Effects of extracts from goji berry *(Lycium barbarum)*, green tea *(Camellia sinensis)*, and persimmon (*Diospyros kaki*) leaves in a dry eye model of mice

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Effects of extracts from goji berry *(Lycium barbarum)***, green tea** *(Camellia sinensis)***, and persimmon** (*Diospyros kaki*) **leaves in a dry eye model of mice**

Abstract

This study aimed to investigate the effects of goji berry *(Lycium barbarum)* [GB], green tea *(Camellia sinensis)* [GT], and persimmon (*Diospyros kaki*) [DK] leaf extracts in a mouse dry eye (DE) model. Fifty-six eyes from 28 female BALB/c mice were equally divided into the following groups: control, GB, GT, and DK (left eye groups); DE, DE + GB, DE + GT, DE + DK (right eye groups). The DE model was induced in right eye groups by topical administration of 5 µL of 0.2 % benzalkonium chloride twice daily for 14 days. A 1 % extract solution of GB, GT, and DK was instilled in the DE + GB, DE + GT, and DE + DK groups between days 15 and 30, respectively. In the DE group, only 0.9 % NaCl was instilled during this period. All groups were evaluated for aqueous tear production rate, corneal fluorescein staining, and tear break-up time (TBUT). Histopathological and Western blot analyses performed after euthanasia. On day 15, aqueous tear production decreased, corneal fluorescein staining scores increased, and TBUT was shortened in right eye groups compared to left eye groups ($P < 0.05$). At the end of the study, no significant differences were observed among the extract-treated DE groups regarding clinical parameters ($P > 0.05$). However, based on histopathological and molecular analyses, the GT group demonstrated the most beneficial effect without side effects ($P < 0.05$). These results suggest that 0.1 % GT leaf extract may be a potential therapeutic agent for DE.

Keywords: Dry eye; Inflammation; Phytotherapy; Precorneal Tear Film; Tear hyperosmolarity; Treatment.

Study contribution

Tear hyperosmolarity and tear film instability, along with the inflammatory process that triggers these factors, play a key role in the pathogenesis of dry eye. Anti-inflammatory therapy has become a common strategy in the treatment of dry eye disease, as inflammation is the primary factor driving disease development. Many herbal extracts with broad-spectrum effects, including anti-inflammatory, antioxidative, and immunomodulatory properties, remain among the current treatment options for dry eye. This study aimed to compare the therapeutic effects of the topical application of extracts from goji berry, green tea, and *Diospyros kaki* leaves in a mouse dry eye model, in terms of histopathologic and biochemical. At the end of the study, GT was found to have the most beneficial effect among these agents. These results suggest that GT may serve as a potential herbal alternative for the treatment of dry eye.

Introduction

Dry eye (DE) is an inflammatory disease characterized by clinical symptoms, including burning, stinging, and a foreign body sensation, which develop due to tear film instability and hyperosmolarity.[1] Key signaling pathways play a central role in the pathogenesis of DE by stimulating the release of pro-inflammatory cytokines such as nuclear factor kappa B (NF-κB). Excessive cytokine release leads to tear film instability and hyperosmolarity on the ocular surface. In recent years, the inhibition of inflammatory responses has become one of the critical issues that researchers focus on in the treatment of DE.[2–4]

Many herbal extracts with a broad spectrum of effects, including anti-inflammatory, antioxidative, immunomodulatory, and anti-aging properties, remain among the current

treatment options for DE.[3] Goji berry (GB) is a flavonoid-rich plant widely used as a flavoring ingredient in Asian cuisine.[5] GB has also been used as a therapeutic agent in various diseases due to its broad spectrum of biological activities. It has been reported to exert an ameliorative effect when used both topically and orally in DE patients.[6, 7] Green tea (GT) is a low-molecular-weight plant extract containing catechins such as epigallocatechin gallate (EGCG), which exhibits a broad range of biological activities.[8, 9]

EGCG has been reported to reduce inflammation and prevent osmolarity increases by suppressing cytokine release in DE cases.[10, 11] *Diospyros kaki* (DK), a fruit cultivated in Asia for many years, is also known as persimmon.[12] Its leaves contain bioactive compounds such as terpenoids and flavonoids, which possess beneficial anti-inflammatory and antioxidant properties.[13] In a mouse model of DE, orally administered DK has been reported to suppress ocular inflammation and improve clinical signs of the disease.[14] The study aimed was to compare the therapeutic effects of the topical administration of extracts from GB, GT, and DK leaves in an experimentally induced mouse dry eye model, using histopathological and biochemical analyses.

Materials and methods

Ethical statement

This study was conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Fırat University Animal Experiments Local Ethics Committee (No:2018/138).

Preparation of leaf extracts

GB, GT, and DK leaves were dried in air and then ground into powder in the laboratory using an herb grinder. Twenty-five grams of powdered leaves was extracted with 95 % ethanol in a volume of 250 ml under sterile conditions for 12 hours at room temperature. Filtration was then performed using Whatman No. 4 filter paper (GE Healthcare, USA). This step was repeated three times, and the obtained extracts were concentrated to 10 % (w/v) by evaporation under reduced pressure in a rotary evaporator at 40 °C. The extracts, prepared separately for each leaf type, were then diluted with 0.9 % sterile saline solution (SF) to a final concentration of 0.1 % (v/v) and stored in sterile bottles under cold chain condition until use.[15]

Animals

Power analysis was used to estimate the sample size, which was determined to be 28, with a 3 % margin of error at a significance level of 0.05 and an 80 % power value (Type I error = 0.05; statistical power = 0.80). The study included 56 eyes (28 left, 28 right) from 28 female BALB/c mice, aged 6-8 weeks, with an average weight of 16−18 g. All animals were housed in a controlled environment with an ambient temperature of 22 °C ± 1 °C, relative humidity of 60 % ± 10 %, a 12:12 light-dark cycle, and no food or water restriction. Mice deemed healthy based on clinical and ophthalmological examinations were included in the study. One week before the experiment, all mice were transferred to an independent room and housed in conventional mouse cages. The cages had an average size of 265 × 205 × 140 mm and a floor housing space of 350 cm^2 (Euro Type 2 cage type, Tecniplast, Italy). The average weight of the study mice was 25 g, and the recommended housing area for a 25 g mouse in multi-animal housing is 77.4 cm^2. Accordingly, four mice

were placed in each cage (for 350 cm^2 of housing space). During this adaptation period, mice were acclimated to a funnel-shaped restraint made of specially designed rigid plastic to prevent self-injury and ensure researcher safety during measurements. This device restricted the mice's movements, allowing for more precise instillation of topical agents and accurate execution of parametric tests.

Mouse model of Dry eye and instillation of extracts

A total of 56 eyes were divided into control, GB, GT, and DK groups (7 × 4 = 28) for the left eyes, and DE, DE + GB, DE + GT and DE + DK groups (7 × 4 = 28) for the right eyes. All left eye groups received 0.9 % saline (Bioflex/Osel-Istanbul) at a dose of 5 µL twice daily, once at 8:00 am and again at 8:00 pm, for 14 days. Between days 15 and 30, the control group continued to receive 0.9 % saline, to the control group, 0.1 % GT leaf extract to the GT group, 0.1 % GB leaf extract to the GB group and 0.1 % DK leaf extract to the DK group at a dose of 5 µL three times a day (at 9:00 am, 2:00 pm, and 8:00 pm). To induce DE model in mice, benzalkonium chloride (BAC) (Sigma–Aldrich) was prepared in a sterile PBS (8 g NaCl, 0.2 g/L KCl, 1.44 g/L Na2HPO4, pH 7.4) at a concentration of 0.2 % (w/v).[16] All right eyes groups received 0.2 % BAC at a dose of 5 µL twice daily 8:00 am, and at 8:00 pm for the first 14 days to induce the DE. Between days 15 and 30, a microinjector (901 N, Hamilton, USA) was used to instill 0.9% saline in the DE group, 0.1 % GB leaf extract in the DE + GB group, 0.1 % GT leaf extract in the DE + GT group and 0.1 % DK leaf extract in the DE + DK group, at a dose of 5 µL three times daily (at 9:00 am, 2:00 pm, and 8:00 pm). Parameters such as excessive weight loss, hair loss, and a significant reduction in feed and water consumption were established as humane

experimental endpoint criteria, Animals were monitored and recorded accordingly until the end of the study.

Procedure of the clinical parametric tests

In all the groups, the aqueous tear production rate, tear break-up time (TBUT), and corneal fluorescein staining tests were measured at baseline and on days 15, 20, 25, and 30 at the same time points (between 9:00 am and 12:00 pm). During each measurement, any ocular abnormalities (photophobia, ocular discharge and redness, conjunctivitis, keratitis) were also noted.

Aqueous tear production rate: The endodontic absorbent paper point test (EAPPT) was used to determine the aqueous tear production rate. Test strips (Roeko, Germany) were placed in the lower conjunctival fornix of the right and left eyes using forceps and left in place for 1 minute. After removal, the degree of wetness was measured under normal light using a millimetric scale (Zone Quick-Menicon, Nagoya, Japan).[16]

TBUT: 1 % fluorescein sodium solution (Alcon, Germany) was instilled into the right and left eyes of the subjects at a dose of 1 µL using a micro-injector (901 N, Hamilton, USA). After manually closing and opening the eyelids three times, the time to the first tear breakup (the first appearance of dry spots) was recorded using a cobalt blue filter under a slit lamp biomicroscope (XL-1, Shin-Nippon, Osaka, Japan).[16]

Corneal fluorescein staining: This test was performed 90 seconds after TBUT using a slit lamp with a cobalt blue filter. Corneal lesions were photographed under an operating microscope.[17] Lesions were scored as follows:

- 0: no staining on the corneal surface.
- 1: staining area ≤ ⅛ of the corneal surface.

- 2: staining area ≤ ¼ of the corneal surface.
- 3: staining area ≤ ½ of the corneal surface.
- 4: staining area ≤ ½ or covering the entire corneal surface.[18]

Histopathological analysis

At the end of the study, the mice were decapitated using sharp, heavy-duty scissors after deep anesthesia with 3 % isoflurane inhalation. Left and right eye tissues were carefully collected and stored at -80 °C until histopathological analyses were performed. For histopathological analysis, eyes were trimmed in the dorsoventral position near the optic nerve and fixed with 10 % buffered formaldehyde. After fixation, tissues were dehydrated, and embedded in paraffin blocks. 3−5 micrometer sections were stained with hematoxylin and eosin (H&E) and periodic acid-Shiff (PAS) stains. The number of vessels and inflammatory cells in the cornea was evaluated under a light microscope.[14]

Western blot analysis

Eye tissue samples were washed with cold PBS, then cut into small pieces and homogenized in lysis buffer using a mechanical and glass homogenizer (0.5 M Tris; pH:8, EDTA, β-mercaptoethanol, phenylmethylsulphonyl fluoride, NP-40, Proteinase K, Dithiothreitol). Shredded tissue samples were dried at 14 000 rpm for 5 min. The supernatant was collected and stored at -80 °C until use. Primary and secondary antibodies used in Western blotting (TNF-α antibody (52B83) ab8348, IL-1α antibody (ALF-161) sc12741, beta actin E-AB-20094, Nrf-2 antibody ab137550, NF-κB antibody sc-8008 primary antibodies; m-IgGκ BP-HRP sc-516102 and ab97023 secondary antibodies) were obtained from Santa Cruz Biotechnology (Germany), Abcam and

Elabscience (USA). Protein samples were run on a 12 % gel using the SDS-PAGE technique. The proteins were transferred to a nitrocellulose membrane via Western blotting, and their synthesis rates were assessed. Finally, the protein levels observed on the nitrocellulose membrane were quantified using a density determination analysis system.[19, 20]

Statistical analysis

All data were analyzed using the Statistical Package for the Social Sciences (SPSS 21, SPSS Inc, Chicago, IL, USA). A one-way analysis of variance (ANOVA) was used to evaluate the aqueous tear production rate, corneal staining test, and TBUT results within each group. The Friedman test was applied to assess non-parametric repeated measures between groups over time. The Wilcoxon test was used to determine significant time interval differences between groups. To compare groups in the statistical evaluation of the Western blot analysis, a one-way ANOVA followed by a post hoc Duncan test was applied. Differences between groups in the histopathological analysis were assessed using one-way ANOVA, with pairwise comparisons performed using the Tukey post hoc test. A P-value of < 0.05 was considered statistically significant.

Results

Aqueous tear production rate

No statistically significant differences were observed in the aqueous tear production rate between the left and right eyes of mice in any group on day 0 (baseline) (**Table 1**). The mean EAPPTT values of all left eye groups remained stable throughout the study, with no statistically significant differences from baseline to the end of the experiment (**Table 1**).

On day 15, following the induction of the DE model with BAC in the right eye groups, the mean EAPPTT values were significantly reduced compared to baseline measurement (**Table 1**). This reduction in the DE group persisted until the end of the study (**Table 1**). When the mean values of the extract-treated groups were analyzed for therapeutic effect between days 15 and 30, the DE + GT group showed the highest increase. However, this increase was not statistically significant when compared to the other extract-treated groups (**Table 1**).

Table 1. Statistical analysis of EAPPTT data according to measurement times and group variables

Groups	**Measurement time (right eye)**					
	Baseline* (mm/min)	15th day (mm/min)	20th day (mm/min)	25th day (mm/min)	30th day (mm/min)	**P-value**
DE	3.85 ± 0.24^{A}	2.35 ± 0.85^{B}	2.14 ± 0.37^{Ba}	2.64 ± 0.74^{Ba}	2.85 ± 0.69^{AB}	0.020
DE + GB	3.71 ± 0.39^{A}	2.50 ± 0.86^{B}	3.28 ± 0.69^{ABb}	4.00 ± 0.50^{ACb}	3.28 ± 0.75^{ABC}	0.018
DE + GT	4.28 ± 0.39^{A}	2.35 ± 0.98^{B}	3.71 ± 0.56^{ACb}	3.92 ± 0.53^{ACb}	3.50 ± 0.40^{BC}	0.006
DE + DK	4.07 ± 0.44^{A}	2.71 ± 0.90^{B}	2.42 ± 0.83^{Bac}	3.50 ± 1.08^{ABab}	3.35 ± 0.74^{AB}	0.022
P-value	> 0.05	> 0.05	0.000	0.000	> 0.05	
Groups	**Measurement time (left eye)**					
	Baseline* (mm/min)	15th day (mm/min)	20th day (mm/min)	25th day (mm/min)	30th day (mm/min)	**P-value**
DE	4.00 ± 0.00	3.85 ± 0.24	3.85 ± 0.24	3.92 ± 0.18	3.64 ± 0.24	0.053
DE + GB	3.92 ± 0.34	3.92 ± 0.18	4.00 ± 0.00	4.00 ± 0.00	3.64 ± 0.37	0.075
DE + GT	3.92 ± 0.53	4.07 ± 0.18	4.00 ± 0.28	4.07 ± 0.34	3.85 ± 0.24	0.357
DE + DK	3.92 ± 0.34	4.00 ± 0.28	3.78 ± 0.39	4.14 ± 0.24	3.71 ± 0.39	0.178
P-value	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	

* Baseline values of the study, a−c: The difference between groups with different letters in the same column is statistically significant ($P < 0.05$), ANOVA, post hoc Tukey. A−C: The difference between groups with different letters on the same line is important. ($P < 0.05$), Friedman was used for non-parametric tests and Wilcoxon tests for intragroup comparisons. (Mean ± standard deviation)

Tear break-up time (TBUT)

At the beginning of the study, no statistically significant differences were observed in the mean TBUT values between the left and right eyes of all mice (**Table 2**). The mean TBUT values of the left eye groups remained stable throughout the study, with no statistically significant differences from baseline to the end of the experiment (**Table 2**). However, significant differences were found among the right eye groups (**Table 2**). On day 15, the mean TBUT in the right eye groups was significantly shorter than baseline measurements (**Table 2**). When the mean TBUT values of the DE + extract groups were evaluated between days 15 and 30, all groups showed a prolongation of TBUT by the end of the study. However, no statistically significant differences were observed between them, and the group with the longest TBUT was DE + GT (**Table 2**). The shortest TBUT was detected in the DE group at the end of the study.

Table 2. Statistical analysis of TBUT data according to measurement times and group variables

Groups	**Measurement time (right eye)**					
	Baseline* (sec.)	15th day (sec.)	20th day (sec.)	25th day (sec.)	30th day (sec.)	**P-value**
DE	6.95 ± 0.18^{A}	3.07 ± 0.11^{B}	3.25 ± 0.09^{BCa}	3.74 ± 0.20^{CDa}	4.54 ± 0.17^{ADa}	0.000
DE + GB	6.92 ± 0.18^{A}	2.95 ± 0.12^{B}	4.57 ± 0.17^{BDb}	5.40 ± 0.10^{CDb}	5.99 ± 0.11^{ACbcd}	0.000
DE + GT	6.98 ± 0.12^{A}	3.04 ± 0.11^{B}	5.02 ± 0.13^{BCc}	5.96 ± 0.12^{CDd}	6.14 ± 0.08^{ADd}	0.000
DE + DK	6.98 ± 0.10^{A}	2.99 ± 0.11^{B}	4.80 ± 0.18^{BCbc}	5.75 ± 0.19^{CDcd}	5.96 ± 0.12^{ADcd}	0.000
P-value	> 0.05	> 0.05	0.000	0.000	0.000	
Groups	**Measurement time (left eye)**					
	Baseline* (sec.)	15th day (sec.)	20th day (sec.)	25th day (sec.)	30th day (sec.)	**P-value**
DE	7.01 ± 0.09	6.94 ± 0.11	6.96 ± 0.14	6.94 ± 0.14	6.96 ± 0.12	0.613
DE + GB	6.98 ± 0.11	7.04 ± 0.08	6.86 ± 0.11	7.05 ± 0.11	6.98 ± 0.12	0.069
DE + GT	7.00 ± 0.12	6.95 ± 0.11	6.99 ± 0.13	7.00 ± 0.10	7.04 ± 0.09	0.434
DE + DK	7.03 ± 0.05	7.01 ± 0.09	7.01 ± 0.10	7.04 ± 0.10	7.04 ± 0.11	0.741
P-value	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	

*Baseline values of the study, a−d: The difference between groups with different letters in the same column is statistically significant ($P < 0.05$), ANOVA, post hoc Tukey. A−D: The difference between groups with different letters on the same line is important. ($P < 0.05$), Friedman was used for non-parametric tests and Wilcoxon tests for intragroup comparisons. (Mean ± standard deviation)

Corneal fluorescein staining

At baseline, the corneal fluorescein staining scores of both eyes in all groups were recorded as “0” (**Table 3**). The mean corneal fluorescein staining scores of the left eye groups remained stable throughout the study, with no statistically significant differences from baseline to the end of the experiment (**Table 3**). On day 15, the corneal fluorescein staining scores of all right eye groups were significantly higher than baseline (**Table 3**). When the changes in the mean corneal fluorescein staining scores of the DE + extract groups between days 15 and 30 were analyzed, corneal staining was slightly reduced in DE + GT group ($P > 0.05$, compared to other groups), and in the DE group (**Table 3**).

Table 3. Statistical analysis of corneal fluorescein staining test scores according to measurement times and group variables

Groups	**Measurement time (right eye)**					
	Baseline* (score)	15th day (score)	20th day (score)	25th day (score)	30th day (score)	**P-value**
DE	0^{A}	3.00 ± 0.57^{B}	2.42 ± 0.78^{BC}	2.42 ± 0.78^{BC}	2.00 ± 0.57^{C}	0.035
DE + GB	0	3.28 ± 0.75	3.28 ± 0.69	2.28 ± 0.75	2.00 ± 0.57	0.059
DE + GT	0^{A}	3.14 ± 0.37^{B}	3.71 ± 0.56^{BC}	1.42 ± 0.53^{C}	1.42 ± 0.78^{C}	0.003
DE + DK	0^{A}	3.28 ± 0.48^{B}	2.42 ± 0.83^{BC}	2.00 ± 0.57^{C}	1.71 ± 0.75^{C}	0.013
P-value	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	
Groups	**Measurement time (left eye)**					
	Baseline* (score)	15th day (score)	20th day (score)	25th day (score)	30th day (score)	**P-value**
DE	0	0.57 ± 0.53	0.57 ± 0.53	0.42 ± 0.53	0.42 ± 0.53	0.896
DE + GB	0	0.57 ± 0.53	0.57 ± 0.53	0.42 ± 0.53	0.28 ± 0.48	0.724
DE + GT	0	0.57 ± 0.53	0.57 ± 0.53	0.42 ± 0.53	0.14 ± 0.37	0.392
DE + DK	0	0.57 ± 0.53	0.57 ± 0.53	0.42 ± 0.53	0.42 ± 0.53	0.919
P-value	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	

*Baseline values of the study, A−C: The difference between groups with different letters in the same column is statistically significant ($P < 0.05$), ANOVA, *post hoc* Tukey. A−C: The difference between groups with different letters on the same line is important. ($P < 0.05$), Friedman was used for non-parametric tests and Wilcoxon tests for intragroup comparisons. (Mean ± standard deviation)

Histopathological analysis

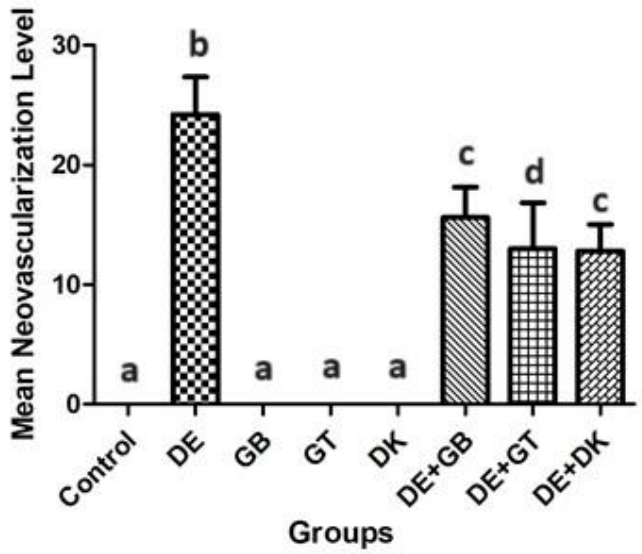
It was determined that corneal neovascularization was more prominent in the DE group compared to all other groups. The differences were statistically significant (**Figure 1A**). There were no significant differences in corneal neovascularization between the control and GB, GT, DK groups (**Figure 1A**). It was determined that there were statistically significant differences between DE + extract groups (**Figure 1A**). The lowest vascularization was detected in DK + GT group. Hemosiderin-laden macrophages were also found as a sign of chronic hemorrhage in cases with severe neovascularization (**Figure 1B**). The highest number of inflammatory cells in the cornea were detected in the DE and DK + GB groups, but the difference between these groups was not statistically significant (**Figure 1C**). The lowest number of inflammatory cells was found in the control, GB, GT, DK groups (**Figure 2B**). DE + GT and DE + DK had the lowest number of inflammatory cells in the DE + extract groups. There was no significant difference between these groups (**Figure 1C**).

Western blot analysis

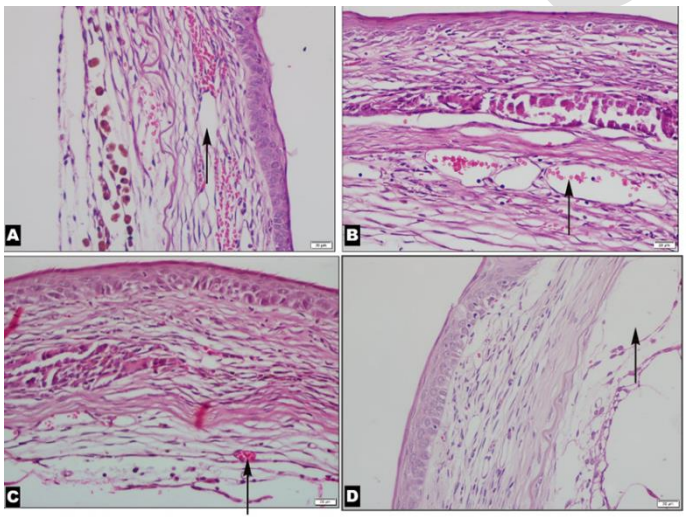
Among all groups, the highest inflammatory expression levels of IL-1α, TNF-α and NF-κB were detected in the DE group (**Figures 2 A–C, E**). In DE + extract groups, the lowest pro-inflammatory expression levels of IL-1α, TNF-α and NF-κB were measured in the DE + GT, while the highest expression levels were measured in the DE + DK groups (**Figures 2 A–C, E**). Regarding Nrf2, the difference in inflammatory expression levels among all groups were statistically significant (**Figures 2A**, and **D**). The highest Nrf2 expression levels were measured in the DE group (**Figure 2D**). In DE + extract groups,

Nrf2 expression levels were highest in the DE + GT and lowest in the DE + DK groups (**Figures 2A and D**).

(A)



(B)



(C)

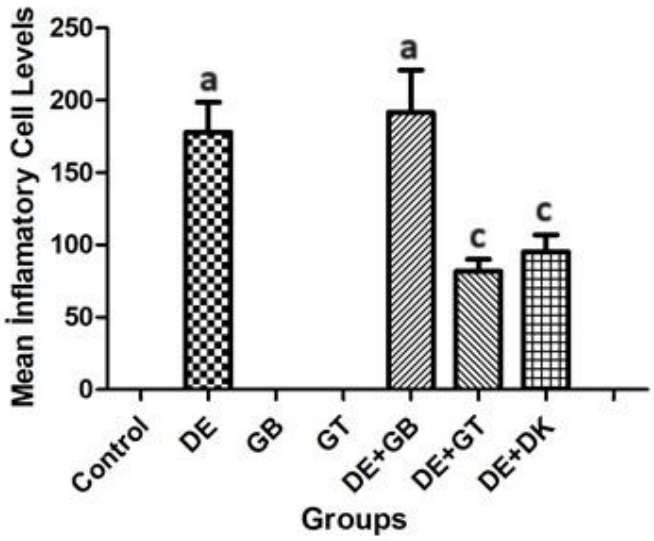
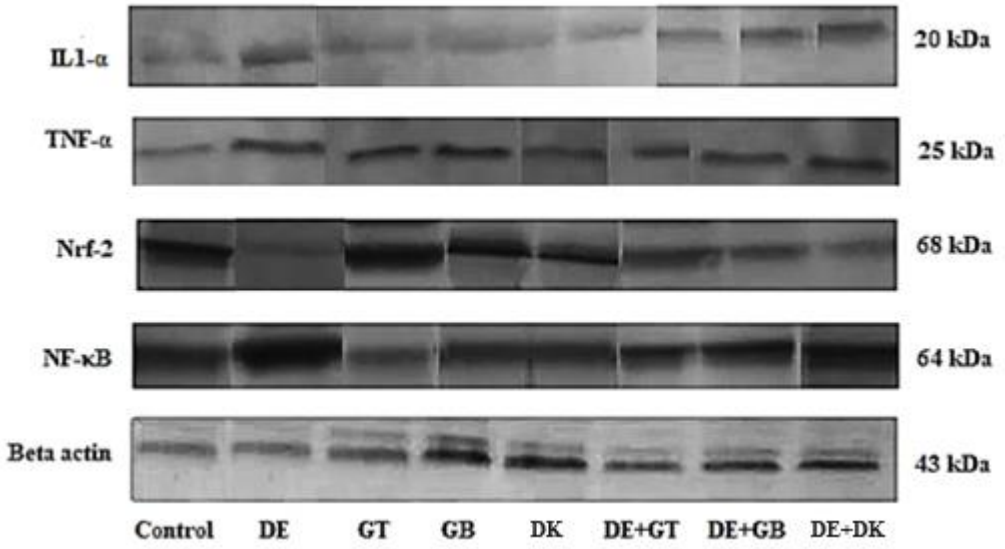
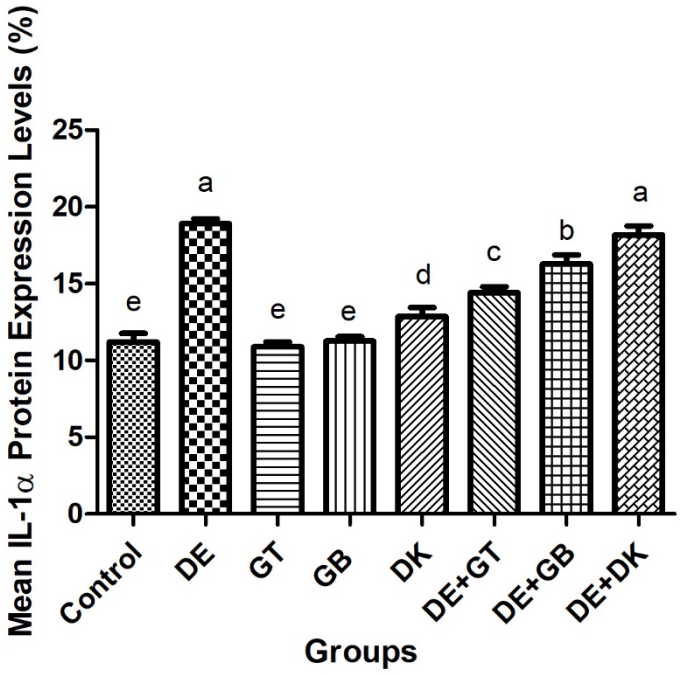


Figure 1. Mean corneal neovascularization and inflammatory cell levels. Mean neovascularization levels **(A).** Corneal neovascularization severity in groups (arrows), H-E, Bar: 20 µm. A) DE group (case no: 3), B) DE + GB group (case no: 2), C) DE + GT group (case no: 4), D) DE + DK group (case no: 4) **(B).** Mean inflammatory cell levels in the cornea in groups. *a-c: Differences between groups are indicated by different letters and this difference is statistically significant ($P < 0.05$). One-way ANOVA post hoc Duncan test **(C)**.

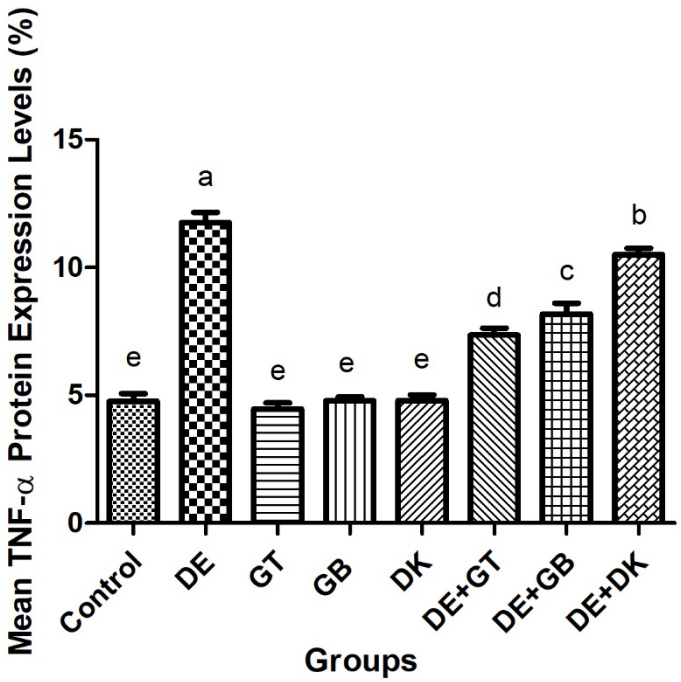
(A)


IL1-α
TNF-α
Nrf-2
NF-κB
Beta actin
20 kDa
25 kDa
68 kDa
64 kDa
43 kDa
Control
DE
GT
GB
DK
DE+GT
DE+GB
DE+DK

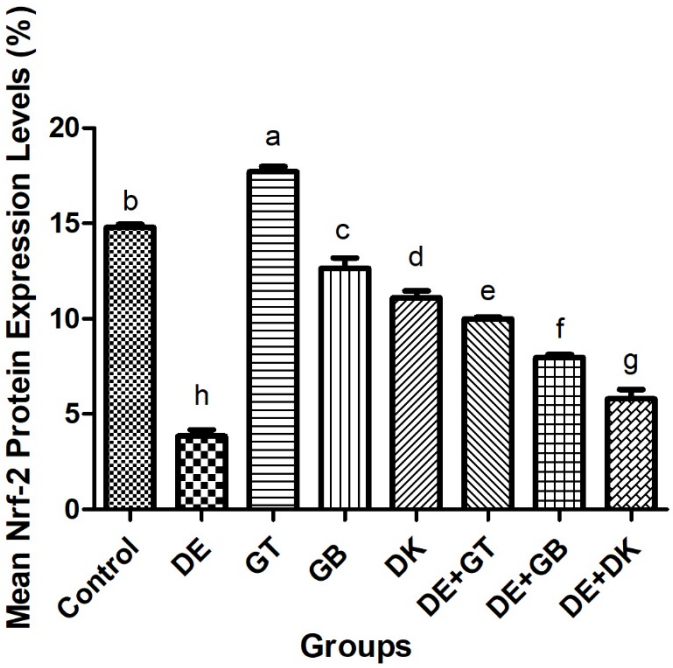
(B)


Mean IL-1α Protein Expression Levels (%)
25
20
15
10
5
0
a
e
e
e
d
c
b
a
Control
DE
GT
GB
DK
DE+GT
DE+GB
DE+DK
Groups

(C)


Mean TNF-α Protein Expression Levels (%)
15
10
5
0
a
b
c
d
e
e
e
e
e
Control
DE
GT
GB
DK
DE+GT
DE+GB
DE+DK
Groups

(D)


Mean Nrf-2 Protein Expression Levels (%)
20
15
10
5
0
a
b
c
d
e
f
g
h
Control
DE
GT
GB
DK
DE+GT
DE+GB
DE+DK
Groups

(E)

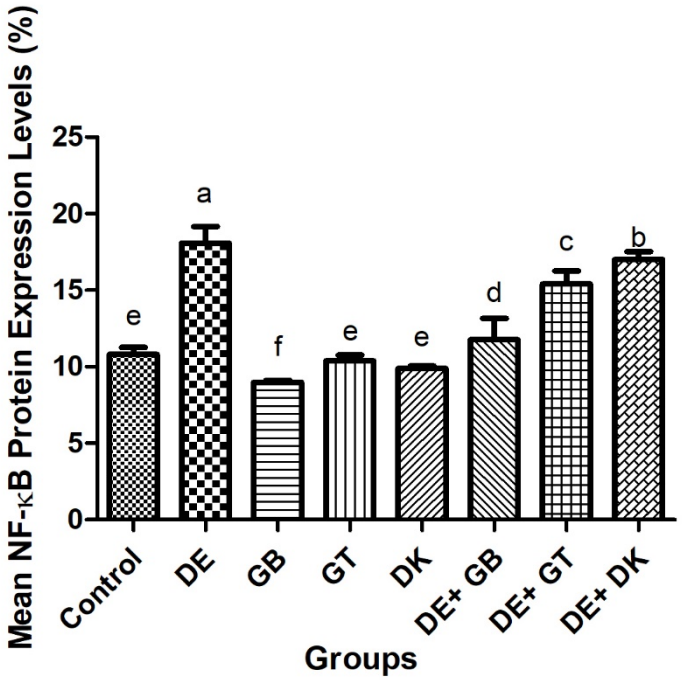


Figure 2. Western-blot analysis results. Western-blot protein bands **(A)**. IL-1α protein expression levels **(B)**. TNF-α protein expression levels **(C)**. Nrf2 protein expression levels **(D)**. NF-Kb protein expression levels **(E).** *a-h: differences between groups are indicated by different letters and this difference is statistically significant ($P < 0.05$). One-Way ANOVA post hoc Duncan test.

Discussion

Dry eye is a multifactorial ocular disease characterized by precorneal tear film (PTF) instability, elevated tear osmolarity, corneal and conjunctival epithelial damage, squamous metaplasia, ocular surface and lacrimal gland dysfunction or inflammation.[1, 2, 21–24] Numerous experimental model studies have been conducted to investigate the complex pathogenesis of DE and its treatment options.[25–31] BAC application is one of these methods.[2, 17, 32–36] Long-term BAC induces foreign body sensation, stinging, redness, corneal edema, and opacity associated with tear evaporation. It has also been reported that BAC causes PTF instability, overexpression of inflammatory cytokines in epithelial cells, and stromal neovascularization.[36–38] In recent years, many researchers have used BAC to induce the DE model due to its cytotoxic effects.[16, 31, 39] In our study, to establish the DE model, 0.2 % BAC was topically applied to the right eyes of the animals at a dose of 5 µL for 14 days.

A decrease in the aqueous tear production rate results in an increase in ocular surface tension and a decrease in the concentration of substances that nourish and protect the eye. This activates the inflammatory process and triggers DE.[40] In many DE models,[14, 16, 41] topical application of 0.2 % BAC twice a day for 14 days has been reported to significantly reduce the aqueous tear production rate. Consistent with these studies, in our study, BAC drops were applied to the animals for 14 days to induce the DE model, and EAPPTT measurements on day 15 showed a decrease compared to baseline data. In contrast, there was no significant difference in the EAPPTT of the animals treated with saline (without BAC) from the beginning to the end of the experiment. In our study, DE + GT showed the highest increase in EAPPTT in eyes treated with DE + extract. The other two groups (DE + DK and DE + GB) also showed similar effects. Previous studies

have reported varying effects of GT on the aqueous tear production rate in patients with DE. In a study investigating the effects of GT extract on the treatment of patients with meibomian gland dysfunction and DE, GT was found to have beneficial effects on clinical parameters.[10] Contrary to these researchers, it has also been reported that GT consumption leads to a decrease in tear composition and PTF quality.[8] In our study, the highest increase in aqueous tear production rate was measured in the DE + GT group (especially on day 25). These results suggest that GT has a beneficial effect on this parameter in eyes with DE.

Fluorescein staining is a standard method for detecting ocular surface damage. Fluorescein sodium is a well-tolerated, minimally irritating, and water-soluble dye. Fluorescein is known to stain areas of epithelial erosions, particularly those containing dead and degenerated cells in the cornea.[42] Many DE studies using this test have reported that BAC induces corneal epithelial cell desquamation, corneal erosions, and ulcerations.[2, 17, 39] Consistent with these findings, our study observed that BAC caused an increase in corneal fluorescein staining scores. In the current study, the most beneficial effect on the corneal staining parameter among the DE + extract groups were measured in the DE + GT group (although there was no significant difference between the other two groups and this group). GT contains a significant amount of EGCG, and one study reported that EGCG reduced the corneal staining score in patients with DE.[11] Additionally, other studies have reported that DK[14, 41, 43] and GB[44] also exhibit beneficial effects similar to GT on corneal fluorescein staining.

Assessing precorneal tear film stability is crucial for detecting mucin and lipid deficiencies as well as meibomian gland dysfunction. Precorneal tear film instability plays a key role in the pathogenesis of DE, and TBUT is a widely used parameter for its

measurement.[42] In DE models using different doses of BAC,[2, 17, 45] this agent has been shown to significantly shorten TBUT by disrupting precorneal tear film stability. In our study, 0.2 % BAC was applied twice daily for 14 days, and TBUT was found to be significantly shortened on day 15 compared to baseline measurements. These results support the hypothesis that BAC disrupts precorneal tear film stability. All three extracts tested in our study had a beneficial effect on TBUT in eyes with DE, with the DE + GT group exhibiting the longest TBUT. Similar to the results of our study, there are many studies reporting beneficial effects of GT,[11] GB,[44], and DK[14, 41, 43] on TBUT.

In numerous *in vivo* studies involving mice,[17, 46] rats,[47] cats,[48] and rabbits;[49] BAC has been reported to induce corneal and conjunctival inflammatory cell infiltration, excessive release of pre-inflammatory mediators, corneal epithelial desquamation, erosions, ulcerations, and corneal neovascularization. In our study, we used the parameters of corneal neovascularization and the number of inflammatory cells, which are histopathological analyses, to determine both the damage caused by BAC and the anti-inflammatory effects of the extracts on eyes with DE. We found that 0.2 % BAC, applied twice daily for 14 days, significantly increased corneal vascularization and the number of inflammatory cells. On the other hand, we did not observe any pathology or abnormality in terms of these parameters in eyes without BAC. These results show that BAC triggers the inflammatory process on the ocular surface, as reported by many researchers.[17, 46−49] Among the DE + extract groups, the groups with the lowest corneal neovascularization and the number of inflammatory cells were DE + GT and DE + DK. DK is a fruit with previously reported anti-inflammatory, antioxidant and antiapoptotic effects.[12, 14, 15] In a study investigating the effect of DK leaf extract in cases of DE, DK was found to suppress the inflammatory response.[14] Additionally, DK has been reported to inhibit corneal

neovascularization in alkali burns.[50] Another experimental study reported that EGCG, the major polyphenol component of green tea leaves, suppressed corneal neovascularization and inflammation.[51] The results obtained from these studies and our study confirm that DK and GT inhibit corneal neovascularization and the number of inflammatory cells on the ocular surface.

There is strong evidence that ocular surface inflammation is key to the pathogenesis of DE. It plays a role in both the initiation and progression of this condition.[2, 17] It has been reported that PTF instability and an increase in tear osmolarity cause an increase in the concentrations of inflammatory cytokines and chemokines in the lacrimal functional unit.[23] Numerous experimental studies have employed various diagnostic techniques to assess the inflammatory changes induced by DE in ocular tissues and to quantify the levels of involved markers.[14, 39, 44] In this study, the levels of IL-1α, TNF- α, Nrf2 and NF-κB in corneoconjunctival tissue samples were analyzed using Western blot. IL-1β and TNF-α are typical proinflammatory cytokines that contribute to ocular surface inflammation.[52]

In several DE models, 0.2 % BAC has been reported to cause significant increases in levels of TNF-α and IL-1α in corneal and conjunctival tissues of animals.[17, 45] NF-κB plays a key role as a transcription factor in the production of these cytokines.[53] It has been suggested that hyperosmolarity in the ocular surface epithelium is associated with an increase in NF-κB and this increase is effective in triggering the inflammatory process.[54] In one study, BAC was reported to increase intracellular NF-kB levels by inducing corneal epithelial toxicity.[55] Nrf2 is a critical nuclear transcription factor that functions as part of the systemic antioxidant defense system against environmental stressors.[56] Activation of the Nrf2/ARE system has been reported to play a significant

role in the disruption of the pro-inflammatory oxidative stress cycle, which causes overproduction of NF-κB and cytokines.[57]

The highest levels of IL-1α, TNF-α, and NF-κB and the lowest levels of Nrf2 eyes of the DE group indicate that BAC induces an inflammatory response and oxidative stress in corneoconjunctival tissues in terms of these markers. Between the DE + extract groups, the DE + GT group had the lowest levels of proinflammatory cytokines (IL-1α, TNF-α) and NF-κB, which plays a significant role in the activation of these cytokines, and the highest levels of Nrf2, an antioxidative transcription factor. In a study investigating the anti-inflammatory and anti-oxidative effects of green tea polyphenol EGCG in human corneal epithelial cells, it was reported that EGCG inhibited NF-κB activation and decreased IL-1α release.[58] In another DE model, both 0.01 % and 0.01 % EGCG were reported to reduce TNF-α and IL-1β release.[11] Similarly, in the current study, NF-κB and proinflammatory cytokine levels were measured in the lowest DE + GT group.

This result suggests that GT may have reduced cytokine levels through NF-κB inhibition. According to western blot analysis, we determined that GB and DK also showed beneficial effects in eyes with DE, but this effect was less than that of GT. Indeed, studies are reporting that both GB and DK have beneficial effects in different models of DE.[14, 59, 60] This study compared the therapeutic effects of extracts from GB, GT, and DK leaves in BAC-induced DE model, we found that all three extracts showed similar and beneficial therapeutic effects in terms of clinical parameters such as aqueous tear production rate, TBUT and corneal fluorescein staining tests. According to corneal neovascularization and the number of inflammatory cell tests and western blot analysis of corneoconjunctival tissues collected at the end of the study, GT showed the most beneficial effect in the DE model.

These results suggest that the use of clinical parametric tests alone is not sufficient in experimental studies comparing therapeutic effects. These tests should be supported by histopathological and molecular analyses. The molecular analyses in our research, in addition to the protein levels of proinflammatory cytokines such as IL-1α and TNF-α, the protein levels of nuclear transcription factors such as NF-κB, which activate these cytokines and plays important roles in the pathogenesis of DE, were also measured. In this way, the role of the NF-κB pathway in the anti-inflammatory effects of the agents was also revealed. In addition to these markers, Nrf2 was also chosen in order to determine whether the effects of the compounds were related to their antioxidant activity. In future studies investigating the therapeutic effects of these extracts, it may be necessary to identify other pathways that play a key role in the pathogenesis of DE and that initiate the inflammatory process, using different molecular techniques.

On the other hand, while there is only one study investigating the effect of topical application of GB leaf extract on DE.[20] The effect of topical application of extracts from GT and DK leaves on DE has not yet been studied. There are studies using only EGCG, which is found in GT. However, GT does not contain only EGCG. This is the first study to investigate the effects of the topical application of GT and DK leaf extracts on DE. In this study, we also used the GB, GT, and DK groups (without the DE model) to determine the possible adverse effects (if any) of the extracts and found that all three extracts had no cause adverse effects on the ocular surface.

Data availability

All relevant data are included in the article and can be shared as a file upon request.

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Conflicts of interest

The authors have no conflict of interest to declare in regard to this publication.

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