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Investigation of the Protective Effect of Thymbra Spicata L. var Spicata and Cyclotrichium Origanifolium on Bladder in Experimental Traumatic Spinal Cord Injury

Deneysel Tavmatik Omurilik Hasarında Thymbra Spicata L. var Spicata ve Cyclotrichium Origanifolium'un Mesane Üzerine Koruyucu Etkisinin Araştırılması

ABSTRACT

Objective:

Spinal cord injuries typically result from trauma-induced fractures, dislocations, ligament injuries, and tears in the vertebral column. Among the most commonly affected organs in these injuries is the bladder. Thymbra spicata L. var spicata (zahter) and Cyclotrichium origanifolium (mountain mint) are known for their high biological potential; their extracts possess antioxidant properties that help mitigate the risk of diseases caused by oxidative stress and play a significant role in treatment. This study aimed to investigate the potential therapeutic and protective effects of zahter and mountain mint extracts on bladder dysfunctions following spinal cord injury.

Material and Methods:

The study included 36 male Wistar Albino rats weighing between 250-300 g, divided into six groups, each containing six rats: Group 1 (control), Group 2 (zahter), Group 3 (mountain mint), Group 4 (trauma), Group 5 (trauma + zahter), and Group 6 (trauma + mountain mint). A trauma model was created in the trauma groups using a weight-drop method on the spinal cord. At the end of the week, urine samples were collected from all rats, and the animals were sacrificed under ketamine anesthesia to obtain bladder tissues. Histopathological changes in the bladder tissues were subsequently examined.

Results:

The experimental study demonstrated that zahter and mountain mint extracts were partially effective against bladder damage caused by spinal cord injury. Zahter exhibited a higher efficacy in reducing oxidative stress and inflammatory markers compared to mountain mint, as evidenced by biochemical and histopathological findings.

Conclusion:

The findings suggest that with the increasing application of experimental models, the therapeutic use of plant extracts may become more widespread and contribute positively to human health.

Key Words:

Spinal cord injury, Bladder, Antioxidant, Thymbra spicata L. var spicata, Cyclotrichium origanifolium

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ÖZ

Amaç:

Spinal kord yaralanmaları, genellikle travma sonrası vertebral kolonda meydana gelen kırıklar, çıkıklar, bağ yaralanmaları ve yırtılmalar sonucunda oluşan hasarları kapsamaktadır. Bu yaralanmalarda komplikasyonların en sık görüldüğü organlardan biri mesanedir. Thymbra spicata L. var spicata (zahter) ve Cyclotrichium origanifolium (dağ nanesi) bitkilerinin yüksek biyolojik potansiyele sahip olduğu; ekstraktlarının antioksidan özellikleri sayesinde oksidatif stresin neden olduğu hastalık riskini azalttığı ve tedavide önemli bir rol oynadığı bilinmektedir. Bu çalışmada, zahter ve dağ nanesi ekstraktlarının spinal kord yaralanması sonrası mesane disfonksiyonları üzerindeki potansiyel tedavi edici ve koruyucu etkilerinin araştırılması amaçlanmıştır.

Gereç ve Yöntemler:

Çalışmada, ağırlıkları 250-300 g arasında değişen toplam 36 adet Wistar Albino erkek sıçan, her grupta altı sıçan olacak şekilde altı gruba ayrılmıştır: Grup 1 (kontrol), Grup 2 (zahter), Grup 3 (dağ nanesi), Grup 4 (travma), Grup 5 (travma + zahter), Grup 6 (travma + dağ nanesi). Travma gruplarında omuriliğe ağırlık düşürme yöntemi uygulanarak travma modeli oluşturulmuştur. Bir haftasının sonunda, tüm sıçanlardan idrar örnekleri alındıktan sonra, ketamin anestezisi altında sakrifiye edilerek mesane dokuları alınmıştır. Mesane dokularında histopatolojik değişiklikler incelenmiştir.

Bulgular:

Deneysel araştırma sonucunda, zahter ve dağ nanesi ekstraktlarının spinal kord yaralanmasına bağlı gelişen mesane hasarına karşı kısmen etkili olduğu saptanmıştır. Zahterin etkinliğinin, biyokimyasal ve histopatolojik bulgular doğrultusunda, oksidatif stres ve inflamatuar belirteçler üzerindeki etkisinin dağ nanesine kıyasla daha yüksek olduğu gözlemlenmiştir.

Sonuç:

Sonuç olarak, deneysel modellerin artmasıyla birlikte bitki ekstraktlarının tedavi amaçlı kullanımının yaygınlaşabileceği ve insan sağlığı üzerinde olumlu etkiler sağlayabileceği düşünülmektedir.

Anahtar Kelimeler:

Spinal kord yaralanması, Mesane, Antioksidan, Thymbra spicata L. var spicata, Cyclotrichium origanifolium

INTRODUCTION

Spinal cord injuries mostly include damage caused by fractures, dislocations, ligament injuries, and tears that occur in the vertebral column after trauma. Spinal cord trauma remains a serious cause of morbidity and mortality today (1).

Primary and secondary damages usually occur in spinal cord injury. Although primary injury is a strong prognostic indicator, it is very important in determining the patient's initial neurological damage. The first findings that appear after this injury usually cause mild hemorrhages in the gray and white matter, axonal degeneration, and membrane damage. In secondary injury, different mechanisms occur at the cellular and molecular level in the next few days after the injury (2). Important conditions such as tissue edema, vascular events such as bleeding and ischemia, neurogenic shock, fluid-electrolyte disorders, mitochondrial disorders, excitotoxicity, apoptosis, and immunological damage occur (3).

Spinal cord injury due to trauma not only creates an acute inflammatory response directly in the medulla spinalis but also can cause secondary injuries in other organs (such as the liver, lung, kidney, and bladder) by affecting the systemic inflammatory response syndrome at the cellular, textural and molecular levels. In this case, many relevant tissues are affected and their functions are impaired in spinal cord injuries (4).

The majority of spinal cord injuries affect bladder and bowel functions. Because the nerves that control the relevant organs begin near the lower end of the spinal cord and lose normal brain input with damage. The bladder is among the organs where complications are most common (4). Studies have shown that spinal cord injuries cause significant structural, physiological, and molecular changes in the bladder. After the spinal shock period following the injury, bladder storage or emptying functions may be impaired due to hyperreflexia of the bladder, and toxic effects may occur as a result of the reactive substances formed. Measurement of microalbuminuria and creatinuria is very important in patients with chronic kidney damage. Microalbuminuria is defined as urinary albumin excretion of 30-300 mg/day or spot urine microalbumin/creatinine ratio of 30-300 mg/g. Microalbuminuria is an important marker of clinical nephropathy and cardiomyopathy.

Neurogenic bladder is a serious disease that often impairs the patient's quality of life and can also affect life. Increased vesico-urinary pressure is threatening as it may cause renal failure. Although the life-threatening effects of the neurogenic bladder have recently diminished with the development of modern therapeutic options, renal complications associated with neurogenic bladder still pose a significant problem in urological practice. Oxidative stress affects many organs in humans. Increased oxidative stress in tissue and organ damage also causes changes in some

markers. The aim of treating neurogenic bladder resulting from spinal cord injury is to suppress detrusor activity and increase the internal bladder volume. Considering the factors that play a role in the pathogenesis of tissue damage due to spinal cord injuries, it is thought that antioxidant and anti-inflammatory agents may have clinical benefits in spinal cord injury (5).

Cyclotrichium origanifolium (mountain mint); has traditionally been used as a sedative, relaxant, carminative, and for the treatment of respiratory ailments. Studies have shown that mountain mint has a strong free radical scavenging activity and therefore has a high antioxidant and antimicrobial effect. In the study conducted by Guzel et al., it was determined that both the prepared extracts and the compounds obtained from these extracts showed very high antioxidant activity (6).

Thymbra spicata L. var spicata (zahter) genus belongs to the Lamiaceae family. In some experimental studies, its antioxidant, antitumor, analgesic, antimicrobial and neuroprotective effects have been demonstrated. In a study conducted by Gedikoğlu et al., in 2019; The essential oil of zahter species was obtained by hydrodistillation method, and its biological activity was examined. It was determined that the essential oil obtained in this study had very high antioxidant and antimicrobial activity (7).

Both zahter and mountain mint have a high biological potential. The antioxidant potential of these plant extracts reduces the risk of diseases caused by oxidative stress and is of particular importance in treatment (8).

Accordingly, after spinal cord injury, many tissues are affected and their functions are impaired. The main ones are complications such as neurogenic bladder, renal failure, and infection that develop after trauma. In addition to medical treatments, alternative medicine treatment methods are tried to prevent these complications, which generally cause permanent damage that impairs the quality of life of patients. It has been shown that zahter and mountain mint, which are widely used for different purposes, have high antioxidant and anti-inflammatory activities and that zahter species also have a protective effect. However, there is not enough information about its effectiveness on bladder dysfunctions due to spinal cord damage. We think that zahter and mountain mint may be healing due to the high effects of oxidative stress on the damage caused by spinal cord injury.

This study aimed to investigate the potential therapeutic and protective effects of zahter and mountain mint on bladder dysfunctions after spinal cord injury.

MATERIAL and METHODS

Animals and Experimental Groups

This study was approved by the Dicle University Animal Experiments Local Ethics Committee under protocol number 2019/08 dated 30/05/2019. The bladder tissues used in this research were obtained from the ethics-approved project but were not utilized in the original project. These tissues were analyzed within the scope of this study. The experimental animals used in this study were obtained from the experimental animal laboratory at Dicle University.

8-10- week-old Wistar Albino male rats weighing between 250-300 g were used in the study. The rats were fed with a normal diet and tap water without any restrictions in stainless steel cages at 22±2 ºC under 12 hours of light and 12 hours of darkness. Ketamine and Xylazine were administered intraperitoneally to the rats that underwent surgery to create spinal cord trauma. The rats were placed in the prone position on cork blocks. Sterilization was achieved with PVD iodine. A midline incision was made between T5 and T12. After the paravertebral muscles were stripped and the laminae were exposed, T7-T8-T9 laminectomy was performed. A steel rod with a diameter of 3mm and a weight of 10g was dropped from a height of 10cm to create a spinal cord injury (9). Following the trauma procedure, the weight was removed, and the muscle and skin incisions were closed with sutures.

Thirty minutes after the induction of trauma, 4000 ppm/kg doses of Thymbra spicata and Cyclotrichium origanifolium extracts were administered intraperitoneally once daily for one week, according to the groups. The control group received an equivalent dose of intraperitoneal physiological saline.

36 Wistar Albino rats were divided into six groups, with six rats in each group:

Group I: Control: The rats in this group were not exposed to trauma. Only placebo saline was applied.

Group II: Zahter: Rats in this group were not exposed to trauma. 4000ppm/kg/day zahter dissolved in 1% ethanol solution was applied IP for 1 week.

Group III: Mountain mint: Rats in this group were not exposed to trauma. 4000 ppm/kg/day mountain mint dissolved in 1% ethanol solution was applied IP for 1 week.

Group IV: Trauma: The rats in this group were traumatized. Only placebo saline was applied.

Group V: Trauma+ Zahter: The rats in this group were traumatized. 4000 ppm /kg/day zahter dissolved in 1% ethanol solution was applied IP for 1 week.

Group VI: Trauma+ Mountain mint: The rats in this group were traumatized. 4000ppm/kg/day mountain mint dissolved in 1% ethanol solution was applied IP for 1 week.

Motor functions of animals given zahter and mountain mint or physiological saline IP according to groups for 1 week, 30 minutes after the trauma; It was evaluated according to the Tarlov scale motor function score method. The evaluation of the motor examination was made by observing and recording the motor examination according to the Tarlov scale before the procedure, at the 1st hour after the procedure, and before decapitation. Motor responses; 0: Completely paralytic; 1: There is minimal movement in the joints; 2: He moves his hind legs well but cannot stand up; 3:Can stand up but cannot walk normally; It was evaluated as 4: He can walk normally. All rats subjected to spinal cord injury exhibited paraplegia after the trauma. On the 8th day, urine samples were collected from all rats before they were sacrificed under ketamine anesthesia. After sacrifice, bladder tissues were collected for histopathological analysis. The urine samples were subjected to biochemical analysis to measure microalbuminuria, creatinuria, and urea levels.

Plant samples and Obtaining Essential Oils

Thymbra spicata L. var. spicata and Mountain mint species were collected from nature and dried in the shade. The aboveground parts of the plants dried in the shade were turned into small particles and boiled with hot water in a distillation device (Clevenger apparatus). The essential oil carried away by the resulting vapor was condensed in the cooler and collected in a container. The essential oils obtained were freed from the water they contained by keeping them in Na2SO4 at +4 ˚C. For application, stock solutions were prepared with ethanol solvent at a concentration 4000 ppm. Mountain mint and Zahter essential oils have evaporation properties. For this reason, closed, air- and light-tight storage containers were used.

Tissue analysis

Bladder tissues were subjected to routine paraffin tissue testing. After fixation (24 hours), tissues were washed (1 night), and passed through increasing alcohol series (50%, 70%, 80%, 90%, 96% , and absolute ethyl alcohol series). After clearing (3x30 minutes in xylene), it was subjected to paraffin infiltration at 58°C. The tissues were then embedded in paraffin blocks. 4-6 µm thick sections were taken from the blocks for hematoxylin-Eosin staining with the help of a microtome (catalog no: Leica RM2265, Wetzlar, Germany).

Hematoxylin-Eosin Staining

Bladder tissue sections taken from paraffin blocks were placed in a double boiler set at 37°C. The sections were kept in an oven at 58-62°C for 6 hours to melt excess paraffin on the slide. Sections were deparaffinized in xylene for 3x15 minutes. The sections were passed through decreasing alcohol series (100%, 96%, 90%, 70%, 50% ethyl alcohol) for 10 minutes each. The sections were transferred to distilled water and kept for 5 minutes. After soaking in Harris Hematoxylin dye for 8 minutes, the sections were washed under running water for 5 minutes. Sections were rinsed and soaked in alcoholic eosin stain for 6 minutes. Sections were quickly immersed in increasing alcohol series (passing through 80%, 90%, and 96% ethyl alcohol series). Sections were kept in absolute alcohol for 2 minutes. Finally, the sections were kept in xylene for 3x15 minutes and covered with a coverslip by dropping Entellan onto the tissue. Sections were examined under an A2 imager Zeiss light microscope.

Device for studying biochemical parameters

Clinical Chemistry tests were performed on Abbot Company's Architech brand C1600 model Autoanalyzer device. Micro-albumin, Creatinine, and urea tests in urine were studied photometrically.

Statistical analysis

While all experimental groups were evaluated, the R 3.6.3 version of the R studio IDE program was used for statistical analysis.

While evaluating the study data, since we could not make parametric test assumptions in all of the data, Kruskal-Wallis, a non-parametric statistical test, was preferred to test differences between more than two groups. Due to the small data volume and the suitability of the data structure for non-parametric tests, Bonferroni correction and Dunn post-hoc tests were used to reduce the risk of Type-I error and increase the power of the test.
The results with p<0. 005 was considered statistically significant.

RESULTS

Parameters Examined in Urine

Microalbuminuria

Kruskal-Wallis test was used to determine the difference between groups for microalbuminuria values in urine and no significant difference was found between groups. (p=0,08) (Figure 1).

Creatinuria

Kruskal-Wallis test was used to determine the difference between groups for urine creatinuria values and the difference between groups was found to be significant (p=0,001) (Figure 2). When we examined which pairwise groups the difference occurred with Dunn's pairwise test, it was found to be significantly lower in the mountain mint (Median=69.33) group than in the trauma group (Median=130.77) (p=0,008). At the same time, it was found to be significantly lower in the trauma+mountain mint (Median=70.13) group than in the trauma (Median=130.77) group (Bonferroni correction p=0.023).

Urea

Kruskal-Wallis test was used to determine the difference between groups for urea values in urine and the difference between groups was found to be significant (p = 0.000) (Figure 3).

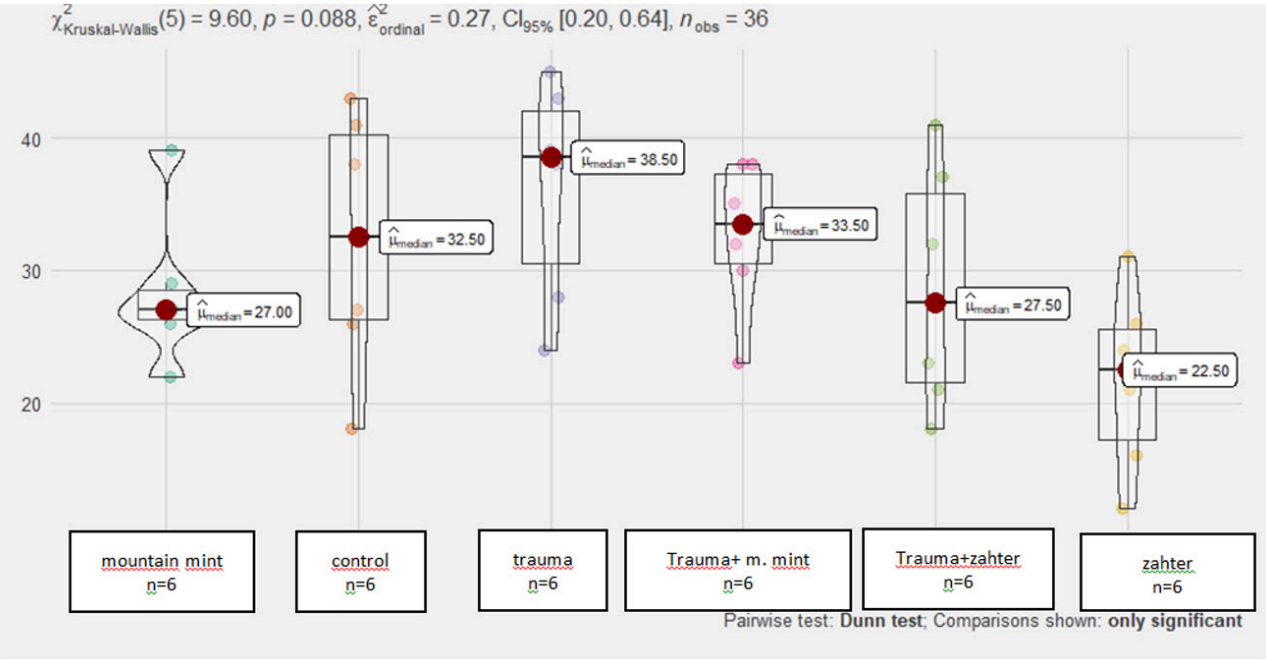

Figure 1: Intergroup distribution and statistics of microalbuminuria(mg/L) value

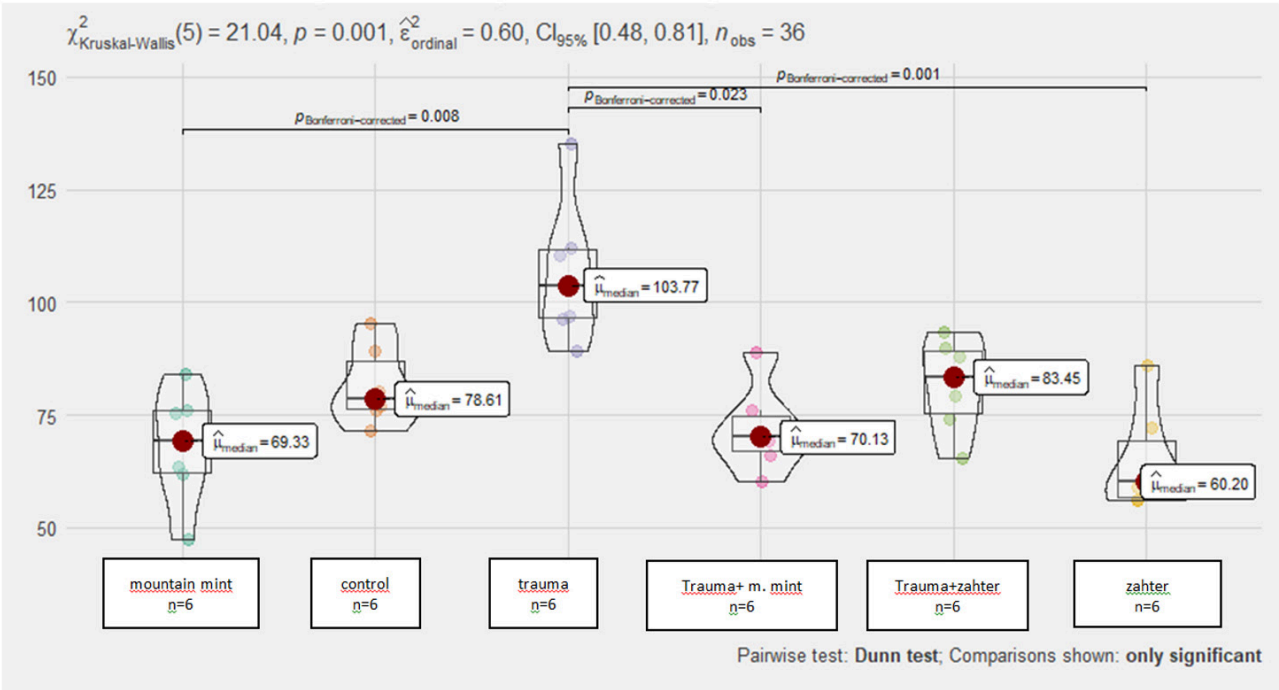

Figure 2: Intergroup distribution and statistics of creatinuria (mg/dL) value

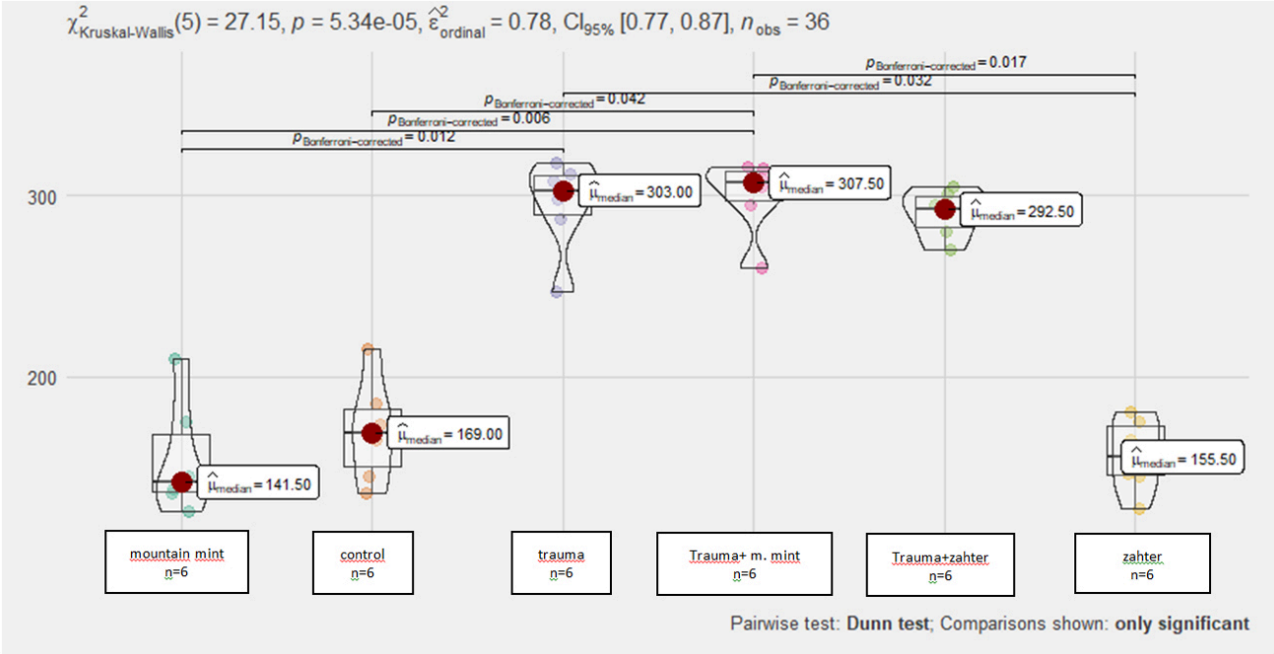

Figure 3: Distribution and statistics of urea(mg/dL) value between groups

For the urea values, significance was observed in the following five pairs.

It was observed that urea levels was significantly higher (Bonferroni correction p=0.012) in the trauma (Median=303.00) group than in the mountain mint (Median=141.50) group.

It was observed that urea levels was significantly higher (Bonferroni correction p=0.006) in the trauma+mountain mint (Median=307.50) group than in the mountain mint (Median=141.50) group.

It was found to be significantly (Bonferroni correction p=0.042) higher in the trauma+mountain mint (Median=307.50) group than the control (Median=169.00) group.

It was observed that urea levels was significantly higher (Bonferroni correction p=0.017) in the trauma+mountain mint (Median=307.50) group than in the zahter (Median=155.50) group.

It was observed that urea levels was significantly higher (Bonferroni correction p=0.032) in the trauma (Median=303.00) group than in the zahter (Median=155.50) group.

Histopathological examination

In the control group, it was observed that the epithelial layer was multilayered, collagen fibers appeared regular in the underlying connective tissue, and the vessels were in normal course. No pathology was found in the sections in this group (Figure 4A).

In the Zahter group, thinning and apoptotic nuclei (arrow) in the epithelial layer, and a regular course of underlying connective tissue collagen fibers were observed. Solitary erythrocyte distribution and small-scale mononuclear cell infiltration (yellow arrow) were observed in the connective tissue under the epithelial layer (Figure 4B).

In the Mountain Mint group, local degeneration (arrow) was observed in the epithelial layer, and local collagen fibers were observed to be degenerated (asterisk) in the underlying connective tissue.

Except for the occasional observation of erythrocytes in the connective tissue area, a near-normal image was observed in the sections in this group (Figure 4C).

In the trauma group, compared to the control group, thinning and ruptures in the epithelial layer (black arrow), apoptosis in the epithelial cells (yellow arrow), secondary degeneration in the underlying connective tissue layer (red asterisk), and occasional lymphocyte accumulation (yellow asterisk) were observed (Figure 4D).

In the trauma+zahter group, it was observed that there was an improvement in the epithelial tissue compared to the trauma group, but degeneration and thinning of the epithelium (black arrow) continued in some places. The connective tissue layer was observed to be regular, but in addition to congestion and dilatation (red star) in the vessels, the presence of solitary lymphocytes (yellow asterisk) was observed here and there (Figure 4E).

In the trauma + mountain mint group, it was observed that there was an improvement in the epithelial tissue compared to the trauma group, but degeneration and thinning of the epithelium (black arrow) continued in some places. Ruptures and degeneration in connective tissue collagen fibers (yellow asterisk), as well as congestion and dilatation in vessels (red asterisk), were detected (Figure 4F).

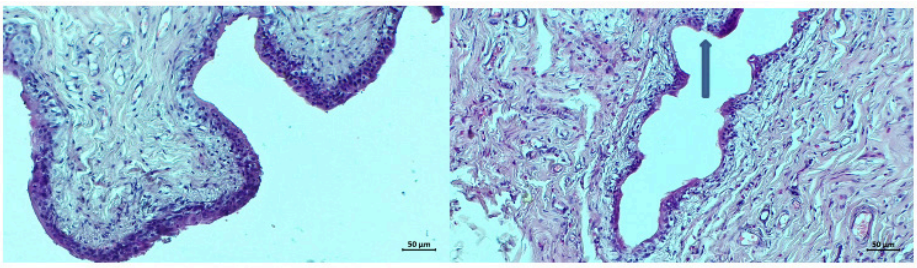

Figure 4: A) control B) mountain mint

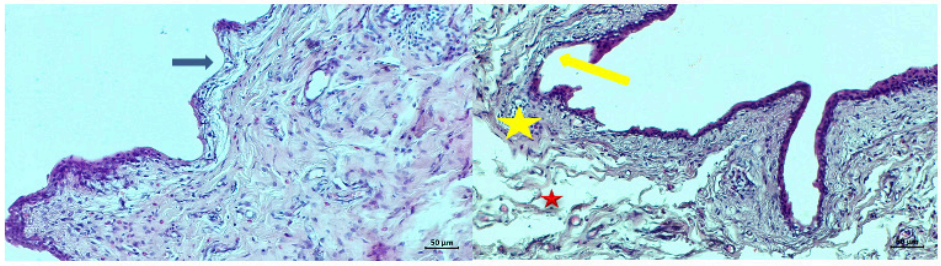

Figure 4: C) zahter D) trauma

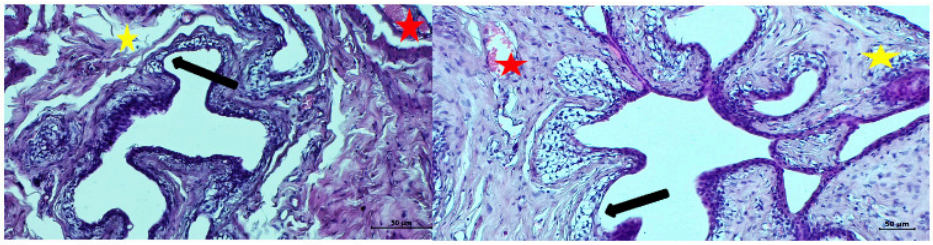

Figure 4: E) trauma + mountain mint F) trauma + zahter
Hematoxylin - Eosin staining, Bar: 50µm.

DISCUSSION

Spinal cord injury is frequently encountered as an important life-threatening health problem that does not have a definitive treatment method (1).

Severe neurological damage and multiple organ dysfunction occur in patients after spinal cord injury. Although the primary effect of trauma is on the spinal cord, dysfunctions occur in distant organs due to oxidative stress and activation of inflammatory pathways after neurogenic damage. Some of these are cardiovascular problems, pulmonary edema, spasticity and atrophy in skeletal muscles, bowel dysfunction, and neurogenic bladder and urinary tract infection as a urinary system complication (4).

The primary function of the bladder includes storing urine at low pressures and emptying it in the appropriate environment. During mixing, the detrusor muscle in the bladder contracts, while the bladder neck and external sphincter relax simultaneously. The upper center that provides coordination of these functions of the bladder and urethra is the pontine urination center located in the pons in the brain. This center also functions in harmony with the sub-center at the level of the sacral spinal cord (S2-4). As a result of disruption of this balance between the bladder and sphincter, hyperreflex or areflexic bladder occurs. When the connection between the pontine voiding center and the sacral spinal cord is interrupted in spinal cord injury, the harmony between the bladder and the urethral sphincter is lost and detrusor external sphincter dyssynergia occurs. In these patients, the bladder cannot empty, and may damage the upper urinary tract tissues by increasing the intra-bladder pressure more than necessary (1).

Many therapeutic approaches have been explored to address neurogenic bladder dysfunction, enhance quality of life, and extend life expectancy in spinal cord injury patients. In this study, we investigated the potential protective and therapeutic effects of Thymbra spicata L. var spicata and Cyclotrichium origanifolium on bladder dysfunction following spinal cord injury.

Ischemic reperfusion injury is recognized as a major factor in tissue damage associated with spinal cord injuries, largely due to the increased production of free radicals that lead to lipid peroxidation (10). Lipid peroxidation is particularly harmful because it initiates a self-perpetuating cascade of oxidative reactions, impairing membrane fluidity, disrupting membrane permeability, and causing irreversible structural damage (11). Studies have reported a positive correlation between the severity of disease and oxidative stress markers, as well as a negative correlation between antioxidant markers (12). This highlights the central role of oxidative stress in the pathogenesis of spinal cord injury and its complications.

In our study, a significant difference was observed in serum creatinine levels between the trauma and mountain mint groups. Rats treated with mountain mint extract following trauma exhibited lower creatinine levels compared to the trauma-only group. Although the exact mechanism remains unclear, this reduction may be associated with the mitigation of multiple organ damage caused by spinal cord injury. These findings align with previous research emphasizing the role of oxidative stress in renal complications and the potential benefits of antioxidant-based interventions.

In their study on bilateral renal ischemia-reperfusion injury, Öztürk, and colleagues determined that Carvakrol, the most powerful component of the zahter, had positive effects on urinary functions, they found that creatinine and BUN values increased due to ischemia, and in the treatment group, these biochemical parameters decreased with the effect of Carvakrol (13). In our study, the creatinine value had a significant difference between the groups and was found to be lower in the mountain mint and zahter groups after the application. Additionally, Cengiz et al., In a study using zahter oil, it was observed that Carvacrol could prevent the toxic effects of cancer drugs and contribute to the healing of damage, especially after and during chemotherapy (14). Dalkılıç et al., In a study conducted on zahter, it was observed that different concentrations of zahter extract in different solvents had antibacterial properties (15).

In a study, the role of a-LA, a powerful antioxidant, on bladder dysfunction and histopathological parameters caused by MetS was investigated. It has been experimentally predicted that a-LA may be useful in the treatment of detrusor overactivity caused by MetS (16, 17). In a study, it was determined that Rutin, a flavonoid with strong antioxidant and anti-inflammatory effects found in the structure of many plants, had a protective effect on bladder contractility and histopathology in CP-induced hemorrhagic cystitis in rats (18). According to the histopathological evaluation results obtained in our study; While it was observed that the bladder tissue was deteriorated in the trauma group, in the control group, there was thinning and ruptures in the epithelial layer, apoptosis in the epithelial cells, homework degeneration in the underlying connective tissue layer, and lymphocyte accumulation in some places. It was observed that in the trauma+zahter group, there was an improvement in the epithelial tissue, but degeneration and thinning of the epithelium continued in some places. It was observed that mononuclear cell infiltration improved in some places, and zahter application partially corrected the pathological effects caused by the trauma.

In a study conducted by Khalil et al. using Thymbra spicata L. extracts, it was found that it could be effective in preventing non-alcoholic liver diseases and endothelial dysfunction (19). In our study, we think that zahter may be an alternative treatment method. However, more studies with different doses and durations are needed to recommend zahter application as an alternative method.

CONCLUSION

According to our histopathological results, it was observed that mountain mint application played a low role in healing the pathological effects of trauma. More studies are needed to recommend mountain mint application as an alternative method to prevent pathological damage after trauma.

As a result, zahter and mountain mint extracts are partially effective against bladder damage resulting from spinal cord injury. The effectiveness of zahter is higher than that of mountain mint on oxidative stress and inflammatory markers, as seen in both our biochemical and histopathological findings. We believe that with the increase in experimental models, the use of plant extracts for therapeutic purposes will become widespread and may have positive effects on human health.

Since the low anti-inflammatory and antioxidant effects may depend on the duration of the experiment, and the dose and application method of the plant extracts, we think that more comprehensive studies should be conducted to understand the effects of these factors.

Ethics Committee Approval:
This study was approved by the Dicle University Animal Experiments Local Ethics Committee under protocol number 2019/08 dated 30/05/2019. The bladder tissues used in this research were obtained from the ethics-approved project but were not utilized in the original project. These tissues were analyzed within the scope of this study. The research was conducted in full compliance with ethical principles and scientific standards.

Informed Consent:
All the participants' rights were protected and written informed consent was obtained before the procedures according to the Helsinki Declaration.

Author Contributions:
Concept – F.K.D., M.C.T., E.D.; Design – F.K.D., M.C.T., E.D.; Supervision - F.K.D., Resources – S.A.K., F.K.D., D.A.K.; Materials - S.A.K., F.K.D; Data Collection and/ or Processing - S.C.D., F.K.D; Analysis and/ or Interpretation –C.N..,B.Y.,D.A.K, S.C.D; Literature Search - S.C.D, F.K.D.; Writing Manuscript - S.C.D, F.K.D.; Critical Review - S.A.K, S.C.D., M.C.T.

Conflict of Interest:
The authors have no conflict of interest to declare.

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