Polish Journal of Veterinary Sciences Vol. 26, No. 2 (2023), 249–255

DOI 10.24425/pjvs.2023.145029

Original article

Protective effectiveness of anise against testicular ischemia and reperfusion injury: An experimental study in rats

E. Yildizhan[1], M. Akkus[1], B.V. Ulger[2], F. Asır[1], S. Söker[1], E. Gündüz[3], M. Rençber[4], M. Barçin[2]

[1] Dicle University, Faculty of Medicine, Department of Histology and Embryology, Kıtılbıl Mah. 21280, Diyarbakır, Turkey
[2] Dicle University, Faculty of Medicine, Department of General Surgery, Kıtılbıl Mah. 21280, Diyarbakır, Turkey
[3] Dicle University, Faculty of Medicine, Department of Emergency Medicine, Kıtılbıl Mah. 21280, Diyarbakır, Turkey
[4] Department of General Surgery, Viransehir State Hospital, Ceylanpınar Yolu No:3, 63700 Viranşehir/Şanlıurfa, Turkey

Abstract

Testicular torsion is a frequently encountered clinical condition that requires urgent treatment. The aim of this study is to investigate the efficacy of Anise (Pimpinella anisum L.) in treating the pathological condition due to ischemia and reperfusion injury by using biochemical, histopathological and immunohistochemical methods.

A total of 6 groups were formed with 8 male Wistar Albino rats in each group. Group 1 (n=8): control group, Group 2 (n=8): Anise aqueous solution was given orally 5 ml/kg by gavage for 30 days. Group 3 (n=8): Ischemia and Reperfusion (I/R) group, bilateral testicles were rotated 270° and reperfused after 30 minutes of ischemia. Group 4 (n=8): I/R+ Anise group, Group 5 (n=8): Anise+ I/R group and Group 6 (n=8): Anise+ I/R+ Anise group.

The results of the Anise group and the Control group were similar. However, the damage in the I/R group was considerably more severe than in any of the other study groups. While it was observed that spermatogenic cells started to regenerate in the I/R+Anise group, edema and congestion were observed in the Anise+I/R group. In the Anise+I/R+Anise group, all histological findings and biochemical parameters were similar to those of the control group.

It was observed that anise had protective effects in ischemia and reperfusion injury in rat testicles.

Keywords: Anise, testicle, ischemia, reperfusion, immunohistochemistry

Correspondence to: Yildizhan E., e-mail: blgc_eda@hotmail.com, tel.: +90 412 2488001- 4032, fax: +90 412 2488523

Introduction

The testicles are highly prone to ischemia, and the probability of developing arterial embolism, inflammation and hypoxia during ischemia is quite high (Sharp et al. 2013). Testicular torsion is one of the emergencies requiring surgical intervention, characterized by the obstruction of blood flow to the testicles as a result of twisting of the spermatic cord, and as a result the development of testicular atrophy (Moslemi et al. 2014). There are two types of torsion, extravaginal and intravaginal (Karaguzel et al. 2014). While extravaginal torsion is frequently seen in the perinatal period and causes abnormal motility of the testicles within the scrotum, intravaginal torsion is caused by the mesorchium seen in adolescence (Bowlin et al. 2017).

Reperfusion occurs when blood flow is restored and reoxygenation occurs (Eltzschigand et al. 2011). With the realization of reperfusion, an increase in free oxygen radicals (ROS) and proinflammatory cytokines such as Interleukin-1β (Il-1β) and Tumor necrosis factor-alpha (TNF-α) may occur (Turner et al. 2004).

Anise (Pimpinella anisum L.) seeds contain anethole, a phytoestrogen also known as anise berries (Sibeko et al. 2021). Previous studies have shown that anethole has antitimetastatic (Choo et al. 2011), antioxidant, anti-inflammatory and gastroprotective properties (Freire et al. 2005), and in hepatic ischemia and reperfusion studies in mice, it has protective properties against damage (Cho et al. 2013). While India, China, Mexico, Russia and Iran are among the main production places of anise seeds or fruits, it is also produced in Turkey (Doğan et al. 2015).

In this study our aim was to examine the protective efficacy of Anise in the treatment and prevention of biochemical, histopathological and immunohistochemical changes that occur in case of ischemia and reperfusion in testicular tissues.

Materials and Methods

This study was approved by the Dicle University Animal Experiments Local Ethics Committee (DUHADEK) (30/09/2020-2020/29).

Preparation of aqueous extract of anise

Anise seeds used in our study were purchased from a local vendor (Turkey, Diyarbakır). It was confirmed to be aniseed by Prof Dr Özlem TONÇER, who works at Dicle University, Vocational School of Agriculture, Department of Organic Agriculture. 100 grams of anise seeds were infused in 1 liter (70°C) distilled water for 30 minutes (Gamberini et al. 2015). After the obtained extract was filtered, it was given orally with the help of gavage at a dose of 5 ml/kg.

Application of testicular ischemia and reperfusion

To protect animal welfare during the experiment, 90 mg/kg Ketamine Hydrochloride (Ketalar; Prizer) + 10 mg/kg Xylazine Hydrochloride (Rompun; Bayer) was administered intraperitoneally (i.p.) to all animals, and sedation was controlled by finger pinching. Cefriaxone at a dose of 50 mg/kg was administered intramuscularly (i.m.) to the groups to be operated before starting the procedures. After placing the rats in the supine position on the operating table, the scrotum area was shaved, cleaned with 10% povidone-iodine, and the scrotal skin was incised in the midline. Then the peritoneum was opened and the testicles were taken out of the scrotum. Bilateral testicles were rotated around the long axis of 270° and ischemia was applied for 30 minutes. It was then detorsioned and reperfused for 30 min. Afterwards, the testicles were reintroduced into the abdomen and the peritoneum was closed with 3/0 Vicryl and the skin with 3/0 prolene (Fig. 1). After the procedures, 20 mg/kg acetominophen was administered p.o. to the animals for analgesia and daily dressings were made for 7 days.

Formation of experimental groups

In the study, 48 Wistar Albino male rats, 8-10 weeks old and weighing between 320-400 gr, were used. The rats were fed in stainless steel cages at 22±2°C, 60% humidity, with a normal diet without any movement or feed/water restrictions. Each rat was weighed separately and their weight was determined, and the drug doses calculated in accordance with the literature were determined.

Group 1 (n=8): the control group and no invasive procedure was applied to the rats throughout the experiment. **Group 2 (n=8):** the anise group, and the aqueous extract of anise was given orally by gavage at a daily dose of 5 mL/kg for 30 days. **Group 3 (n=8):** Ischemia/Reperfusion (I/R) group, on the first day of the experiment the rats were sacrificed after I/R was performed. **Group 4 (n=8):** I/R+ Anise group, Aqueous extract of Anise was given for 30 days after I/R was performed on the first day of the experiment. **Group 5 (n=8):** Anise+ I/R group, I/R was performed after 30 days of anise administration to rats. **Group 6 (n=8):** Anise+ I/R+ Anise group.

After the rats were sacrificed by exanguination method, total antioxidant capacity (TAS) and total oxidant capacity (TOS) were measured from the blood samples taken. Blood samples were taken into EDTA

tubes and centrifuged at 3000 rpm for 10 minutes and sera was obtained. The obtained serum samples were stored at -80°C. Testicular tissues were taken into 10% formaldehyde and sent to Dicle University Histology/ Embryology laboratory.

TAS and TOS measurements

The methods developed by Erel for measuring the total antioxidant and oxidant capacities of the body against free radicals, were used (Erel 2004, Erel 2005).

Routine histological follow-up

After the testis samples taken from the sacrificed animals were fixed in 10% formalin (Sigma) for 24 hours, the shrunken tissues were kept in formalin for another 24 hours. Tissue samples washed in tap water for 12 hours after fixation were passed through increasing alcohol series for dehydration. Tissues were stained with hematoxylin and eosin (HE) for routine histological examinations.

The stained tissues were evaluated with a light microscope and Johnsen testicular biopsy scoring was performed on 10 randomly selected seminiferous tubules from each group (Johnsen 1970). According to the Johnsen Testicular Biopsy Score, regular and complete spermatogenesis and tubule structure were scored as grade 10 and the absence of Germ cells or Sertoli cells as grade 1.

Immunohistochemical studies

After routine histological tissue follow-up, sections were stained with the proinflammatory cytokine interleukin-6 (Il-6) and the proapoptic marker Bcl-2 antibodies.

Measurement of body and organ weights

At the end of the study, bilateral testicular weights and body weights were obtained and recorded. Total testicular weight indices (TWI) were calculated by multiplying bilateral testicular weights (grams) by 100 and divide it by body weights (g) (Sahinturk et al. 2007).

Statistical analysis

Data analyzes were performed using SPSS 20 (SPSS Inc. Chicago, IL, USA). One-Way Analysis of Variance (ANOVA) test was used for normally distributed data, and Post-Hoc Tamhane's T2 and Tukey tests were used to find the different group. Kruskal Wallis-H test was applied to the data that did not show normal distribution, and Mann-Whitney U-test was used to find the different group. Significance level was accepted as significant in case of $p<0.05$ value.

Results

TAS and TOS results

We observed that the TAS level of the Anise group was higher than all other groups ($p<0.05$), while there was no difference between the other study groups in terms of TAS levels ($p>0.05$). In terms of TOS levels, the TOS level of the I/R group was higher than the other study groups ($p<0.05$) (Table 1).

Body and testicular weight results

At the beginning of the study male rats with an average weight of 320-400 g were used. The mean values±standard deviations of the rats whose mean body weights, bilateral testicular weights and total testicular weight indices (TAI) were calculated at the beginning and end of the experiment and shown in Table 1.

When the differences between body weights were examined, we observed that the highest weight gain was in the Anise group. In terms of testicular weights, we observed that there was no statistically significant difference ($p>0.05$) between the Control group and Anise and Anise+ I/R groups (Table 1).

Histopathological results

In the histopathological examinations of the tissues stained with HE, the tissues were evaluated under the light microscope and 10 seminiferous tubules were counted randomly from each group and were subjected to Johnsen testicular biopsy scoring.

In the pairwise comparison between the groups, the scores of the Control and Anise groups were similar ($p>0.05$), while degeneration, pycnosis and atrophic tubules developed in the seminiferous tubules in the tissues of the I/R group (Fig. 1). In the anise+ I/R group, the degeneration was milder than the I/R group, but the edema continued. Damage grading of the I/R group was scored higher than the Anise+ I/R group. We observed that the seminiferous tubules in the tissues of the anise+I/R+anise group had a similar appearance to the control group, and in the statistical analysis, the damage score was lower than the I/R, Anise+ I/R and I/R+ Anise groups ($p<0.05$) (Fig. 1) (Table 1).

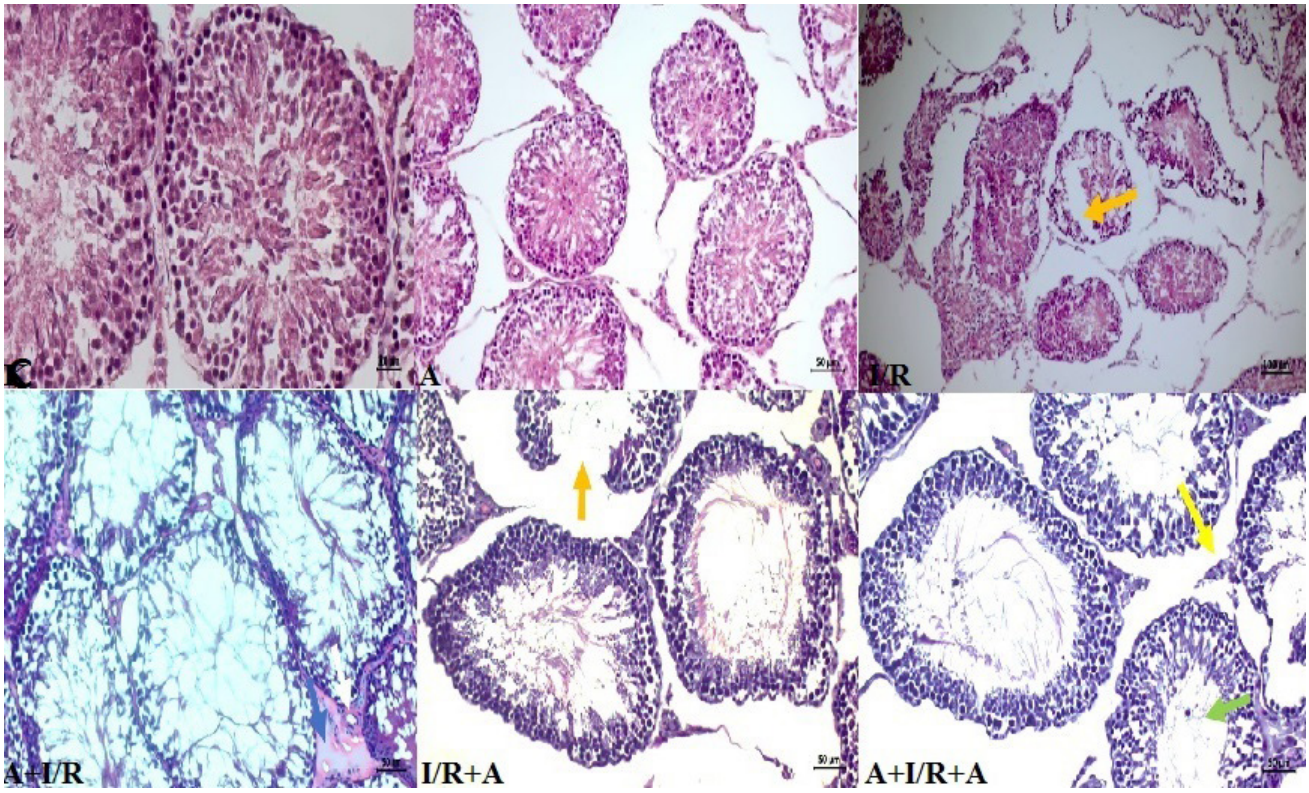

Fig. 1. C: control group, A: Anise group, I/R: Ischemia and Reperfusion group of rats. Orange arrow: disruption of seminiferous integrity and pycnotic cells, White arrow: congestion, Blue arrow: interstitial edema, Green arrow: spermium circulating in the lumen, Yellow arrow: normal-looking Leydig cells (hematoxylin and eosin (HE) staining; bar=50 µm).

Table 1. Mean±standard deviation values of total antioxidant capacity (TAS) (µmol/L), total oxidant capacity (TOS) (mmol/L) and Johnsen Scorings, body weights and TWI data.

GROUPS	TAS (µmol H_2O_2 equivalent/L)	TOS (mmol Trolox equivalent/L)	Johnsen Scoring (n x tubule count)	Initial Body Weight (g)	Final Body Weight (g)	Body Weight Difference (g)	Testicular weight (g)	TWI (g)
Control (n:8)	2.93±0.47	23.07±3.12	98.87±1.12	337.37±15.29	344.00±21.28	6.62±15.88	2.67±0.16	0.77±0.04
Anise (n:8)	6.15±1.55	22.21±3.61	99.25±0.88	336.62±11.99	362.12±14.29	25.87±24.50	3.02±0.26	0.83±0.09
I/R (n:8)	3.05±0.67	94.11±22.23	11.00±1.06	352.87±12.79	352.87±12.79	0.00±0.00	2.11±0.15	0.59±0.05
I/R+Anise (n:8)	2.95±0.47	39.67±9.08	59.12±4.48	343.62±20.80	326.00±20.16	17.75±9.57	1.91±0.15	0.58±0.07
Anise+I/R (n:8)	2.77±0.73	43.41±6.48	72.37±2.87	355.75±13.62	373.75±13.19	18.00±5.75	2.57±0.21	0.68±0.05
Anise+I/R+Anise (n:8)	3.99±1.01	21.13±4.44	82.12±3.22	345.12±21.49	356.87±14.98	11.75±12.18	1.90±0.20	0.54±0.06

TAS; total antioxidant capacity (µmol H_2O_2 equivalent/L), TOS; total oxidant capacity (mmol Trolox equivalent/L), TWI; Testicular weight index.

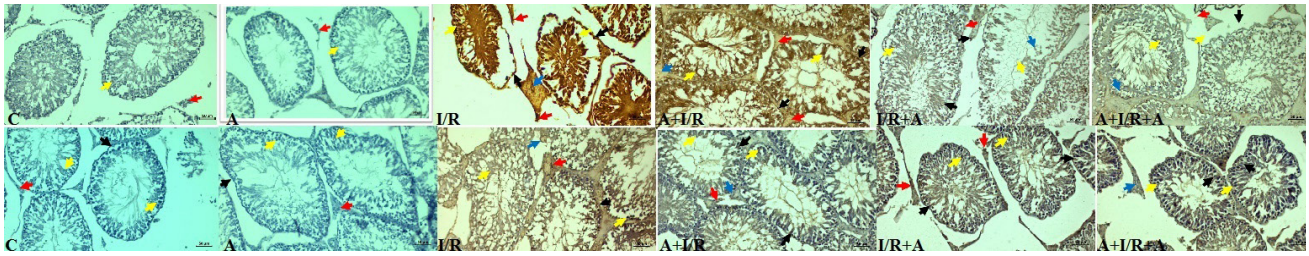

Fig. 2. C: Control group, A: Anise group, I/R: Ischemia and Reperfusion group of rats. Yellow arrow: spermatogenic cells, Black arrow: Sertoli cells, Blue arrow: vascular endothelium, Red arrow: Leydig cells. First line: Interleukin-6, Second line: Bcl-2 (reverse staining: hematoxylin; bar=50 µm).

Immunohistochemical results

IL-6 Results

Intense positive IL-6 expressions were observed in seminiferous tubules, spermatogenic cells and Sertoli cells in the I/R group. We also observed a decrease in the number of inflammatory cells in the anise+I/R group compared to the I/R group. Moderately positive IL-6 expression was observed in spermatogenic cells and Sertoli cells. In the Anise+I/R+Anise group, sporadic positive IL-6 expressions were observed in spermatogenic cells, while expressions continued in Sertoli cells. Administration of Anise both before and after I/R was found to significantly improve inflammatory cell pathways (Fig. 2).

Bcl-2 results

Intense positive Bcl-2 expression was observed in spermatogenic cells and Sertoli cells in the transversal section of the I/R group. In addition, intense positive BCL-2 expression was observed in the capillary vessel endothelium and Leydig cells in the interstitial space. While positive Bcl-2 expressions were observed in very few spermatogenic cells in the Anise+I/R+Anise group, we observed that the expressions continued at a mild level in Sertoli cells. Bcl-2 expressions in the vascular endothelium were quite mild (Fig. 2).

Discussion

Phytochemical studies are studies that have continued from the past to the present and form the basis of medicine. Anethole found in anise seeds is a phytoestrogen widely used as a sweetener in cosmetics, perfume, pharmaceutical industry and food manufacturing. Studies conducted to date have shown that anethole has various pharmacological effects such as anticarcinogenic, anti-inflammatory, hepatoprotective, antidiabetic and neuroprotective properties (Sheikh et al. 2015). Ritter et al. showed that anise seeds did not cause any toxic effects on the liver and did not harm the liver (Ritter et al. 2013). According to the toxicity classification, the lethal dose (LD50) of anise was determined as 5 g/kg (Hosseinzadeh et al. 2014).

Our study revealed that while anise seeds decreased the levels of oxidants in the serum, they increased the antioxidant levels. Moshkelani et al. found that rats with testicular ischemia and reperfusion injury had an increase in reactive oxygen products (ROS) and decreased levels of antioxidant-effective enzymes such as glutathione peroxidase (GP) and superoxide dismutase (SOD) (Moshkelani et al. 2020). Fakouri et al. on the other hand, showed that while malondialdehyde (MDA) levels were significantly increased in rats that underwent ischemia and reperfusion, there was a decrease in MDA levels when folic acid was given to rats (Fakouri et al. 2017). A significant increase in serum TOS levels of the groups which underwent testicular I/R was observed in our study. However, we observed that TOS levels decreased and TAS levels increased when anise was given to rats undergoing testicular I/R.

In their study, Ritter et al. showed that anetheol intake reduces proinflammatory cytokines such as TNF-α, Il-1β and Il-6 that occur during ischemia, and also causes a decrease in ROS levels that occur during reperfusion (Ritter et al. 2013). While Qin et al. observed a significant increase in the number of apoptotic cells in the testis I/R group (Qin et al. 2019), Doğan et al. also observed intense Bcl-2 expressions in the spermatogenic cells of the testis I/R group and strong mitotic activity in the basal layers of the seminiferous tubules (Doğan et al. 2020). Parallel to these findings, in our study, intense IL-6 expression in testicular tissues of the I/R group and inflammatory cells in the basement membrane were observed. In addition, Bcl-2 expressions were intensely positive in the I/R group, but decreased in the Anise+I/R+Aniseed group. Histopathologically, it was observed that anise seeds showed a anti-apoptotic effect.

In their study, Wei et al. reported a significant decrease in testicular weight, seminiferous tubule diameter, number of germ cell layers and Johnsen testicular injury scoring as a result of testicular I/R injury com-

pared to the control group, but these findings were improved when they were treated with sesame (Wei et al. 2020). There are studies showing that anise has positive effects on body weight. It is known that anise has high nutritional values (18% protein, 8-23% fat, 2-7% essential oil, 3-5% sugar and 12-15% crude fiber) (Bekara et al. 2015). In our study, we observed that the I/R group had the lowest testicular damage score, and the damage score of the Anise+I/R+Aniseed group was close to the control group. In addition, it was observed that the body weight of the Anise group was the highest. Doaa et al. investigated the effects of vitamin D3 against testicular torsion and measured the body weight, relative testicular weight and absolute testicular weight of all rats in their study. As a result of their study, they found that the final body weight of the I/R+Vit D3 group was significantly increased compared to the control and sham groups (Doaa et al. 2021). We, on the other hand, observed that there was a decrease in body weight of the I/R+Aniseed group, but an increase in body weight of the Anise+I/R+Aniseed group and Anise+I/R group. We found that anise has positive effects on the preservation of body and testicular weights in testicular ischemia and reperfusion.

Conclusion

In our study, we determined that anise has anti-inflammatory and anti-apoptotic effects as a result of histopathological evaluation. In addition, we observed that the oxidative stress in the serum resulting from I/R decreased after anise administration. Our study revealed that anise has a positive effect on histopathological, immunohistochemical and biochemical parameters in testicular I/R damage.

Acknowledgements

We would like to thank to Prof. Özlem TONÇER (Dicle University Agricultural Vocational School, Organic Agriculture Department) for her help during this study.

References

Bekara A, Hamadouche NA, Kahloula K, Harouat S, Dalila TabbasD, Aoues AK **(2015)** Effect of Pimpinella anisum L (Aniseed) aqueous extract against lead (Pb) neurotoxicity: neurobehavioral study. Int J Neurosci Behav Sci 3: 32-40

Bowlin PR, Gatti JM, Murphy JP **(2017)** Pediatric testicular torsion. Surg Clin North Am 97: 161-172.

Cho HI, Kim KM, Kwak JH, Lee SK, Lee SM **(2013)** Protective mechanism of anethole on hepatic ischemia/reperfusion injury in mice. J Nat Prod 76: 1717-1723.

Choo EJ, Rhee YH, Jeong SJ, Lee HJ, Kim HS, Ko HS, Kim JH, Kwon TR, Jung JH, Kim JH, Lee HJ, Lee EO, Kim DK, Chen CY, Kim SH **(2011)** Anethole exerts anti-metatstaic activity via inhibition of matrix metalloproteinase 2/9 and AKT/mitogen-activated kinase/nuclear factor kappa b signaling pathways. Biol Pharm Bull 34: 41-46.

Doaa IM, Doaa AA, Samar FE, Hanaa FA, Hebatallah HAN, Hosam AS, Mehana AE, Saied EM **(2021)** Vitamin D_3 prevents the deleterious effects of testicular torsion on testis by targeting mRNA-145 and ADAM 17: in silico and invivo study. Pharmaceuticals (Basel). 14 (12): 1222.

Doğan G, İpek H **(2020)** The protective effect of Ganoderma lucidum on testicular torsion/detorsion-induced ischemia-reperfusion (I/R) injury. Acta Cir Bras 35: e202000103.

Eltzschigand HK, Eckle T **(2011)** Ischemia and reperfusion from mechanism to translation. Nat Med 17: 1391-1401.

Erel O **(2004)** A novel automated method to measure total antioxidant response against potent free radical reactions. Clin Biochem 37: 112-119.

Erel O **(2005)** A new automated colorimetric method formeasuring total oxidant status. Clin Biochem 38: 1103-1011.

Fakouri A, Asghari A, Akbari G, Mortazavi P **(2017)** Effects of folic acid administration on testicular ischemia/reperfusioninjury in rats. Acta Cir Bras 32: 755-766.

Freire RS, Morais SM, Catunda-Junior FEA, Pinheiro DC **(2005)** Synthesis and antioxidant, anti-inflammatory and gastroprotector activities of anethole and related compounds. Bioorg Med Chem 13: 4353-4358.

Gamberini MT, Rodrigues DS, Rodrigues D, Pontes VB **(2015)** Effects of the aqueous extract of Pimpinella anisum L. seeds on exploratory activity and emotional behavior in rats using the open field an delevated plus maze tests. J Ethnopharmacol 168: 45-49.

Hosseinzadeh H, Tafaghodi M, Abedzadeh S, Taghiabadi E **(2014)** Effect of aqueous and ethanolic extracts of Pimpinella anisum L. seeds on milk production in rats. J Acupunct Meridian Stud 7: 211-216.

Johnsen SG **(1970)** Testicular biopsy score count-a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. Horm Res Paediatr 1: 2-25.

Karaguzel E, Kadihasanoglu M, Kutlu O **(2014)** Mechanisms of testicular torsion and potential protective agents. Nat Rev Urol 11: 391-399

Moshkelani S, Asghari A, Abedi G, Jahandideh A, Mortazavi P **(2020)** Effects of magnesium sulfate administration on testicular ischemia/reperfusion injury in rats. Arch Razi Inst Winter 75: 83-91.

Moslemi MK, Kamalimotlagh S **(2014)** Evaluation of acute scrotum in our consecutive operated cases: a one-center study. Int J Gen Med 7: 75–78

Ritter AM, Domiciano TP, Verri WA, Zarpelon AC, Da Silva LG, Barbosa CP, Natali MR, Cuman RK, Bersani-Amodo CA **(2013)** Antihypernociceptive activity of anethole in experimental inflammatory pain. Inflammopharmacology 21: 187-197.

Sahinturk V, Guclu C, Baycu C **(2007)** Protective effects

of vitamin E on ethane dimethane sulfonate-induced testicular toxicity in rats. Asian J Androl 9: 117-124.

Sharp VJ, Kieran K, Arlen AM **(2013)** Testicular torsion: diagnosis, evaluation, and management. Am Fam Physician 88: 835-840.

Sheikh BA, Pari L, Rathinam A, Chandramohan R **(2015)** Trans-anethole, a terpenoid ameliorates hyperglycemia by regulating key enzymes of carbohydrate metabolism in streptozotocin induced diabetic rats. Biochimie 112: 57-65.

Sibeko L, Johns T **(2021)** Global survey of medicinal plants during lactation and postpartum recovery: Evolutionary perspectives and contemporary health implications. J Ethnopharmacol 270: 113812.

Turner TT, Bang HJ and Lysiak JL **(2004)** The molecular pathology of experimental testicular torsion suggests adjunct therapy to surgical repair. J Urol 172: 2574-2578.

Wei SM, Wang RY, Chen YS **(2020)** Sesamol protects testis from ischemia reperfusion injury through scavenging reactive oxygen species and upregulating expression. Oxid Med Cell Longev 2020: 9043806.

Qin Z, Zhu K, Xue J, Cao P, Xu L, Xu Z, Liang K, Zhu J, Jia R **(2019)** Zinc-induced protective effect for testicular ischemia-reperfusion injury by promoting antioxidation via microRNA-101-3p/Nrf2 pathway. Aging (Albany NY) 11: 9295-9309