo de *B. Serrata* (n=10): *B. Serrata* se administró a una dosis de 500 mg·kg^{-1} por sonda durante 10 días. 3. Grupo de metotrexato (n=10): el metotrexato se administró por vía intraperitoneal en una dosis única de 20 mg·kg^{-1}. 4. Grupo de metotrexato + *B. Serrata* (n = 10): el metotrexato se administró por vía intraperitoneal en una dosis única de 20 mg·kg^{-1}, luego se administró 500 mg·kg^{-1} de *B. Serrata* por sonda durante 10 días. Se determinó que *B. Serrata* aumentó significativamente el nivel de testosterona en suero (P<0,001), el nivel de GSH en los testículos (P<0,001) , la motilidad de los espermatozoides (P<0,001), la concentración de espermatozoides (P<0,001), el peso absoluto de los órganos de la próstata ventral (P<0,001) de las vesículas seminales (P<0,05) en el grupo de metotrexato + *B. Serrata,* La disminución en el nivel de MDA de los testículos (P>0,05) y el aumento en la actividad de la enzima GSH-Px de los testículos (P>0,05) y el peso corporal final (P>0,05) no fueron significativos en el grupo de metotrexato + *B. Serrata* en comparación con el grupo de metotrexato. En conclusión, los efectos negativos del metotrexato en el sistema reproductivo masculino pueden reducirse administrando extracto de *B. Serrata*.

Palabras clave: Extracto de *Boswellia serrata*; metotrexato; lesión testicular; semen; estrés oxidativo

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INTRODUCTION

Methotrexate (MTX) is a drug, which used to treat various neoplasms such as acute lymphoblastic leukemia, osteosarcoma, non hodgkin lymphoma and certain types of cancer. Methotrexate, which has anti-proliferative, anti-inflammatory and immunomodulatory effects, is used in low doses in inflammatory diseases [1, 2, 3, 4]. Chemotherapeutic drugs affect many systems such as gastrointestinal, liver, kidney, respiratory and skin [5, 6, 7]. Also many chemotherapy agents, including MTX, can cause infertility [8, 9, 10]. In terms of gonadal toxicity of antimetabolites, the most studied drug in laboratory animals is MTX. Low and medium doses of MTX were reported to cause oligospermia but not testicular atrophy in rats (*Rattus norvergicus*) [11]. In some studies, it has been determined that MTX causes degeneration by decreasing germinal epithelial thickness, seminiferous tubule diameter and testicular weight. Additionally MTX has a lethal effect on all spermatogenic cells, especially spermatocytes and spermatids, and reduces the size of sertoli and leydig cells [8, 12]. MTX causes Deoxyribonucleic Acid (DNA) damage and subsequent apoptosis in germ cells. Additionally, MTX application causes a decrease in epididymal sperm count and motility, and an increase abnormal sperm rates [10, 13].

Oxidative stress plays an key role in the pathogenesis of MTX induced testicular damage [14]. Methotrexate causes an increase in free radicals due to impairment of antioxidant defenses and differences in the pro-inflammatory cytokine system in testicular tissue. Oxidative stress caused by the increase in free radicals causes damage to the seminiferous tubules and causes a decrease in germ cells [9, 15]. As a result, testicular dysfunction and fertility problems occur.

Since the toxic effects of medicinal plants are less than chemicals, medicinal plants are widely used to prevent tissue damage. *Boswellia* species (Burseraceae) are one of the most used herbal plants worldwide. *Boswellia serrata* is a plant species belonging to the Burseraceae family. Many pharmacological effects of *B. serrata* such as antioxidant, antiinflamatory, anticancer, antidiabetic have been reported [16]. Most of the effects of *B. serrata* are due to the boswellic acids its contains [17]. Aqueous extracts of *B. serrata*, boswellia oil and methanolic leaf extract's antioxidant activity is dose dependent [16, 17, 18]. *Boswellia* species are used all over the world. However, there is limited literature information on the effect of *B. serrata* on the male reproductive system. This aim of this study was to investigate the effect of *B. serrata* on MTX-induced testicular damage by examining the antioxidant effect of *B. serrata*.

MATERIALS AND METHODS

Animals and experimental design

Firat University Local Animal Use Committees (Elazig, Türkiye) approved the experimental protocols of this study with protocol number 2022/18-03. Forty healthy adult male Sprague Dawley rats were procured (10-12 weeks/250-300 g) and maintained from Firat University Experimental Research Centre (Elazig, Türkiye). Polycarbonate cages (Tecniplast Laboratory Animal Equipment, Italy) were used to the animals kepting (a 12 h day night cycle and temperature of 24 ± 3°C). Standard commercial pellet food and fresh drinking water were given to animals as *ad libitum*.

The rats were divided into 4 groups, after a one-week adaptation period. 1. Control Group (n=10): No treatment was applied for 10 days. 2. *B. serrata* Group (n=10): *B. serrata* was given at 500 mg·kg^{-1} dose [19] by gavage for 10 days. 3. Methotrexate Group (n=10): Methotrexate was given by intraperitoneally as a single dose of 20 mg·kg^{-1} [9]. 4. Methotrexate + *B. serrata* Group (n=10): Methotrexate was given by intraperitoneally as a single dose of 20 mg·kg^{-1} [9], then 500 mg·kg^{-1} *B. serrata* [19] was given by gavage for 10 days.

ELISA analysis

The blood samples were taken into serum tubes and centrifuged (Nüve NF800R, Türkiye) (3220 G/10 min), the serums were separated. Serum Testosterone levels of all animals were measured using rat specific enzyme linked immunosorbent assay (ELISA) kits (sunred 201-11-0260) according to the manufacturer's recommendations. The standard curve was used to determined the concentration of Testosterone hormone.

Analysis of malondialdehyde and antioxidant enzyme activity

Testicular tissue samples were weighed and then homogenized with a mechanical homogenization device (IKA Ultra-Turrax T25) using Tris Buffer (dilution ratio: 1/10, g/v) while maintaining their coldness. The homogenates were centrifuged at 3220 G for 60 min and the supernatant was separated [20]. Malondialdehyde (MDA), Glutathione (GSH) levels, Glutathione peroxidase (GSH-Px) enzyme activities and total protein content were measured from the supernatant. Total protein content was determined according to Lowry method [21] by using spectrophotometre (Shimadzu, UV-1700 PharmaSpec, Kyoto Japan). The method described by Placer *et al.* [22] was used for MDA analysis using spectrophotometer. The method described by Sedlak and Lindsay [23] was used for GSH analysis using spectrophotometer. The method described by Lawrence and Burk [24] was used for GSH-Px enzyme analysis using spectrophotometer.

Analysis of spermatological parameters

Reproductive organs (testes, ventral prostate, seminal vesicles and cauda epididymis) were removed and weighed (HR-250AZ,Türkiye). Epididymal sperm concentration was determined using the hemocytometer method. The right epididymis was minced in 1 mL of 0,09% NaCl and incubated at room temperature for 4 h. After incubation semen was drawn up to the 0.5 line of the pipette (red blood cell count pipette) and 2% eosin solution was drawn up to the 101 line of the pipette. The diluted sperm suspension was transferred to Thoma lame counting chambers (the volume is 0,1 mm^3) and counted at 400 (10×40) magnification with using a light microscope (Nikon eclipse Ci-L, Japan). The result was expressed in million/right cauda epididymis. Freshly isolated left cauda epididymal tissue was used for analysis motility of sperm. Percent motility of sperm was assessed using a light microscope (heated stage, 37°C) (Nikon eclipse Ci-L, Japan). For determining abnormal sperm (morphological) percentage, Tris buffer spermatozoa suspension was mixed with eosin nigrosine stain (1.67% eosin, 10% nigrosine and 0.1 M sodium citrate) and peripheral smear slides were prepared, and examined under a light microscope at 400 (10×40) magnification. A total of 200 spermatozoa were examined per slide and the abnormality rates of spermatozoa (total, tail and head) were expressed as a percentage [25, 26].

Statistical analysis

It was determined by Shapiro Wilk normality analysis whether the values obtained as a result of the study showed normal distribution.

As a result of Shapiro Wilk normality analysis, group means normally distributed data were determined by Anova one-way analysis of variance, and differences between groups were determined by Duncan test. Significance level was accepted as $P<0.05$. SPSS package program (IBM SPSS Version 22.0) was used for statistical evaluations. Data were given as Mean ± Standard Deviation ($\bar{x} \pm SD$).

RESULTS AND DISCUSSIONS

Methotrexate is the most usually used anticancer drug due to its significant benefits. However, it also has side effects on all organs of the body. Due to the high mitotic activity of the testicular germinal epithelium, testicular germinal epithelium is very sensitive to damage caused by cytotoxic drugs [27]. In this study the effects of *B. serrata* on testicular damage caused by MTX were investigated by evaluating body weight, serum Testosterone levels, oxidative stress parameters and spermatological parameters.

In this study, the final body weight and serum Testosterone levels of rats were significantly reduced in the Methotrexate groups ($P<0.001$) compared to the control group. However, compared to the Methotrexate group while there was no significant difference in final body weight in the Methotrexate + *B. serrata* group ($P>0.05$), a significant increase in serum Testosterone level was determined ($P<0.001$) (FIG 1). Also there was no difference between the control group and the *B. serrata* group ($P>0.05$) in final body weight and Testosterone levels ($P>0.05$). Testicular MDA levels increased ($P<0.001$) significantly, testicular GSH-Px enzyme activities reduced ($P<0.01$) significantly and there was no significant difference in GSH levels ($P>0.05$) in Methotrexate group compared to the control group. Compared to the Methotrexate group, while there was no significant difference in GSH-Px enzyme activities and malondialdehyde levels ($P>0.05$) in the Methotrexate + *B. serrata* group, a significant increased in GSH level ($P<0.001$) was determined (TABLE I).

When the effect of *B. serrata* on spermatological parameters and reproductive organs weight were examined, it was observed that sperm motility ($P<0.001$), sperm concentration ($P<0.001$), absolute seminal vesicles organ weight ($P<0.05$) and absolute ventral prostate organ weight ($P<0.001$) significantly decreased in the Methotrexate group. When abnormal spermatozoon rates were examined, it was observed that tail and total abnormal spermatozoon rate ($P<0.01$) significantly increased in *B. serrata* and Methotrexate groups compared to the control group. However compared to the Methotrexate group while there was no significant difference in tail and total abnormal spermatozoon rate ($P>0.05$) in the Methotrexate + *B. serrata* group, a significant increase in sperm motility ($P<0.001$), sperm concentration ($P<0.001$), absolute seminal vesicles organ weight ($P<0.05$) and TABLES II and III). The study showed that MTX causes testicular damage by increasing oxidative stress and MTX causes deterioration in sperm parametres and Testosteron levels. This is similar to several study on MTX-induced testicular damage [9, 28, 29, 30]

Al-Yahya *et al.* [31] reported that the up to 1000 mg·kg^{-1} boswellic acid was safe in rats. Singh *et al.* [19] stated that no side effects were observed at the 500 mg·kg^{-1} dose of *B. serrata*. Sami *et al.* [32] declared that a dose of 500 mg·kg^{-1} *B. serrata* was more ameliorative than the lowest dose. Therefore, in this study, *B. serrata* dose was chosen as 500 mg·kg^{-1}. Nusier *et al.* reported that doses of *B. serrata* differed in their effects on the rat reproductive system [33].

Some studies [34, 35] have reported that MTX administration causes a decrease in body weight. Padmanabhan *et al.* reported that MTX administration (10 and 20 mg·kg^{-1}, once a week, 10 weeks) resulted in a reduction in final body weight [34]. Armağan *et al.* [35] reported that a 20 mg·kg^{-1} single dose of MTX administered on the first day caused a decrease in final body weight. This study is parallel to many studies on MTX [8, 33, 34, 35]. However, the increase in final body weight was not statistically significant in the Methotrexate + *B. serrata* group compared to the MTX group (FİG. 1). Singh *et al.* explained that the 500 mg·kg^{-1} dose group had no effect on the body weights of rats. This study is similar to the study by Singh *et al.* [19].

Oxidative stress is an important factor in male infertility [36]. Therefore, studies suggest that testicular MTX toxicity can be prevented or reduced by the use of antioxidant agents (enzymatic and non-enzymatic) in studies. Different doses of MTX cause oxidative stress, and can have harmful effects on the testicular tissue [8, 34, 37]. In this study, the high MDA levels and low GSH-Px enzyme activities in the MTX group are similar to many studies investigating MTX induced

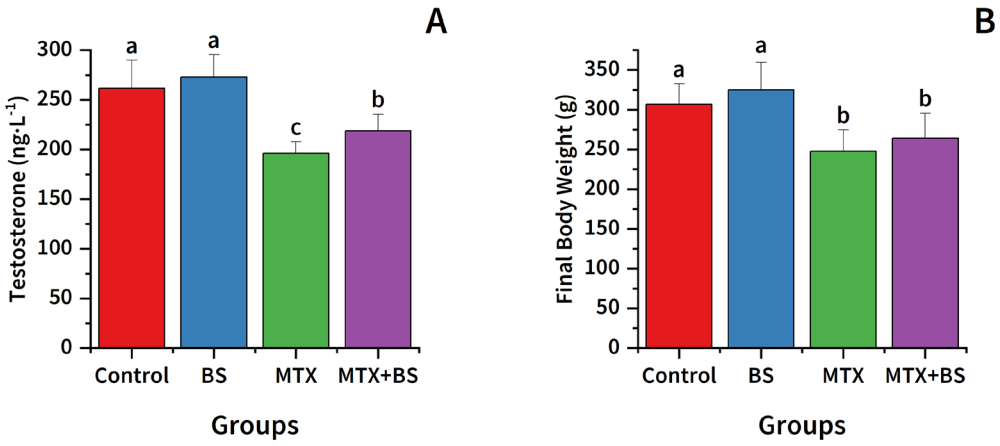

FIGURE 1. (A) Serum Testosterone levels of groups ($P<0.001$), (B) Body Weight ($P<0.001$). Values were expressed as mean and standard deviation ($\bar{x} \pm SD$)

TABLE I
Malondialdehyde Levels, Glutathione Levels and Glutathione Peroxidase Enzyme Activity in Testes Tissue (Mean ± SD)

Groups	MDA (nmol·g^{-1} tissue)	GSH (nmol·g^{-1} tissue)	GSH–Px (IU·gr prot^{-1})
Control	72.64 ± 10.02^{b}	1.84 ± 0.29^{b}	122.58 ± 15.47^{a}
BS	77.12 ± 18.70^{b}	1.91 ± 0.35^{b}	133.73 ± 11.05^{a}
MTX	137.27 ± 41.11^{a}	1.86 ± 0.11^{b}	97.25 ± 17.40^{b}
MTX+BS	118.71 ± 38.78^{a}	2.55 ± 0.18^{a}	100.10 ± 29.12^{b}
Significant	*P*<0.001	*P*<0.001	*P*<0.01

oxidative stress in testicular tissue [9, 37, 38, 39]. MTX causes cells to become sensitive to ROS and reduces the antioxidant enzyme system effectiveness in the cells. In this study, the decrease in MDA levels and increase GSH-Px enzyme activity level in the Methotrexate + *B. serrata* group wasn't significant compared to the Methotrexate group (TABLE I). But increased GSH levels in testicular tissue were statistically significant in Methotrexate + *B. serrata* group (TABLE I). Doaee *et al.* suggest that *B. serrata* resin extract plays a role as an antiinflammatory and antioxidant agent, protecting nigrostriatal dopaminergic neurons and ameliorating motor disorders in rat with Parkinson's disease [40]. Tohamy *et al.* reported that *B. serrata* showed a protective effect against testicular damage caused by Fipronil poisoning on the male rat model for 60 days [41]. In this study, significant difference in antioxidant properties of *B. serrata* was determined only in GSH levels (*P*<0.001) (TABLE I). Gupta *et al.* in a study, which they examined the relationship between the antioxidant, antiproliferative and antimicrobial activities of topographically collected *B. serrata* oleo gum resin and the boswellic acid concentration, showed that the extracts antioxidant activities could be because of the percentage of boswellic acids (BAs)[42].

MTX induced oxidative stress caused testicular damage and reduced Testosterone levels by directly effect spermatogenic, sertoli and leydig cells and therefore reduced Testosterone levels [43]. The sperm is highly susceptible to damage caused by oxidative stress. This leads to loss of sperm motility and reduced sperm count. In this study, the concentration of sperm, motility of sperm (TABLE II) and Testosterone concentration of serum (FIG. 1A) was decreased in the Methotrexate group. The concomitant administration of *B. serrata* with MTX significantly increased motility of sperm, concentration of sperm and Testosterone levels of serum as shown in FIG. 1A and TABLE II.

These results are parallel to the study of Tohamy *et al.* [41], in which they reported the protective effect of BA on motility of sperm, concentration of sperm and Testosterone level of serum. In this study the tail and total abnormal spermatozoon rate (*P*<0.01) increased in *B. serrata*, Methotrexate and Methotrexate + *B. serrata* groups (TABLE II) compared to the control group. The decrease in the abnormal spermatozoa rate was not statistically significant in the Methotrexate + *B. serrata* group (*P*>0.05)(TABLE II). However, while *B. serrata* reduced this adverse effect, the *B. serrata* group had the highest in the rate of anormal spermatozoon compared to the control group (*P*<0.01)(TABLE II). In the study by Tohamy *et al.* [41] in which they invigestigated the effect of boswellic acid on Fipronil induced male reproductive toxicity, it was observed that the rate of coiled tail and bent head sperm in the boswellic acid group at a dose of 500 mg·kg^{-1} was higher than the control group.

Additionally, MTX caused a decrease in ventral prostate weight (*P*<0.001) and seminal vesicles weights (*P*<0.05). However, in this study, the decrease in testes, epididymis, cauda epididymis organ weights as a result of MTX application was not found to be significant (*P*>0.05) (TABLE III). There are different results regarding male reproductive organ weights in studies conducted with a dose of 20 mg·kg^{-1} Methotrexate. Yüncü *et al.* reported that intraperitoneal MTX caused a decrease in testicular weight [43]. Aslankoc *et al.* reported that intraperitoneal MTX did not affect testes weight, but MTX caused a decrease in epididiymis organ weights [44]. Güvenç *et al.* reported that intraperitoneal MTX did not affect weights of testes, epididiymis, and cauda epididymis organs, but MTX administration led to a decrease in absolute ventral prostate weight and seminal vesicles weight [9]. This study is similar to the study of Güvenç *et al.* [9]. In this study; *B. serrata* showed a positive effect on the MTX induced reduction in seminal vesicles (*P*<0.05) and ventral prostate weight (*P*<0.001), (TABLE III). This study is similar to the study of Tohamy *et al.* [41].

TABLE III
Effects of *Boswellia serrata* on Absolute Reproductive Organ Weights (g) (Mean ± SD)

	Absolute Reproductive Organ Weights (g)				
Groups	Testes (Right+Left)/2	Epididymis (Right+Left)/2	Cauda Epididymis	Seminal Vesicles	Ventral Prostate
Control	1,36 ± 0,15	0,49 ± 0,02	0,20 ± 0,03	1,28 ± 0,16^{a}	0,42 ± 0,07^{a}
BS	1,43 ± 0,09	0,48 ± 0,03	0,20 ± 0,01	1,30 ± 0,36^{a}	0,45 ± 0,09^{a}
MTX	1,35 ± 0,12	0,46 ± 0,05	0,18 ± 0,02	0,96 ± 0,30^{b}	0,17 ± 0,03^{c}
MTX+BS	1,41 ± 0,15	0,49 ± 0,07	0,20 ± 0,03	1,24 ± 0,20^{a}	0,24 ± 0,07^{b}
Significant	*P*>0.05	*P*>0.05	*P*>0.05	*P*<0.05	*P*<0.001

TABLE II
Effects of *Boswellia serrata* on Spermatological Parametres (Mean ± SD)

	Spermatological Parameters				
Groups	Motility (%)	Sperm concentration (million / cauda epididymis)	Abnormal spermatozoon rate (%)		
			Head	Tail	Total
Control	75.18 ± 12.38^{a}	127.90 ± 50.97^{a}	3.01 ± 1.37	2.44 ± 0.68^{b}	5.40 ± 1.57^{b}
BS	68.66 ± 13.98^{a}	127.90 ± 30.22^{a}	3.57 ± 0.64	4.90 ± 1.66^{a}	8.14 ± 0.87^{a}
MTX	29.16 ± 16.97^{b}	47.75 ± 21.09^{c}	4.11 ± 1.96	5.00 ± 1.5^{a}	8.37 ± 2.73^{a}
MTX+BS	68.14 ± 23.83^{a}	90.25 ± 29.14^{b}	3.00 ± 0.94	4.11 ± 1.59^{a}	6.80 ± 1.81^{ab}
Significant	*P*<0.001	*P*<0.001	*P*>0.05	*P*<0.01	*P*<0.01

CONCLUSION

In conclusion, significant improvement was determined in motility of sperm, count of sperm, Testosterone level of serum and GSH level of testes. However no significant difference was detected in abnormal spermatozoon rate, MDA level and GSH-Px enzyme activity after *B. serrata*. Different doses of *B. serrata* should be investigated through different mechanisms in MTX induced testicular damage. At the same time, it was thought that *B. serrata* application should be studied at different times.

Limitations of the study

We were not able evaluate the amount of boswellic acids in *B. serrata* extract and histology of the testis due to the budget constraint and lack of equipment.

Conflict of interest

The authors declare no conflicts of interest.

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