ORIGINAL ARTICLE

Veterinary Research Forum. 2020; 11 (1) 27 – 33
doi: 10.30466/vrf.2019.102182.2432
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Antioxidant property of *Plantago major* leaf extracts reduces testicular torsion/detorsion-induced ischemia/reperfusion injury in rats

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Article Info

Article history:

Received: 19 January 2019
Accepted: 07 May 2019
Available online: 15 March 2020

Keywords:

Antioxidant
Ischemia/reperfusion injury
Plantago major
Rat
Testicular torsion

Abstract

The present study was aimed to determine the protective effects of *Plantago major* L (PM*)* leaf extracts on the testicular torsion/detorsion (T/D)-induced ischemia/reperfusion (I/R) injury in rats. Twenty-four mature male Sprague-Dawley rats, weighing 200-220 g, were selected. They were randomly divided into four groups of six animals each: Sham (sham-operated rats; all the surgical steps were performed but T/D was not induced), TDC (Control group; T/D was induced and the right testicular torsion of 720° lasting two hours was followed by detorsion), TDP50 (T/D-operated rats received 50.00 mg kg^{-1} of PM extract daily for seven days intraperitoneally after detorsion) and TDP100 (T/D-operated rats received 100 mg kg^{-1} of PM extract daily for seven days intraperitoneally after detorsion). After seven days of treatment, the right testicles were collected. Histopathological and biochemical analyses including levels of malondialdehyde (MDA) and catalase (CAT) and peroxidase activities were determined in testicular tissues of the rats. Tissue sections were taken from testis, Hematoxylin-Eosin staining was done, and the slides were examined by a light microscope. The level of MDA was significantly increased in the testes of the TDC group. The CAT activity levels were decreased significantly after I/R. The post-torsion treatment with PM, particularly at 100 mg kg^{-1}, prevented the increase in lipid peroxidation and reduced the CAT activity levels. The PM also prevented I/R-induced cellular damage and histological changes in the testicular tissues. According to the results of the current study, PM leaf extracts had significant positive effects on the testicular T/D-induced I/R injury. The possible mechanism of reduction in biochemical and histological injuries by PM extracts could be due to antioxidant property.

Introduction

Inadequate blood supply to an organ is called ischemia, which can cause many health problems such as peripheral vascular insufficiency, myocardial infarction, hypovolemic shock, and strokes. The recirculation of blood within an ischemic organ is essential to prevent permanent cellular damage, however, reperfusion may aggravate tissue injury.[1] One of the causes of tissue ischemia is testicular torsion. This emergency condition is regarded as one of the most prevalent urologic disorders affecting newborns, children, and adolescents. It occurs when the spermatic cord twists. As a result, the venous blood circulation is impaired by edema and bleeding and the arterial circulation is blocked.[2] Testicular torsion/detorsion (T/D) can cause an ischemia/reperfusion (I/R)-induced injury to testes. This syndrome is one of the causes of infertility. It is reported that excessive production of reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, and hydroxyl radicals is a crucial factor in the genesis of the I/R injury.[3] In fact, ROS constitutes the basic pathophysiological processes of the I/R injury. Neutrophils produce high levels of ROS. Surgery is vital for individuals with testicular torsion. Ideally, it should be performed within 6-8 hr subsequent to the onset of symptoms.[1-3] A delay in treatment may decrease fertility. There is no consensus on environmental factors responsible for testicular torsion. Some researchers believe that low

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temperature and humidity increase the risk of testicular torsion. Nevertheless, others do not find any association between this condition and seasonal changes. In general, testicular torsion appears to happen with increased frequency, when it is cold.[4,5] Risk factors for testicular torsion include testicular tumors, increased testicular volume, a spermatic cord with a long intrascrotal portion, and a history of cryptorchidism.[6] It is indicated that the administration of exogenous antioxidants or ROS scavenger agents can minimize or prevent the I/R-induced oxidative injury to testicular tissues of rats.[6] Drugs such as sildenafil,[7] carvedilol,[8] and benidipine[9] have profound inhibitory effects on the I/R injury to testes of rats. Furthermore, herbal medicine is a leading contributor to the treatment of the injury. Plant-derived antioxidants are effective therapeutic agents which can help cope with oxidative stress. Extracts of *Crocus sativus*,[10] *Echinacea*,[11] *Dracocephalum moldavica*,[12] *Thymus vulgaris*, and *Lavandula multifida*[13] possess antioxidant properties. *Plantago major* (PM) belongs to the family *Plantaginaceae* and the genus *Plantago*.[14] The leaves have several bioactive compounds, such as terpenoids, flavonoids, pectin, iridoid glycosides, and tannins, which have anti-inflammatory and antioxidant properties.[14] Generally, PM has anesthetic, antifungal, antiviral, anti-inflammatory, and anthelmintic properties.[15] Moreover, water-soluble compounds in PM increase the proliferation of human lymphocytes.[16] The PM is rich in polysaccharides and polyphenols. Phenolic compounds have antiviral effects.[17] Furthermore, polyphenols remove hazardous oxidants, thereby, eliminated detrimental effects of free radicals. They also chelate metal ions.[18] The PM leaves have antiulcerogenic effects.[19] PM bears anti-inflammatory and immune-regulatory properties and can maximize the production of nitric oxide and tumor necrosis factor-alpha (TNF-α), consequently, it strengthens the immune system against tumors and infections.[14,19] Antioxidant effects of the plant have been reported. Baicalein- (a flavonoid) and aucubin (an iridoid glycoside) are among the most biologically active components of PM. They have antioxidant, anti-inflammatory, and anticancer properties.[20] Hussan *et al*. have showed that leaf extracts of PM had anti-inflammatory effects on the inflammatory response during acetaminophen hepatotoxicity.[21] To the best knowledge of the authors, there is no report representing any ameliorative and/or protective effect of PM against torsion and/or reperfusion-induced impact on spermatogenesis. Thus, the current study was conducted to investigate the possible ameliorative effect of PM following reperfusion on spermatocytogenesis, spermatogenesis and testicular general structure. For this purpose, the Johnson score (as a well-known method for spermatogenesis) and histopathological changes of testicles were analyzed.

Materials and Methods

Animals. Twenty-four male Sprague-Dawley rats weighing 200-220 g were used in this study. Before conducting the experiment, the rats were acclimatized to laboratory conditions for one week. They were housed in a temperature-controlled environment at 22.00 ± 2.00 ˚C with a relative humidity of 60.00% and under light-dark cycles of 12:12 hr. In order to carry out the study, guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (IASP) were followed.[22] University of Tabriz Research Ethics Committee approved all the experiments (Act No. d/27/131).

Extract preparation. The PM plants were collected from Urmia, Iran, in 2017. They were recorded in the Herbarium of the Urmia Agriculture Research Center (code: 9708). The dried powder of PM leaves (100 g) was suspended in a mixture of ethanol and water solution (500 mL). The ratio was 1:1. It was loaded into the Soxhlet extractor within 5 hr. The extract remained at 40.00 ˚C after the solvent was filtered and evaporated. It weighed 30.10 g. The yield level was 30.10%.

Determination of the total phenolic content. To determine the total phenolic content, the Folin-Ciocalteu method was slightly modified.[23] In this method, first, the extract was at 40.00 ˚C for 30 min after being subjected to the Folin-Ciocalteu reagent (FCR). Next, the absorbance of the sample was measured at 765 nm. A standard diagram depicted different levels of pyrocatechol. Finally, the total phenolic content was expressed as µg of pyrocatechol equivalent (PE) per mg of extract.

Determination of the total flavonoid content. To determine the total flavonoid content, the method proposed by Jia *et al.* was used.[24] In this method, sodium nitrite (NaNO2) and aluminum chloride (AlCl3) are used in the alkaline environment of sodium hydroxide (NaOH). Quercetin standard curve (1-200 µg mL^{-1}) with equation of $Y = 0.0160 x + 0.001$, $R^2 = 0.99$ was used to determine the total flavonoid content. The absorbance of total flavonoids was monitored at 420 nm by using a UV/Visible PC spectrophotometer (Unico, Shanghai, China). Results were expressed as µg of quercetin equivalent (QE) per mg of extract.

Determination of total antioxidant capacity. To determine the total antioxidant capacity of the hydroalcoholic extract of PM, the ferric reducing ability of plasma (FRAP) method was used.[25] The hydroalcoholic extract of PM (10.00 mL) was added to the FRAP solution (1.00 mL). Absorbance changes were measured after 4 min at 592 nm. Results were expressed as equivalents of butylated hydroxytoluene (BHT) as a potent antioxidant.

Experimental design. The rats were randomly divided into four groups (n = six per group): Sham (Sham-operated rats; all the surgical steps were performed,

however, T/D was not induced), TDC (control group; T/D was induced and the right testicular torsion of 720° lasting two hours was followed by detorsion), TDP50 (T/D-operated rats received 50 mg kg^{-1} of PM extract daily for seven days intraperitoneally after detorsion), and TDP100 (T/D-operated rats received 100 mg kg^{-1} PM extract daily for seven days intraperitoneally after detorsion).[20]

Experimental testicular T/D procedure. The animals were anesthetized with an intramuscular injection of 80.00 mg kg^{-1} ketamine (Alfasan, Woerden, The Netherlands) and 10.00 mg kg^{-1} xylazine (Alfasan). The skin of their scrotum was shaved and scrubbed. All procedures were conducted under sterile conditions. The scrotum was entered through a right vertical paramedian incision, the tunica vaginalis was opened, and the right testis was delivered to the surgical field. Torsion was induced by rotating the right testis 720° clockwise and maintained by replacing the testis into the scrotum and fixing there with a 4/0 silk suture (Supa, Tehran, Iran). The incised scrotum was closed. After two hours of torsion, the spermatic cord was counter-rotated and the testis was reperfused. The PM extract was intraperitoneally injected into the TDP50 (50.00 mg kg^{-1}) and TDP100 (100 mg kg^{-1}) groups. The sham and TDC groups were injected with distilled water. They were injected for one week. After the I/R procedure, the rats were undergone right orchiectomy. They were euthanized using an anesthetic overdose. Histopathological and biochemical analyses were conducted regarding the right testis.

Histopathological analysis. The right testis of each animal was fixed in a 10.00% neutral buffered formalin, dehydrated in a graded ethanol series, cleared in xylene, embedded in paraffin wax, and cut in 5.00 µm sections, which were mounted on slides, stained with Hematoxylin and Eosin (H&E), and examined using a light microscope (BX60; Olympus, Tokyo, Japan). Histopathological changes in the testes were scored according to the modified version of the four-point grading system proposed by Cosentino *et al*.[26] Furthermore, spermatogenesis in the tissue specimens of the testes was evaluated according to the scoring system proposed by Johnsen.[27] I/R caused alterations in spermatogenesis with varying levels of severity observed in four random areas of each testis with a magnification of 40×. The final score for each testis was determined by calculating the average of all the grades.

Preparation of tissue homogenate. Each portion of the testicular tissue (1.00 g) was first cut in liquid nitrogen and mixed in a 0.10 M phosphate buffer, pH 7.20 (10 times the tissue volume). Next, the mixture was centrifuged at 12,000 rpm at a temperature of 4.00 ˚C for 10 min. The supernatant solution was used for the assay.

Lipid peroxidation assays. Malondialdehyde (MDA) levels in testicular tissues were measured using the thiobarbituric acid-reactive substances (TBARs) assay.[28] The tissue homogenate (0.20 mL) was combined with the TCA (Trichloroacetic acid 15.00%; Merck, Darmstadt, Germany) and TBA (Thiobarbituric acid 0.67%; Merck) solutions and loaded at 100 ˚C for 15 min. The supernatant absorbance was measured at 532 nm.

Enzyme assays. Catalase (CAT) activity was determined by the change in absorbance at 240 nm in the presence of hydrogen peroxide.[29] One unit of catalase activity was defined as the amount of enzyme necessary for reducing 1.00 µmol of H_2O_2 per min. The peroxidase activity level was measured according to the change in absorbance at 460 nm due to o-dianisidine oxidation in the presence of hydrogen peroxide.[30] One unit of peroxidase activity was defined as the amount of enzyme necessary for the oxidation of 1.00 µmol of o-dianisidine per min.

Total protein determination. The protein concentration was determined by the method of Lowry, with bovine serum albumin (BSA; Merck) as the standard. This method is based on the reaction of Cu^{+}, produced by the oxidation of peptide bonds, with Folin–Ciocalteu reagent (Merck).[31]

Statistical analysis. The data were expressed as mean ± SD. The analytical results were evaluated using the SPSS (version 22.0; SPSS Inc., Chicago, USA). A one-way analysis of variance (ANOVA) was used for the statistical analysis of the parametric data. Individual groups were compared using Tukey's multiple comparison tests. The multivariate Kruskal-Wallis test and Dunn's post-test were used to analyze the non-parametric data. Differences less than 0.05 were considered statistically significant ($p < 0.05$).

Results

Total phenol and total flavonoid of PM hydro-alcoholic extracts. The results of the present study showed that the total phenolic content and total flavonoid were 136.58 ± 0.31 (µg PE mg^{-1}), 2.55 ± 0.19 (µg QE mg^{-1}).

Total antioxidant level of PM hydroalcoholic extracts. Figure 1 shows the total antioxidant level of the PM hydroalcoholic extracts at different concentrations. At the concentration of 20.00 mg mL^{-1}, the total antioxidant level was 5.93 dibutyl hydroxyl toluene (BHT). Then, by increasing the concentration from 20.00 to 40.00 and then to 80.00 mg mL^{-1}, the total antioxidant level was increased to 10.60 and 20.33 BHT, respectively. Thus, a two-fold increase in the concentration of extract resulted in a two-fold increase in total antioxidant level. The total anti-oxidant level was increased as the extract concentrations were increased in a dose-dependent manner.

Histopathological studies. Figure 2 shows histo-logical changes in the testicular tissues of the rats. Sham group had a normal testicular architecture with normal germinal cells and tubules. In TDC group, severe interstitial edema, hemorrhage and coagulative necrosis of the seminiferous tubules and germinal cells were observed (mean grade: 3.62; range: 3.50-4.00).

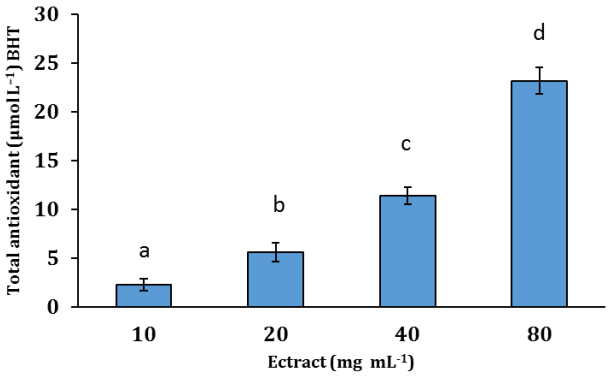

Fig. 1. The total antioxidant level of the PM hydroalcoholic at different concentrations. All data are presented in Mean ± SD. [abcd] Different letters are presenting significant differences among groups ($p < 0.05$).

In TDP50 group, there were moderate interstitial edema, hemorrhage and coagulative necrosis of seminiferous tubules and germinal cells (mean grade: 2.62; range: 2.25-4.00). In TDP100 group, non-cohesive germinal cells and seminiferous tubules with mild interstitial edema and necrosis were observed (mean grade: 1.43; range: 1.50-2.00). The comparison of the grades showed that there was a significant difference between group 2 and the other groups ($p < 0.05$). Also, spermatogenesis was evaluated according to the scoring system proposed by Johnsen. Sham group showed normal spermatogenesis (mean score: 9.56). There was severe spermatogenic damage in TDC group (mean score: 2.62). TDP50 group demonstrated moderate spermatogenic damage (mean score: 5.31). Mild spermatogenic damage was observed in TDP100 group (mean score: 8.62). There were several spermatocytes in some sections in most of the tubules. Furthermore, the ratio between spermatids and spermatozoa was high. The evaluation of spermatogenesis revealed a significant difference between the four groups ($p < 0.05$). Treatment with PM, particularly at 100 mg kg^{-1}, can exert ameliorative effects on the testicular T/D induced I/R injury.

Testicular lipid peroxidation, CAT, and peroxidase activities. There was a significant increase in the lipid peroxidation level in TDC group, compared to the Sham group ($p < 0.05$). However, there was a significant decrease in the lipid peroxidation level in the TDP50 group and TDP100 group, compared with the TDC group ($p < 0.05$). Furthermore, the CAT activity level in TDC group was lower than that in Sham group. An increase in PM extract concentrations, particularly at 100 mg kg^{-1}, resulted in an increase in CAT activity level. Furthermore, the peroxidase activity level in TDC group was higher than that in Sham group. In the presence of PM extracts, particularly at 100 mg kg^{-1}, the peroxidase activity returned to a normal level (Table 1).

Table 1. The level of lipid peroxidation, peroxidase and catalase at the experimental groups.

Groups	CAT (U per mg protein)	Peroxidase (U per mg protein)	MDA (nmol dL^{-1})
Sham	13157.00 ± 153.00[a]	2529.00 ± 49.00[a]	3.11 ± 0.12[a]
TDC	8485.00 ± 87.00[b]	2866.00± 55.00[b]	6.27 ± 0.37[b]
TDP50	8285.00 ± 93.00[b]	2269.00 ± 27.00[c]	5.38 ± 0.35[c]
TDP100	9437.00 ± 101.00[c]	2530.00 ± 38.00[a]	4.69 ± 0.23[d]

CAT: Catalase, MDA: Malondialdehyde, Sham: sham-operated rats in which all the surgical steps were performed but T/D was not induced, TDC group: torsion/detorsion was followed by detorsion after 2 hr, TDP50: torsion/detorsion was induced and 50.00 mg kg^{-1} of *Plantago major* leaf extract was administered once daily for seven days intraperitoneally, and TDP100: torsion/detorsion was induced and 100 mg kg^{-1} of *Plantago major* leaf extract was administered daily for seven days intraperitoneally after detorsion.
[abcd] Different letters indicate significant differences among the groups ($p < 0.05$).

Discussion

The results of our study showed that total phenolic content and total flavonoid of PM extracts were 136.58 ± 0.31 (µg PE mg^{-1}), and 2.55 ± 0.19 (µg QE mg^{-1}), respectively. This study demonstrated that PM extracts

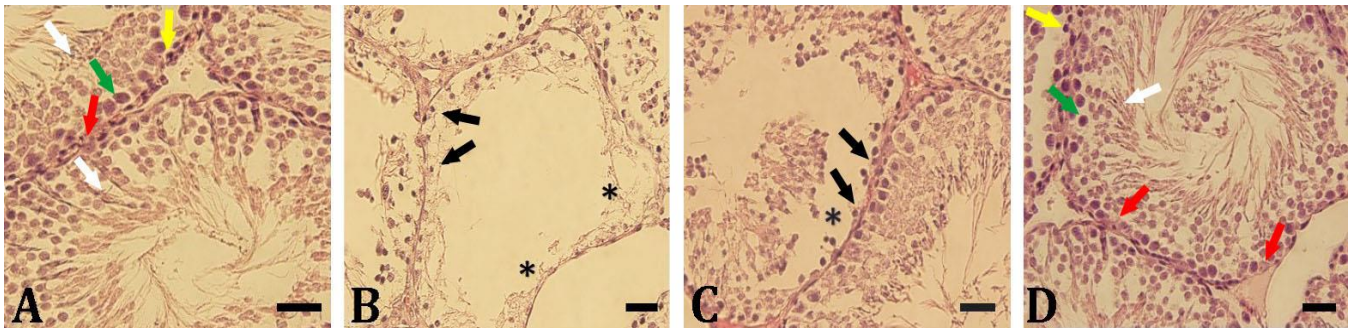

Fig. 2. Histological findings of rat testicular tissue in sham (A), TDC (B), TDP50 (C) and TDP100 (D) groups. Spermatogonia (yellow arrows), spermatocyte (green arrow), spermatid (white arrows), Sertoli cell (red arrows), cell necrosis (black arrows) and depletion of germ cell (stars). **A)** Normal structure of testicular tissue. **B)** Severe testicular degeneration and necrosis in the seminiferous tubules and germinal cells. **C)** Moderate testicular degeneration and necrosis in the seminiferous tubules and germinal cells. **D)** Mild pathological changes were found and the severity of the lesions reduced which almost was similar to the Sham group, (H&E; Scale bar = 30 µm).

exhibited significant antioxidant activity. Similar results were reported by Nazarizadeh *et al*.[14] Many pathological mechanisms play a significant role in severe testicular T/D-induced ischemia/reperfusion injury. In the present study, testicular torsion increased the peroxidase level. It also increased the degree of lipid peroxidation, estimated by measuring MDA levels. The results revealed that I/R injury caused testicular oxidative stress, however, PM prevented this I/R-induced injury. Mammalian testes are highly sensitive to oxidative stress because there are high levels of polyunsaturated fatty acids in their plasma membranes.[32] Fatty acids are essential for male germinal cells to maintain sperm mobility. The I/R-induced injury in testes is able to result in DNA damage, protein synthesis inhibition, and spermatogenesis arrest, thereby, result in impaired sperm production.[33] Based on previous findings, in the current study, we tried to find out the protective effect of PM. The PM was able to significantly protect spermatogenesis, down-regulate I/R-induced edema, hemorrhage, as well as coagulative necrosis. Hence, it could be concluded that the PM could significantly exhibit a protective effect. A decrease in the blood supply to tissue causes hypoxia during ischemia, leading to increased levels of lipid peroxidation products such as hypoxanthine, lactic acid, and thiobarbituric acid.[34] However, after ischemia, when the blood supply to the tissue increases, large amounts of oxygen- and/or nitrogen-derived free radicals are formed and result in further damage to the ischemic tissue. The I/R injury causes oxidative stress. The activated neutrophil recruitment and increased oxidative stress are properties of an inflammatory response.[35] Neutrophils increase the activity of nicotinamide adenine dinucleotide phosphate (NADPH) in ischemia. Hence, they are regarded as important sources of ROS.[36] Furthermore, lipid peroxidation activates nitrogen-activated protein kinases (MAPKs), which are required for signal transduction pathways of germinal cells apoptosis.[37] The MDA, a reliable parameter of lipid peroxidation and oxidative stress, was increased in the rat testes due to the I/R injury.[38] The oxidative stress is defined as high oxidative status and/or a low antioxidative status in the damaged tissue.[8] Parlaktas *et al*. reported that the MDA augmentation correlated directly with the lesion severity while peroxidase was decreased.[8] The mechanisms underlying reperfusion injury are complex. Numerous studies indicate that there is a relationship between oxidative stress and inflammation.[39] There was a significant decrease in the levels of lipid peroxidation and peroxidase activities, however, a significant increase in the CAT activity levels in the treatment groups, compared to TDC Group, showed the significant protective effect of PM particularly at 100 mg kg^{-1}. Moreover, it is likely that these ameliorative effects result from the anti-inflammatory properties of PM via the inhibition of ROS, leukocyte infiltration, and inflammatory cytokines.[39] The TNF-α and interleukin-1beta (IL-1b) are produced in the primary phase of inflammation and have various functions, particularly releasing free radicals.[40] Türel *et al*. have reported that methanol extracts of PM seeds exert anti-inflammatory effects on the carrageenan-induced paw edema in rats and inhibited pro-inflammatory cytokines, such as TNF-α and IL-1b.[41] According to a study by Samuelsen, anti-inflammatory effects of PM arise from iridoid glycosides, such as aucubin, and flavonoids, such as baicalein and hispidulin.[14] Hispidulin is effective as a 5-Lipoxygenase inhibitor.[42] The inhibition of the cyclo-oxygenase (COX)-catalyzed prostaglandin biosynthesis may account for the anti-inflammatory effects.[43] Minding the I/R-induced oxidative stress and considering the PM-induced antioxidant and anti-inflammatory properties and finally, to answer the question that how PM was able to induce protective effect the antioxidant status of testicles were considered in the current study. For this purpose, the testicular catalase and MDA contents were investigated. In the PM-treated group, the catalase and MDA contents were increased and decreased, respectively. These biochemical changes occurred in association with histological amelioration. Furthermore, the evaluation of spermatogenesis by Johnsen's scoring system[27] demonstrated abnormal spermatogenesis in the TDC group, however, moderate and mild spermatogenic damage in the treatment groups. These results showed that the protective effects of PM extracts were dose-dependent.

The quality of spermatogenesis in the treatment groups was different from that in the Sham-operated group. This suggested that the PM by up-regulating the testicular antioxidant status promoted spermatogenesis and/or germ cell survival. The higher dose of PM (100 mg kg^{-1}) exhibited higher catalase, lower MDA and improved histological features versus low dose (50.00 mg kg^{-1})-received group. These findings represented that the PM-induced impacts were dose-dependently changeable. Similar histopathological results were reported by Ozbal *et al*. on the I/R injury to rat testes.[44] Likewise, Taati *et al*. indicated that necrosis and apoptosis in testicular tissues were caused as a result of I/R.[45] The highest level of the anti-inflammatory activity was found in the methanol extract followed by the ethanol extract. This result is in line with that of a study by Beara *et al*.[46] It is said that flavonoids, such as baicalein and hispidulin, and iridoid glycosides, such as aucubin, contribute to the anti-inflammatory activity. Stef *et al*. have analyzed the total antioxidant and scavenging capacity of PM, among many other herbal medicines.[47] According to their study, PM can be regarded as an antioxidant compound due to its reducing power and radical scavenging capacity.[47] Likewise, Kumarasamy *et al*. have reported that PM seed extracts exhibited high levels of free radical scavenging activity and inhibited lipid peroxidation.[48] In addition, according to the results of some other studies, PM had anti-

inflammatory effects on the ROS, leukocyte infiltration, and inflammatory cytokines.[47-49] Testicular torsion exerts its effects through oxidative stress. Hence, immediate treatment should be administered to prevent testicular atrophy and male infertility. However, testicular death is not an uncommon condition. Due to the involvement of oxidative stress, it is better to use antioxidant compounds. Moreover, the results showed that treatment with PM, particularly at 100 mg kg^{-1} could activate antioxidant mechanisms and minimize the I/R injury to testes.

According to the results of the present study, treatment with PM, particularly at 100 mg kg^{-1}, could exert ameliorative effects on the testicular T/D induced I/R injury by decreasing lipid peroxidation and peroxidase, increasing the activity level of CAT and overcoming the injury to testicular tissues. The possible mechanism of reduction in biochemical and histological injuries by PM extracts could be due to antioxidant activity.

Acknowledgments

The authors would like to thank the Research Affairs of University of Tabriz and Faculty of Veterinary Medicine, for financially supporting this work.

Conflict of interest

The authors have no competing interests to declare.

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