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Research Article

INVESTIGATION OF THE PROTECTIVE EFFECTS OF POMEGRANATE (*Punica granatum* L.) PEEL EXTRACT ON LIPOPOLYSACCHARIDE-INDUCED UVEITIS IN RATS

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Abstract: Pomegranate peel contains bioactive ingredients such as flavonoids, ellagitannins, phenolics and proanthocyanidin compounds with high antioxidant activity. Pomegranate peel has antiapoptotic, antioxidant and anti-inflammatory effects due to its high punicalagin content. We aimed to determine the effect of pomegranate peel extract (PPE) on lipopolysaccharide (LPS)-induced uveitis. Sixty rats were seperated randomly into twelve groups (n = 5). The healthy group received intraperitoneal normal saline, the uveitis group received 200 µg/kg LPS, the dexamethasone (DEX) group received 200 µg/kg LPS plus 1 mg/kg DEX, the PPE100, PPE300 and PPE500 groups received 200 µg/kg LPS plus 100, 300 and 500 mg/kg PPE, respectively. The eye tissues were collected at 3rd and 24th hour. and investigated molecularly (Relative quantification of gene expression), biochemically (Superoxide dismutase activity, Glutathione and Malondialdehyde levels) and histopathologically (staining with Harris Hematoxylin and Eosin Y). Tumor Necrosis Factor-α, vascular endothelial growth factor, and Caspase-3 levels markedly decreased in a dose-dependent manner in the uveitic rats following PPE administration. PPE administration significantly ameliorated uveitic disorders in oxidative stress factors including Glutathione, Superoxide dismutase and Malondialdehyde, with its effects raising in a dose-dependent manner. PPE eliminated histopathological changes in eye tissues due to uveitis. PPE can be a promising agent by contributing to alternative preventive treatment methods for uveitis with its anti-inflammatory, antioxidative, antiapoptotic and antiangiogenic effects.

Özet: Nar kabuğu, yüksek antioksidan aktiviteye sahip flavonoitler, elajitanenler, fenolikler ve proantosiyanidin bileşikleri gibi biyoaktif bileşenler içermesi nedeniyle en faydalı parçalardan biridir. Nar kabuğundaki yüksek punikalagin içeriği, antienflamatuvar, antioksidan ve antiapoptotik etkiler üretmesini sağlar. Bu çalışmada deneysel lipopolisakkarit (LPS) kaynaklı üveit modelinde nar kabuğu ekstresinin (NKE) koruyucu etkilerini değerlendirmeyi amaçladık. 60 sıçan rastgele 12 gruba ayrıldı (n = 5). Sağlıklı grup intraperitoneal normal salin; üveit grubu 200 µg/kg LPS; deksametazon (DEX) grubu 200 µg/kg LPS ve 1 mg/kg DEX; NKE100, NKE300 ve NKE500 grupları ise 200 µg/kg LPS ile birlikte sırasıyla 100, 300 ve 500 mg/kg NKE aldı. Göz dokuları 3. ve 24. saatte toplandı ve moleküler, biyokimyasal ve histopatolojik olarak incelendi. NKE uygulamasını takiben üveitik sıçanlarda tümör nekroz faktörü-α, vasküler endotelyal büyüme faktörü ve kaspaz-3 seviyeleri doza bağlı olarak belirgin şekilde azaldı. NKE uygulaması, doza bağlı olarak artan etkileri ile Glutatyon, Süperoksit dismutaz ve Malondialdehit gibi oksidatif strese bağlı faktörlerdeki üveitik bozuklukları önemli ölçüde iyileştirdi. NKE ayrıca üveit ile ilişkili göz dokularındaki histopatolojik değişiklikleri de ortadan kaldırdı. NKE, antienflamatuvar, antioksidan, antiapoptotik ve antianjiyojenik etkileri ile üveit için alternatif koruyucu tedavi yöntemlerine katkıda bulunarak umut verici bir ajan olabilir.

Introduction

Uveitis is an intraocular inflammatory disease affecting the choroid, ciliary body and iris (Guly & Forrester 2010) and people suffer from it regardless of their race, age or gender (Read 2006). It can result from metabolic and chemical injuries, complications of autoimmune diseases, and viral or bacterial infections causing ocular inflammation. There are also clinical cases defined as idiopathic uveitis (Suttorp-Schulten & Rothova 1996).

Corticosteroids are currently being used in the treatment of uveitis. However, long-term use of corticosteroids may cause many undesirable ocular effects such as increased intraocular pressure and accelerated cataract formation, undesirable systemic effects such as Cushing's syndrome, hypertension, diabetes, and adverse effects such as osteoporotic bones and metabolic disorders (LeHoang 2012). Therefore, research for determination of more effective agents in the treatment of uveitis is an important need for human health.

Pomegranate (*Punica granatum* L.) is commonly grown in various countries and consumed as juice or in fruit form. Pomegranate products have been used for centuries for medicinal purposes. Pomegranate peel, wich is one of the most useful part of the fruit, contains bioactive substances such as flavonoids, ellagitannins, phenolics and proanthocyanidin compounds with high antioxidant activity (Konsoula 2016). Punicalagin, the main antioxidant compound of pomegranate (Yaidikar *et al.* 2014, Les *et al.* 2015, Xu *et al.* 2015) is found in high amounts in the peel, and provides anti-inflammatory, antioxidant, anti-apoptotic and many other beneficial biological effects (Gil *et al.* 2000, Xu *et al.* 2005). BenSaad *et al.* (2017) reported that gallic acid, ellagic acid and punicalagin were the substances liable for the anti-inflammatory effect of pomegranate. As a part of the *in-vitro* and *in-vivo* study by Lin *et al.* (2021), it was demonstrated that punicalagin decreased the mRNA expression and protein level of proinflammatory factors and it was useful in the therapy of murine fungal keratitis (Lin *et al.* 2021).

Gallic acid has biological activities including antibacterial, antiviral, anti-inflammatory, antitumorigenic, antimelanogenic, and antioxidant effects (Kaur *et al.* 2009). Therefore, it is of great importance in both biomedical and pharmaceutical branches. Stoddard *et al.* (2013) reported that gallic acid protects the corneal epithelium against oxidative injury suppressing ROS in human corneal limbal epithelial cells. Khan *et al.* (2021) indicated that gallic acid coated contact lenses were very useful for scavenging free radicals, inhibiting protein adhesion, and killing pathogenic microbial species. These lenses could be used to treat ocular surface infections including inflammatory events, fungal and bacterial keratitis (Khan *et al.* 2021).

Pomegranate peel extract (PPE) has previously been shown to have protective activity in severe inflammatory conditions such as systemic sepsis (Kamboh 2016, Ugan *et al.* 2020) In addition, punicalagin and gallic acid, both PPE components, have significant effects against ocular diseases, which increased their importance in alternative medicine products. However, the effect of PPE against inflammatory eye diseases, such as uveitis, is unknown. Based on all this information, this study aimed to determine the protective effects of PPE in lipopolysaccharide-induced uveitis in rats.

Materials and Methods

Sixty male Albino Wistar rats (4-6 months, weighing 300-330 g) were used in the experiments. The rats were provided by the Atatürk University Medical Experimental Research Center. The care and use of the rats were confirmed by the Atatürk University Institutional Animal Care and Use Committee. All experiments confirmed by the Ataturk University Animal Experiments Local Ethics Committee (Protocol number: 77040475-641.04-E.2000135871) were applied in accordance with international guidelines.

Chemical substances

Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4, synthesized from the cell barrier of the Gram-negative bacteria, was provided from Sigma-Aldrich, USA. Thiopental sodium was purchased from Ibrahim Ethem ULAGAY AS (İstanbul, Türkiye) and all other chemical substances were obtained from Sigma and Merck (Germany). Dexamethasone (DEX) was obtained from a local pharmacy.

Analysis and preparation of the pomegranate peel extract

Pomegranate was obtained from Mersin province in Türkiye and its content analysis was performed (Table 1). The peels of the fruits were peeled off and left drying by laying sparsely in an environment, where dry air flow was provided without any direct sunlight. The dried peels were ground with the help of a grinder. 50 grams of the ground peels were taken and put into a cartridge that was cleaned with ethanol as the extraction dissolvent. The cartridge was placed in a 500 mL Soxhlet extractor and 650 mL dissolvent was added to the boiling flask. It was extracted until the dissolvent became clear (20-30 siphons). The acquired liquid extract was filtered with blue band filter paper, and the spalls were ejected. Then, the dissolvent was vaporized with a rotary evaporator at 50-55°C. The extract, which was completely removed from its dissolvent, was weighed and placed in a storage box and stored at +4ºC (Wang & Weller 2006).

Lipopolysaccharide - induced uveitis and treatments

Sixty rats were randomly split into 12 groups, with 5 rats in each group (Table 2).

Table 1. Ingredients and their amounts in 1 g of peel extract.

Substance Name	Substance Amount (mg/g extract)
Gallic Acid	14.45±0.53
Punicalagin A	191.56± 0.36
Punicalagin B	189.48± 0.62
Ellagic Acid	68.02± 0.42

Table 2. Definitions of the experimental groups.

Group	Group Code (n=5 for each)	Administration	Time to end the experiment
1	Healthy 3rd h	Only intraperitoneally normal saline	3 h after LPS administration
2	Uveit 3rd h	200 µg/kg LPS	
3	DEX 3rd h	200 µg/kg LPS+1 mg/kg DEX	
4	PPE 100 3rd h	200 µg/kg LPS+100 mg/kg PPE	
5	PPE 300 3rd h	200 µg/kg LPS+300 mg/kg PPE	
6	PPE 500 3rd h	200 µg/kg LPS+500 mg/kg PPE	
7	Healthy 24th h	Only ip normal saline	
8	Uveit 24th h	200 µg/kg LPS	24 h after LPS administration
9	DEX 24th h	200 µg/kg LPS+1 mg/kg DEX	
10	PPE 100 24th h	200 µg/kg LPS+100 mg/kg PPE	
11	PPE 300 24th h	200 µg/kg LPS+300 mg/kg PPE	
12	PPE 500 24th h	200 µg/kg LPS+500 mg/kg PPE	

LPS-induced uveitis model was established by a single subcutaneous (sc) injection of 200 µg/kg LPS (Yadav *et al.* 2009, Keles *et al.* 2014). Pomegranate peel extract at doses of 100, 300 and 500 mg/kg were prepared (Sadeghipour *et al.* 2014). 1 mg/kg DEX was administered intraperitoneally half an hour before the LPS injection, simultaneously as the LPS injection, and 30 min after the LPS injection (Keles *et al.* 2014). Three different PPE doses (PPE100, PPE300 and PPE500) were each administrated to its respective group orally for 7 days before LPS injection. The eye tissues were collected at two time points, 3 and 24 h after LPS injection, and stored under appropriate conditions for biochemical, molecular and histopathological analysis (Keles *et al.* 2014).

Biochemical analyses

100 mg of each eye tissue was homogenized with 1 ml PBS and ground in liquid nitrogen with Tissue Lyser II (Qiagen). Following the grinding process, all tissues were centrifuged at 5000 rpm for 10 min. Superoxide dismutase (SOD) activity, Glutathione (GSH), and Malondialdehyde (MDA) levels were measured manually and expressed as U/mg protein for SOD activity, nmol/mg protein for GSH and MDA similar to the previous studies (Ugan & Un 2020). Total protein amounts were measured manually using the Lowry method (Lowry *et al.* 1951). The mean absorbances of each sample and the standard curve were calculated. All data were shown as mean±standard deviation (SD) relative to each mg protein.

Molecular analyses

Relative quantification of gene expression (real-time reverse transcriptase-polymerase chain reaction)

The relative Caspase-3 (CASP-3), Caspase-9 (CASP-9), tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor (VEGF) expression analyses were performed with StepOnePlus Real-Time PCR System technology (Applied Biosystems) using cDNA synthesized from rat RNA as previously described (Yayla *et al.* 2020). All primers and probes were purchased as TaqMan Gene Expression Assays: TNF-α (JN157530), VEGF (JN177806), Caspase-3 (JN174232) and Caspase-9 (JN174233). The expression data for β-actin ((JN174235) in each eye tissue was used as the housekeeping gene, and the primers and probes for the β-actin were designed by Primer Design. Ct values were automatically transformed into delta delta Ct ($2^{-\Delta\Delta Ct}$).

Histopathological analyses

Preparation of solutions, dehydration and clearing procedures of tissue samples, preparation of sections and staining with Harris Hematoxylin and Eosin Y were carried out in line with previous studies for histopathological evaluation (Akpinar *et al.* 2022). The eye tissue samples obtained from rats for histopathological evaluation were fixed in 10% formalin solution for 48 h.

Staining of sections were conducted with Harris Hematoxylin for three minutes. And then eye tissue sections were rinsed with tap water for five minutes to remove excess paint and kept in Eosin Y solution for two minutes for counter staining. Slides were slowly immersed in 96% ethyl alcohol five times to remove excess paint, they were kept in 99% ethyl alcohol and three series of xylene for two minutes each and then mounted with Entellan. For histopathological examinations, stromal lamellar detachments, edema and dilatation areas, polymorphonuclear leukocyte aggregations and infiltrations in eye tissues were evaluated by light microscope. At least 5 areas in every tissue slide at ×100 enlargement were evaluated and appointed to define the violence of the alterations using scores on a scale including absent or almost absent: -/+, grade 1: + (mild positive), grade 2: ++ (moderate positive), grade 3: +++ (severe positive) (Un *et al.* 2022).

Statistical analysis

Data are expressed as means±standard deviation (SD). Statistical analyzes were performed with One-way ANOVA and Duncan's multiple comparison tests using the IBM SPSS 21.0 package program. *p* value less than 0.05 was considered statistically significant.

Results

The results of biochemical analyses

Oxidative stress-related factors including MDA, SOD and GSH were measured in all rat eyes. Levels of MDA increased in eye tissues of the uveitis group. In the PPE administered groups, there was statistically significant decreased MDA levels crosschecked to the uveitic rats (p<0.001) (Fig. 1a). SOD activity and GSH level significantly redused due to oxidative damage in the eye tissues of the uveitis group. PPE administration regulated oxidative stress in a dose-dependent manner. PPE remarkably increased SOD activities (p<0.001) and GSH levels (p<0.001) depending on the dose check against uveitic rats (Figs 1b-c).

The results of molecular analyses

In order to evaluate whether 100, 300 and 500 mg/kg PPE alleviated LPS-induced uveitis, mRNA expression levels including TNF-α, VEGF, Caspase-3 and Caspase-9 in the eye tissue of rats were analyzed. The mRNA expression levels of TNF-α and VEGF mRNA increased in the eye tissue of uveitis group. PPE administration significantly decreased TNF-α (p<0.05) and VEGF (p<0.05) mRNA expression levels induced by uveitis. In the uveitis group, although there was no significantly change in Caspase-9 levels (p>0.0742), Caspase-3 levels remarkably increased (p<0.05). Caspase-3 mRNA expression levels stimulated by uveitis was significantly reduced in the PPE 100 (p<0.05), 300 (p=0.0685) and 500 (p<0.01) mg/kg groups, compared to the uveitis group. (Figs 2a-d).

The results of histopathological analyses

The histopathologic effects of PPE on the pretreatment of uveitis were shown in Figs 3a-b, and the histopathologic scores were given in Table 3.

No pathological marks were observed in eye samples of the healthy group for the 3rd and 24th h (Figs 3a-b). Signs of severe uveitis were observed in the tissues of the uveitis groups with serious stromal lamellae separations in the cornea. Significant dilatations, polymorphonuclear leukocyte (PMNL) infiltrations and PMNL aggregations were also observed in the iris and major dilatations and edema areas were observed in the ciliary body.

It was observed that the histopathological damage caused by uveitis improved in PPE-administered groups depending on the dose. In addition, the histopathological appearance of PPE 500 administered group was similar to the healthy group (Figs 3a-b).

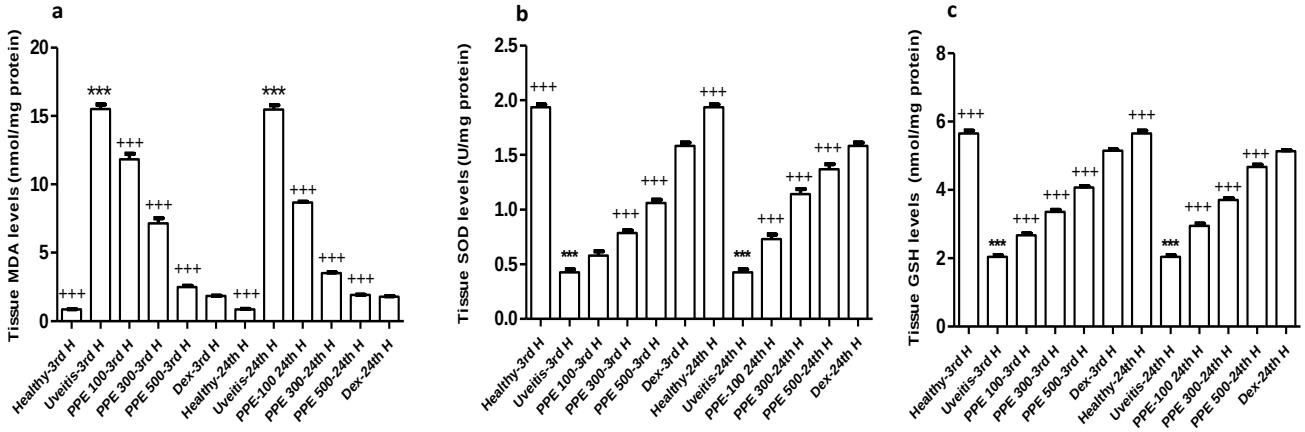

Fig. 1. The results of biochemical analyses in rat eye tissues at 3rd and 24th hours after LPS injection. **a.** MDA levels (nmol/mg protein), **b.** SOD activity (U/mg protein), **c** GSH levels (nmol/mg protein). +compared to uveitis group, *: compared to healthy group.

Table 3. Histopathologic scores of experimental groups.

	Groups	Stromal Lamella Separation	Edema and Dilatation	PMNL Aggregation and Infiltration
3rd hour	Healthy	-	-	-
	Uveitis	+++	++	+++
	Dex	-/+	-/+	-/+
	PPE-100	-	-/+	+
	PPE-300	-/+	-/+	+
	PPE-500	-	-	-/+
24th hour	Healthy	-	-	-
	Uveitis	++	+	++
	Dex	+	-/+	+
	PPE-100	-/+	-/+	-/+
	PPE-300	+	+	+
	PPE-500	-	-	+

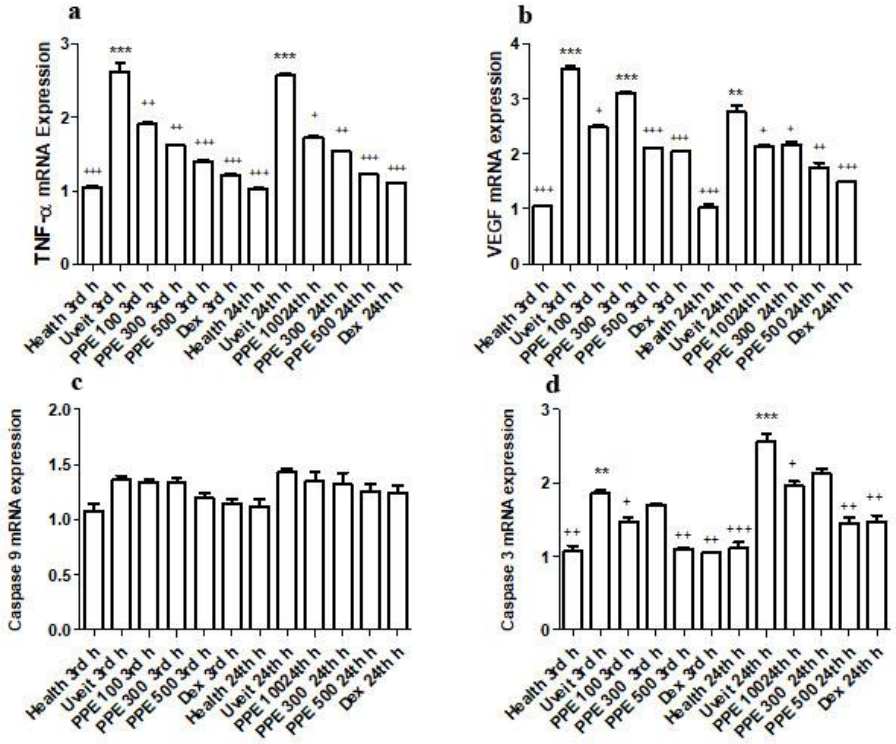

Fig. 2. Molecular findings in eye at 3rd and 24th hours after LPS injection. **a.** TNF-α mRNA expression levels, **b.** VEGF mRNA expression levels, **c.** Caspase 9 mRNA expression level, **d.** Caspase 3 mRNA expression levels. +: compared with uveitis groups; + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$, *: compared to healthy group; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Discussion

Uveitis is defined as a specific type of inflammation in the uvea and can affect any part of the uveal layer (Guly & Forrester 2010, Seve *et al.* 2017). LPS-induced uveitis occurs by systemic injection of one time non-lethal dose of LPS, and the maximum inflammatory reaction is reached 24th h after the LPS injection (Rosenbaum *et al.* 1980, Chang *et al.* 2008).

Cytokines have an important role in the development of uveitis (Zhang *et al.* 2017). The serious increase in cytokines and chemokine levels due to inflammation of the uvea causes changes in both the activation of intracellular signal cascades in the ocular tissues and in the expression of various inflammatory genes (Curnow & Murray 2006, Kim & Moudgil 2008). Therefore, changes in these markers are important for inflammatory eye disease and, also, for the follow-up of the disease. In the light of this information, the effects of PPE in LPS-induced uveitis model were evaluated molecularly, biochemically and histopathologically.

It was determined that PPE reduced TNF-α mRNA expression. During uveitis, TNF-α, which is an important regulator of immune and inflammatory reactions (Sugita *et al.* 2007) collects leukocytes, increases leukocyte adhesion to the vascular endothelium, activates macrophages and strains T cells, and promotes apoptosis of leaking and established cells (Dick *et al.* 2004). Park *et al.* (2016) reported that PPE caused anti-inflammatory effect and decreased TNF-α levels in THP-1 cells exposed to particulate matter PM10. In addition, Wang *et al.* (2018) showed that PPE caused anti-inflammatory effect and decreased TNF-α levels in concanavalin A-induced autoimmune hepatitis in mice. Also, increased TNF-α levels increase free radicals (Cinar *et al.* 2019, Kutlu *et al.* 2020). PPE ingredients such as ellagitannin, punicalin, ellagic acid and gallagic acid were associated with antioxidant, antimicrobial, antidiabetic, anticarcinogenic, anti-inflammatory and cardiovascular protective activities (Alexandre *et al.* 2019). Mastrogiovanni *et al.* (2020) stated that PPE reduced the oxidative stress and inflammatory status induced bovine mammary epithelial cells BME-UV1. Amer *et al.* (2015) demonstrated that pomegranate peel had antioxidant and anti-inflammatory effects on Eimeria papillata-induced infection in mice. Consistent with the previous studies, this study found that PPE reduced TNF-α levels.

The present results also showed that PPE corrected changes in the oxidant and antioxidant parameters including GSH, SOD and MDA due to uveitis, indicating the strong antioxidant feature of PPE. Oxidative stress plays a causal role in the complications of uveitis (Yadav *et al.* 2011). El-Bouhy *et al.* (2021) indicated that PPE up-regulated GSH levels and caused antioxidant effects. Similar to these results, this study showed that PPE administration regulated GSH level, depending on the dose while GSH levels decreased in the uveitis group. In a study conducted by Doostan *et al.* (2017) PPE modulated SOD activity and protective effects againts oxidative stress induced by methotrexate in rats. Hala *et al.* (2017) reported that pomegranate pretreatment played

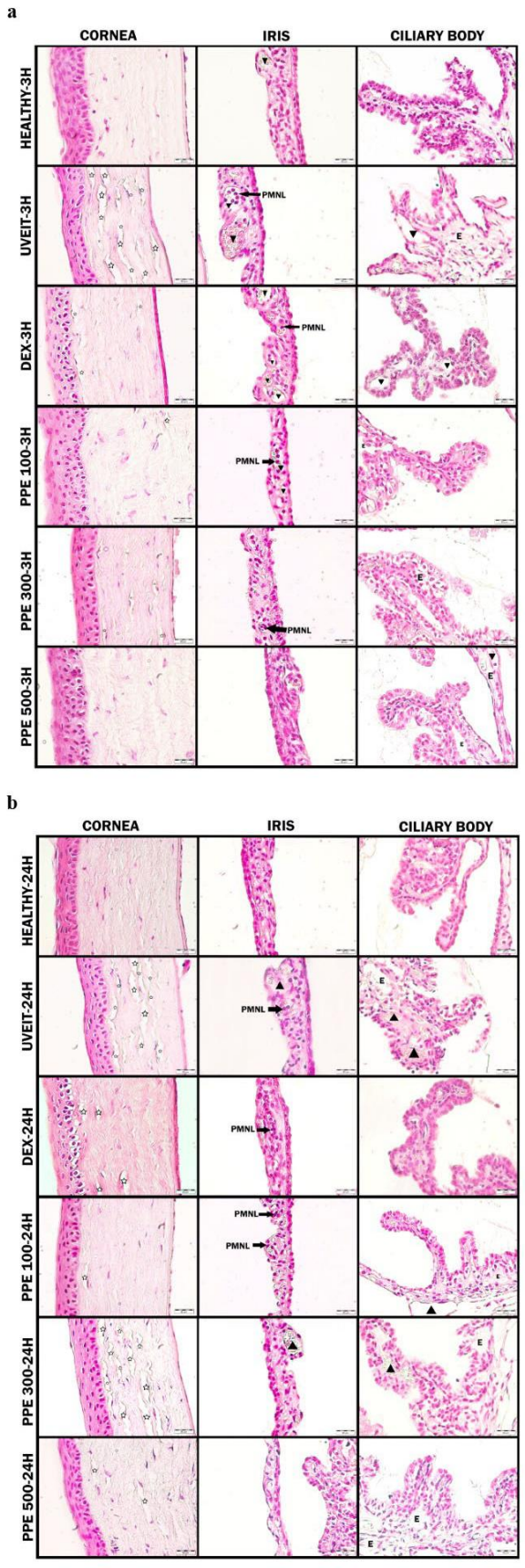

Fig. 3. Histopathological appearance of eyes of each group at **a.** 3rd hours and **b.** 24th hours after LPS injection (Star: Stromal lamellae separations, Triangle: Dilatation, E: Edema, PMNL: Polymorphonuclear leukocytes).

a protective role against oxidative stress in retinal damage. In addition, they stated that pomegranate stimulated extraordinary healing of retinal morphology and function and reduced oxidative stress by raising antioxidant enzymes such as GSH-Px and SOD in the retina. Elwej *et al.* (2016) suggested that PPE decreased MDA level in renal tissue and improved barium-induced renal oxidative damage in adult rats. Bagheri *et al.* (2021) demonstrated that PPE modulated MDA level in renal tissue in a rat model of alloxan-induced diabetes. Our findings are consistent with these previous studies.

VEGF is a strong stimulator of angiogenesis and endothelial activation and primarily expressed in glial cells and retinal neurons and is present in small numbers in blood vessels (Famiglietti *et al.* 2003). Hypoxic or ischemic conditions, production and retinal expression of VEGF significantly climb-up degeneration (Yoshida *et al.* 2009, Do *et al.* 2015). VEGF plays a role in the formation of retinal neovascularization in ischemic retinopathies like age-related macular degeneration (Do *et al.* 2015). Dana *et al.* (2015) reported that PPE decreased VEGF mRNA expression levels and led to antiangiogenic and antiproliferative effects on melanoma cell line. Ai *et al.* (2019) stated that treatment with Jikan Mingmu Drops containing gallic acid and ellagic acid ameliorates dry eye syndrome in diabetic mice. Further, they showed that Jikan Mingmu Drops treatment significantly redintegrated the morphology and structure of conjunctival epithelial cells, and importantly decreased the levels of TNF-α, and VEGF in the conjunctiva. In line with the previous studies, this study found that PPE reduced VEGF levels.

LPS-induced inflammation causes severe apoptosis (Scholz *et al.* 2015). Activation of Caspase-9 increases Caspase-3 that stimulates proteolytic corruption of diverse cellular goals and stimulation of endonucleases, which ultimately causes cellular death (Chang *et al.* 2011). Earlier studies indicated that Caspase-3 expression, one of the apoptotic and angiogenic markers, increased in uveitis (Tagirasa *et al.* 2017). Emam *et al.* (2020) argued that PPE decreased Caspase-3 levels and suppressed nephrotoxicity in mice. El Bohi *et al.* (2021) reported that PPE decreased Caspase-3 levels and modulated the expression of apoptosis-related proteins as well as oxidative damage, and inflammation in liver and kidney tissues injury in rats. In line with the previous studies, this study also found that PPE declined Caspase-3 levels.

Uveitis was reported to cause grave histological change in the eye tissue (Labsi *et al.* 2021). Earlier studies indicated that uveitis created histopathological injury in the eye tissue including ciliary body, retina (Garcia-Otero *et al.* 2021), cornea, iris (Crabtree *et al.* 2022). In addition, Hashem *et al.* (2017) showed with their histological studies that pomegranate had a protective effect on experimental ischemia/reperfusion retinal damage in rats. This found that PPE corrected histopathologic injury in the eye tissue due to uveitis.

In conclusion, it was determined in the present study that PPE reduced the molecular, biochemical and histopathological injury seen in eye tissues of uveitic rats. This effect was associated with the antioxidant and anti-inflammatory features of PPE.

Conclusion

PPE ameliorated the alteration in anti-inflammatory and antiapoptotic parameters owing to uveitis. PPE improved the modification in oxidant and antioxidant parameters stimulated by uveitis. PPE ameliorated histopathological damage of eye tissues caused LPS-induced uveitis. PPE could be a promising agent by contributing to alternative preventive treatment methods for uveitis with its anti-inflammatory, antioxidative and antiapoptotic effects. Also, PPE can play a significant role in the production of new preventive medicines for uveitis with its natural ingredient.

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Ethics Committee Approval: The care and use of the rats were confirmed by the Atatürk University Institutional Animal Care and Use Committee. All experiments confirmed by the Ataturk University Animal Experiments Local Ethics Committee (Protocol number: 77040475-641.04-E.2000135871) were applied in accordance with international guidelines.

Data Sharing Statement: All data are available within the study.

Author Contributions: Concept: T.N.Y., M.Y., D.K., P.A.K., E.C., Z.H., Design: T.N.Y., M.Y., D.K., P.A.K., Execution: T.N.Y., M.Y., D.K., Material supplying: T.N.Y., M.Y., D.K., P.A.K., Data acquisition: T.N.Y., M.Y., R.A.U., E.T., Data analysis/interpretation: T.N.Y., M.Y., R.A.U., E.T., Writing: T.N.Y., M.Y., E.C., Z.H., Critical review: T.N.Y., M.Y., D.K., P.A.K., E.C., Z.H.

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