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Preclinical evaluation of thymoquinone and black cumin seed oil formulations for experimental dry eye disease in rats: Comparative efficacy and safety per ARRIVE

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Objective: To test whether topical thymoquinone and black cumin seed oil formulations improve signs of experimental dry eye in rats, and to compare their histopathology with standard treatment of dry eye disease (DED)
Methods: Seventy-two adult male Wistar-Albino rats were included in this study randomly divided into 12 groups with 6 subjects in each group. DED models were induced for all groups except the control group, using the most accepted cage method. One group remained untreated, the remaining groups were treated with balanced salt solution (BSS), cyclosporine 0.05% (CYC), CYC 0.05%+hyaluronic acid (CYC+HA), fluorometholone 0.01% (FML), TQ solution, TQ emulsion, TQ hydrogel, CO solution, CO emulsion. The Schirmer test for 1-min and Oxford corneal staining tests performed by a single masked grader, were conducted at the end of the first and third weeks. All tests were performed on each of the 12 groups of six rabbits at the end of the first and third weeks.
Results: There was no statistically significant difference in the ocular surface tests during the first-week among the groups. When the degree of ocular staining at third week obtained it was found to be significantly higher in the cases treated with BSS and CYC+HA compared to controls (respectively, Z=-2.298, p=0.022; Z=-2.298, p=0.022). TQ solution and CO hydrogel showed a significant decrease compared to week 1 in staining score (respectively, t=5.0, p=0.004; t=3.162, p=0.025). In the third week, ocular surface tests revealed a statistically significant improvement in the group treated with TQ emulsion compared to the control group (Z=-2.127, p=0.033).
Conclusions: Thymoquinone solution improved tear and staining metrics in this model. Emulsions and hydrogels showed mixed efficacy and higher histopathologic injury. These findings support reformulation and vehicle-controlled testing before translation. The observed improvement in ocular surface parameters may be attributed to the anti-inflammatory properties of TQ and cytotoxic effects may be ameliorated by different formulation types or doses.

Dry eye disease (DED) is a chronic and frequent pathology that prevalence varies widely depending on diagnostic criteria, ranging from approximately 5%–60% of adults [1,2]. Various factors contribute to the development of this condition, including age, hormonal disorders, autoimmune diseases, surgery, and some medications [2-4]. Chronic immune-mediated inflammatory changes lead to dry eye with progressive damage to the conjunctival epithelium and lacrimal gland. Increased inflammatory cytokines have been demonstrated in the epithelium of the tear, lacrimal gland, and conjunctiva [5]. Dysfunction of the lacrimal functional unit and tear instability result in the increase of ocular surface inflammation [3]. The inflammation cascade enters a vicious circle. Although many different treatments are used today, a definitive treatment has not been defined yet [6]. Despite various treatment options, such as anti-inflammatory agents, corticosteroids, cyclosporine, and tear preparations, a definitive cure remains elusive [7]. Immunosuppressive agents usually are not preferred because of their side effects.

Nigella sativa L., commonly known as “Black cumin,” is an annual flowering plant found in regions in our country [8]. It has been reported that black cumin seed oil has anti-inflammatory, antioxidant, antimicrobial, and anticancer activities in complementary and alternative medicine [9-15].

Structurally, black cumin seeds have mainly saturated / unsaturated fatty acids (31.0%–35.5%, linoleic, linolenic, oleic acids, etc), essential oils (0.4%–0.45%), carbohydrates (33.0%–34.0%), proteins (% 16.0–19.9), amino acids, alkaloids, tannins, saponins, fibers, minerals (calcium, zinc, phosphate), and vitamins (ascorbic acid, thiamine, niacin, pyridoxine and folic acid) [8,9,16]. Thymoquinone (TQ; C10H12O2, 2-isopropyl-5-methyl 1, 4-benzoquinone) is the

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most important bioactive component found in black cumin essential oil at a rate of 3.48–10.10 mg/g essential oil [17]. Quinone compounds, particularly thymoquinone, are responsible for many of the pharmacological effects of black cumin seeds [12,15,18]. TQ solution can rapidly distribute across the tear film and reach the corneal epithelium, conjunctiva, and lacrimal functional unit with minimal interference. By contrast, emulsions and hydrogels may alter tear film dynamics due to their higher viscosity or lipid content, potentially reducing uniform drug dispersion. Better penetration of the solution enhances TQ's anti-inflammatory and antioxidant actions at the ocular surface, improving tear metrics and reducing epithelial staining. TQ solution quickly reaches target tissues, scavenging ROS and supporting antioxidant defenses (glutathione, catalase). Emulsions and hydrogels, because of their viscosity, may cause tear stagnation, which increases local hyperosmolarity and ROS accumulation. Better oxidative stress reduction occurs with the solution, explaining improvements in staining.

Crude black cumin seed oil and its fractions (neutral lipids, glycolipids and phospholipids) showed potent free radical scavenging activity in another study [19]. It has been shown in a study that TQ inhibit the inflammation chain at some stages. Given its anti-inflammatory properties, this study aims to investigate whether TQ can effectively treat DED. TFOS DEWS III provided an evidence-based review of current strategies to manage DED. Novel pharmacological treatments are offered potential future options. New treatment options are usually related with antiinflammatory effects [2,4]. We hypothesized that the TQ solution would improve staining and Schirmer outcomes compared with controls, and would cause less histopathologic change than emulsion or hydrogel formulations.

In line with this objective, we have designed this study to evaluate the efficacy of different forms of TQ and black cumin seed oil, including solutions, emulsions, and hydrogels, in the treatment of DED through animal experiments. Our goal is to provide a new therapeutic alternative for DED treatment in clinical practice.

METHODS

Animals and experiment design: All animal procedures in this study complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Seventy-two Wistar albino male rats aged between 8 and 10 weeks weighing 250 to 300 gr were used. The study was designed to guideline ARRIVE 2.0. Rats were randomly divided into twelve groups (6 rats per group) as follows: the control group, the untreated group, the balanced salt solution (BSS) group, the cyclosporine 0.05% (CYC) group, the cyclosporine 0.05% + hyaluronic acid (CYC+HA) group, the fluorometholone 0.01% (FML) group, the TQ solution 0.1% group, the TQ emulsion 0.1% group, the TQ hydrogel 0.1% group, the black cumin seed oil (CO) solution group, the CO emulsion group and the CO hydrogel group.

Disease model and treatment controls: 72 Wistar-Albino male rats were randomly divided into 12 groups with 6 rats in each group. The DED model was created for all groups except randomly selected control group using the widely accepted cage method, consistent with existing literature. The rats were housed in cages with controlled conditions, including high airflow (2.4 m/sec), a temperature of 28.5 °C, and relative humidity (RH) at 15%, for one week. DED was also induced systemically by administering scopolamine hydrobromide subcutaneously. 0.5 mg/0.2 ml of scopolamine hydrobromide was injected subcutaneously 3 times/day at 8 AM, 12 PM, and 5 PM on only the first day of experiment to potentiate the effect of environmental factors [20]. A block randomization procedure was used to distribute rats across cages and treatment groups to minimize cage effects. At the end of the first and third weeks, the Schirmer test and ocular surface staining tests were performed on all rats and the degree of ocular surface staining was recorded by the same examiner who was unaware of which group the rats belonged to. Oxford grading system was used for corneal staining. Following the instillation of 1 µl fluorescein, the ocular staining pattern was divided into six groups according to severity from 0 (absent) to 5 (severe) [21]. Scoring was performed by a single masked observer. Schirmer test was performed by Whatman 41 filter paper (trimmed by about 1/4 to fit rat eyes) placed in the palpebral conjunctiva of the lower fornix. After one minute, the distance wetted (mm) was measured. All measurements were repeated at the end of the three-week follow-up time. However, tears could not be collected due to the limited volume and production rate in rats. The success of the DED model induction was assessed by comparing the findings of the control group with those of the experimental groups. The mean Schirmer test result in the control group (no dry-eye induction with no treatment) was 13.50±2.07. It is dramatically lower in other groups, demonstrating the success of the dry eye model. The predetermined treatments were applied to both eyes of the rats three times a day for three-week according to the group to which they belonged, except the control group (no dry-eye induction with no treatment) and the untreated group (experimental dry-eye model with no treatment).

Histopathological evaluations: At the end of the three-week treatment period, both eyes of the rats were enucleated after

decapitation. All eyes were fixed in 10% buffered formalin for 36 h. The samples were washed with distilled water, dehydrated in graded alcohol and cleared with xylene on an automated tissue processor (Leica TP1020 Tissue Processor, Leica Biosystems, Nussloch, Germany). They were embedded in paraffin blocks (Leica EC Embedding Center, Nussloch, Germany) and made ready for sectioning. Sections of 4 µm thickness were cut and deparaffinized and incubated at 50 °C overnight. The samples were treated with xylene and graded alcohols followed by staining with hematoxylin-eosin (H&E) and coverslip using Canadian balm. Histological evaluations were made with a Leica DM 4000 B light microscope (Leica Microsystems GmbH, Wetzlar, Germany), and images of the samples were acquired by using the Leica Application System program (LAS Version 4.2.0, Leica Microsystems GmbH, Wetzlar, Germany). Semiquantitative scoring for the lesions in the cornea were assessed including the structural changes in each layer based on a scoring system [22,23]. Evaluation of samples was performed semiquantitatively by graded assessment at 400x magnification [22-24].

Histological changes between the control and treatment groups were assessed, considering the structural changes in each layer. Evaluation was conducted separately for the three corneal layers: superficial squamous cells of the corneal epithelium, middle multilayered stroma, and the endothelium. Criteria included structural changes like cell loss in the epithelium (decrease in cell layers, thinning), nuclear or cytoplasmic vacuolization, nuclear condensation (pycnosis), and separation or loss in Bowman's membrane as a sign of cytotoxicity. Each finding was assigned a score. Pathological scores were also assigned based on whether these changes were in the upper or lower half of the epithelium. Evaluation of the stroma included assessing the arrangement of collagen fibers, staining pattern of stromal cells (keratocytes), stromal edema, and whether these findings were in the upper and lower halves of the stroma. Finally, presence/absence of the corneal endothelium, separation from the stroma, and cell vacuolization were evaluated and a score was assigned to each finding [22-24]. For each group in the study, 6 different samples were scored and the average of them was obtained by the same histologist who was unaware of which group they belonged to. Two masked pathologists graded predefined epithelial, stromal, and endothelial criteria. Disagreements were resolved by consensus. Scores were analyzed as ordinal outcomes. The final score for each group was evaluated as no histopathological change (0), mild change (<5 points), moderate change (5–8 points), and severe change (>8 points).

Preparation of solutions: Two ml of black cumin seed oil containing thymoquinone at a concentration of 3mg/ml (total of 6 mg thymoquinone) was mixed with 4 ml glycerin. Similarly, 6 mg thymoquinone (powder) was dissolved in 2 mL mineral oil for the thymoquinone control group and mixed with 4 ml glycerin. pH values of all solutions were between 6.0 and 7.5.

Preparation of hydrogels: Pluronic F-127 was used as a polymer for the preparation of thermosensitive hydrogels. Three different Pluronic F-127 concentrations of 10%, 12%, and 15% were tested to ensure optimum gelling. Pluronic F-127 polymers providing the concentrations specified in the preparation of the formulations (0.4 g for 10%, 0.48 g for 12%, 0.6 g for 15%), were mixed with 4 ml of water at +4 °C overnight. Then, 2 ml of black cumin seed oil containing thymoquinone at a concentration of 3 mg/ml (total of 6 mg thymoquinone) was added to these mixtures. Similarly, 6 mg thymoquinone was dissolved in 2 ml mineral oil for the thymoquinone control group and mixed with 4 ml Pluronic F-127 solution.

Preparation of emulsion formulations: The oil phase was used at a rate of 33% so that the amount of thymoquinone could be 1 mg/ml. The experimental design was created with water, span 80 and Tween-80. Two ml of black cumin seed oil containing thymoquinone at a concentration of 3 mg/mL was used in the preparation of the formulations. Similarly, 6 mg thymoquinone was dissolved in 2 ml mineral oil for the thymoquinone control group. To this, 4 ml of water/span80/Tween-80 mixture was added in the ratios indicated in Table 1. A coarse emulsion was obtained by mixing this mixture at 300 rpm for 5 min in a magnetic stirrer. Afterward, this coarse emulsion was homogenized in a Microfluidics LV1 device at 1400 bar pressure for 3 cycles. pH values of all emulsion formulations were between 6.0 and 7.5.

Viscosity of hydrogels: For the viscosity measurement studies, a Brookfield viscometer (DV2T-RV) equipped with a CP52 spindle was used. Viscosities of hydrogels containing Pluronic F-127 in 3 different concentrations as 10%, 12%, and 15% and formulations prepared by adding mineral oil were given in Figure 1. When the results were examined, it was seen that the most suitable Pluronic F-127 concentration was 12%. It remained liquid at room temperature and gelation was achieved as it approached body temperature. pH values of all hydrogel formulations were between 6.0 and 7.5.

Statistical analysis: Statistical analysis was performed with IBM SPSS Statics for Windows v22.0 (IBM corp., Armonk). Schirmer test measurements and ocular surface staining scores are expressed as mean ± standard deviation). The Kolmogorov–Smirnov test was used to test a normal distribution. First and third-week results were compared with paired sample *t* test or Wilcoxon test. A Student *t* test or

Table 1. The amounts of water, span 80 and Tween-80 used in the experimental design.

No	Water (%)	Span 80 (%)	Tween-80 (%)	Water (ml)	Span 80 (ml)	Tween-80 (ml)
1	94.00	3.00	3.00	3.76	0.12	0.12
2	97.00	0.75	2.25	3.88	0.03	0.09
3	94.00	1.50	4.50	3.76	0.06	0.18
4	94.75	0.75	4.50	3.79	0.03	0.18
5	97.00	1.50	1.50	3.88	0.06	0.06
6	95.50	3.00	1.50	3.82	0.12	0.06
7	95.38	1.75	2.88	3.82	0.07	0.12
8	94.69	2.38	2.94	3.79	0.10	0.12
9	96.19	1.25	2.56	3.85	0.05	0.10
10	94.69	1.63	3.69	3.79	0.07	0.15
11	95.06	1.25	3.69	3.80	0.05	0.15
12	96.19	1.63	2.19	3.85	0.07	0.09
13	95.44	2.38	2.19	3.82	0.10	0.09

Mann–Whitney U test was used to determine the statistical significance between groups of the Schirmer test measurements and ocular surface staining scores. Sample size was performed by Gpower 3.1, the sample size at least 80% power level, a large effect size (d=1.80, Cohen, J) and a 95% confidence level was determined. Statistical significance was set at $p<0.05$.

RESULTS

Seventy-two Wistar-Albino male rats were randomly divided into 12 groups with 6 rats in each group. Table 2 summarizes the mean of Schirmer test results and corneal staining scores in the first and third weeks. In the groups treated with BSS, TQ solution, and CO hydrogel, the Schirmer measurements obtained in the third-week were significantly higher than in the first week (respectively $p=0.026$; $p<0.001$; $p<0.001$) and also the degree of ocular surface staining was significantly lower than in the first week (respectively $p=0.004$; $p=0.004$; $p=0.025$).

There was no statistically significant difference in the ocular surface tests at the first-week between the untreated group and both the control group and the drug-administered groups. According to the results of the comparison between the control group and the drug-administered groups, the ocular surface tests at the third-week were found statistically significantly higher in the subjects treated with only TQ Emulsion compared to the control group ($p=0.033$).

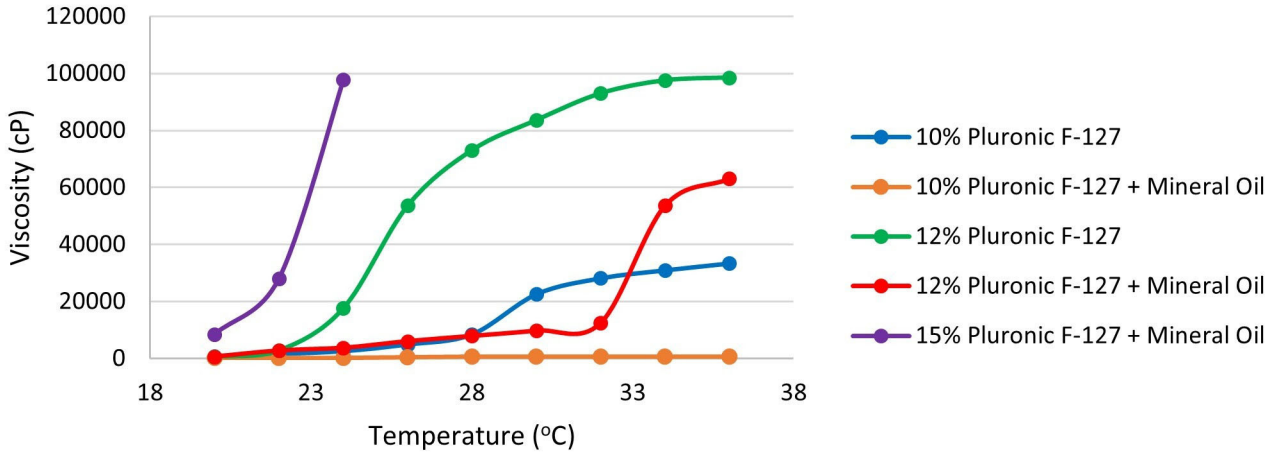

Figure 1. Graph of viscosity versus temperature of prepared formulations.

Table 2. Mean and median of Schirmer test results and corneal staining scores.

Various	First week		Third week		t/Z	p
	± *SD*	Med (IQR)	± *SD*	Med (IQR)		
Untreated						
Schirmer test	3.67±1.63	4 (1)	12.67±2.73	12 (0)	−2.214	**0.027** β
Staining	2.50±1.38	2.5 (3)	1.83±0.98	1.5 (2)	3.162	**0.025***
Control						
Schimer test	13.50±2.07	14.5 (3)	12.33±1.97	13 (4)	0.806	0.457*
Staining	-		-			-
FML						
Schimer test	4.50±1.87	5 (2)	14.50±3.89	13.5 (6)	−7.071	**0.001***
Staining	1.67±0.82	1.5 (1)	1.17±0.41	1 (0)	−1.732	0.083 β
BSS						
Schimer test	2.33±1.03	2 (1)	10.17±2.56	10 (2)	−2.226	**0.026** β
Staining	2.83±1.17	3 (2)	2.00±0.89	2 (2)	5.000	**0.004***
TQ solution						
Schimer test	2.00±1.26	1.5 (2)	13.33±1.51	13 (3)	−14.910	**<0.001***
Staining	2.50±1.05	2.5 (1)	1.67±0.82	1.5 (1)	5.000	**0.004***
TQ emulsion						
Schimer test	3.67±1.97	3 (4)	16.50±3.56	18 (3)	−2.207	**0.027** β
Staining	1.50±0.84	1 (1)	1.17±0.41	1 (0)	−1.414	0.157 β
TQ hydrogel						
Schimer test	3.50±1.64	3.5 (1)	10.33±3.14	10.5 (4)	−4.880	**0.005***
Staining	1.50±0.84	1 (1)	1.17±0.41	1 (0)	−1.414	0.157 β
CO emulsion						
Schimer test	3.17±1.17	3.5 (1)	15.67±3.44	16 (6)	−10.628	**<0.001***
Staining	2.33±1.37	2 (3)	1.33±0.52	1 (1)	−1.857	0.063 β
CO hydrogel						
Schimer test	2.67±1.63	2.5 (3)	13.50±3.99	13 (7)	−8.013	**<0.001***
Staining	2.33±1.21	2.5 (2)	1.67±0.82	1.5 (1)	3.162	**0.025***
CO solution						
Schimer test	2.83±0.75	3 (1)	12.00±2.45	11 (5)	−8.057	**<0.001***
Staining	1.33±0.52	1 (1)	1.00±0.00	1 (0)	−1.414	0.157 β
CYC HA						
Schimer test	2.17±1.33	2 (2)	12.67±1.21	12 (1)	−2.207	**0.027** β
Staining	2.67±1.51	3 (3)	2.00±0.89	2 (2)	2.000	0.102*
			CYC			
Schimer test	2.67±1.37	3 (3)	11.33±0.82	11.5 (1)	−10.796	**<0.001***
Staining	2.33±1.37	2 (3)	1.50±0.84	1 (1)	−1.633	0.102 β

Continuous variables were expressed as either the mean ± standard deviation (SD) and median (interquartile range). Continuous variables were compared with paired sample *t* test* or wilcoxon test β. Statistically significant p values are in bold. FML: Fluorometalone. BSS: Balanced salt solution. TQ: Thymoquinone. CO: Black cumin oil. CYC: Cyclosporine. CYC HA: Cyclosporine+ hyaluronic acid

Table 3. Selected water/span 80/Tween-80 ratio with particle size and PDI results.

n	Water (%)	Span 80 (%)	Tween-80 (%)	Particle size (nm)	PDI	Zeta
n=1	96.25	2.25	1.5	113.2	0.477	-6.5
n=2	96.25	2.25	1.5	115.2	0.481	-7.1
n=3	96.25	2.25	1.5	116.5	0.481	-6.4
Avg	96.25	2.25	1.5	115.0±1.6	0.480±0.002	-6.6±0.3

FML, TQ emulsion, TQ hydrogel, CO emulsion, CO solution, CYC+HA, and CYC applied groups showed a significant increase only in the Schirmer test in the third-week compared to the first-week (respectively p=0.001; p=0.027; p=0.005; p<0.001; p<0.001; p=0.027; p=0.001). In all cases treated with the drug, changes in the ocular surface tests' results of the third-week/first-week were significantly higher than in the control group. When the degree of ocular surface staining obtained in the third-week of the control and drug-administered groups was compared, it was found significantly higher in the cases treated with BSS and CYC+HA (respectively p=0.022, p=0.022).

Results of emulsion experimental design: The particle size distributions and zeta potentials of the emulsions were determined using a Zetasizer Nano ZS instrument with a DTS1070 Folded Capillary Zeta Cell. The graphics obtained as a result of the experimental design were shown in Figure 2. Considering the particle size and PDI, it was decided to use the ratios marked with purple. Particle size and PDI values of the selected ratio were presented in Table 3. The average particle size, PDI value and zeta potential value of the emulsion prepared at selected ratios were found to be 115.0±1.6 nm, 0.481±0.004 and −6.6±0.3 mV respectively.

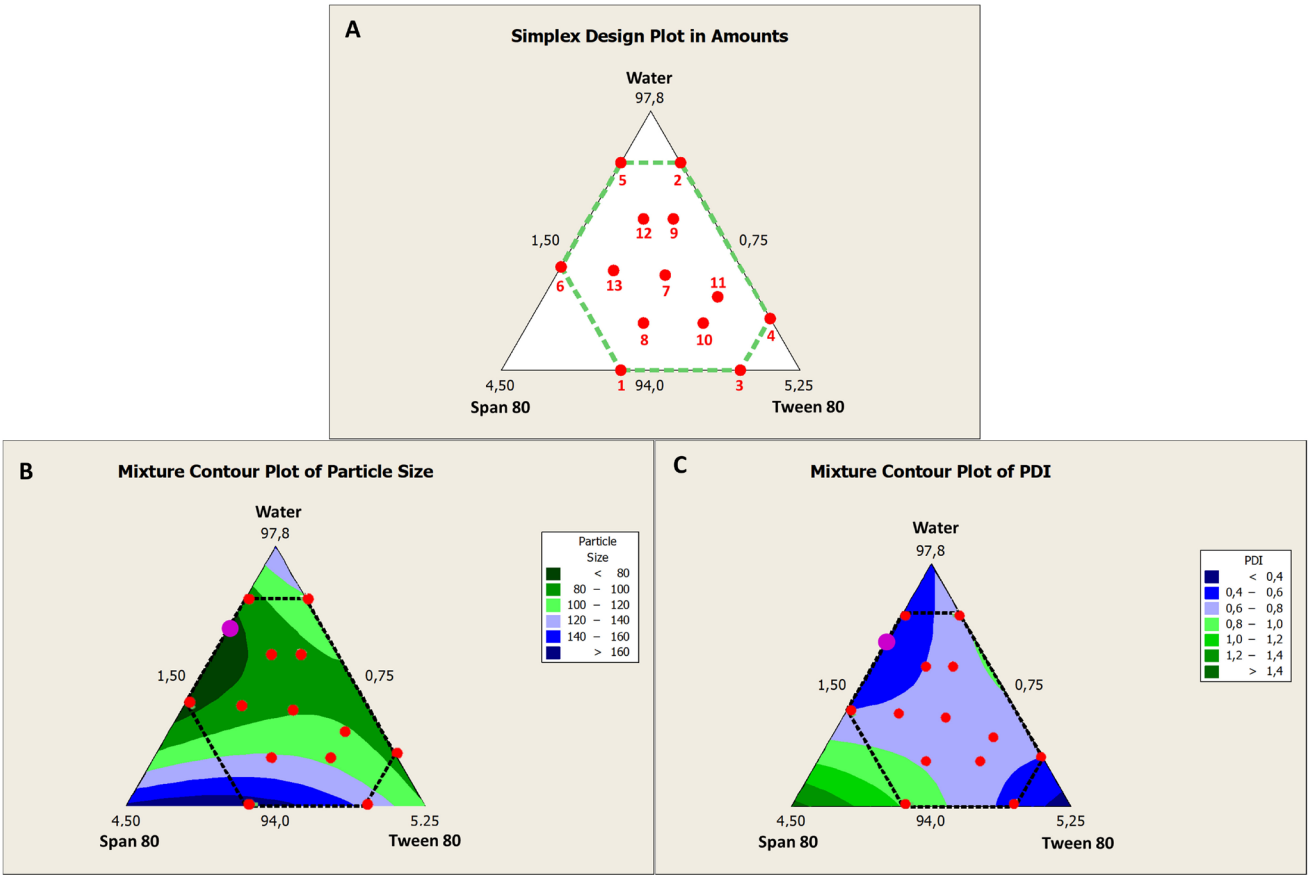

Figure 2. Representation of formulation numbers on the contour plots (A), particle size results on contour plot (B) and PDI results on contour plot (C).

Histopathological results: In the histological examinations conducted as previously described, we observed higher cytotoxicity levels in the TQ emulsion, CO emulsion, and hydrogel groups, as illustrated in Figure 3. A summary of histopathological changes as scores is presented in Table 4.

In the epithelial layer and stroma of the control group, localized intracytoplasmic vacuoles were observed in the upper half. No cell loss was observed in the epithelium in this group. The anterior limiting membrane was intact and firmly attached to the epithelium.

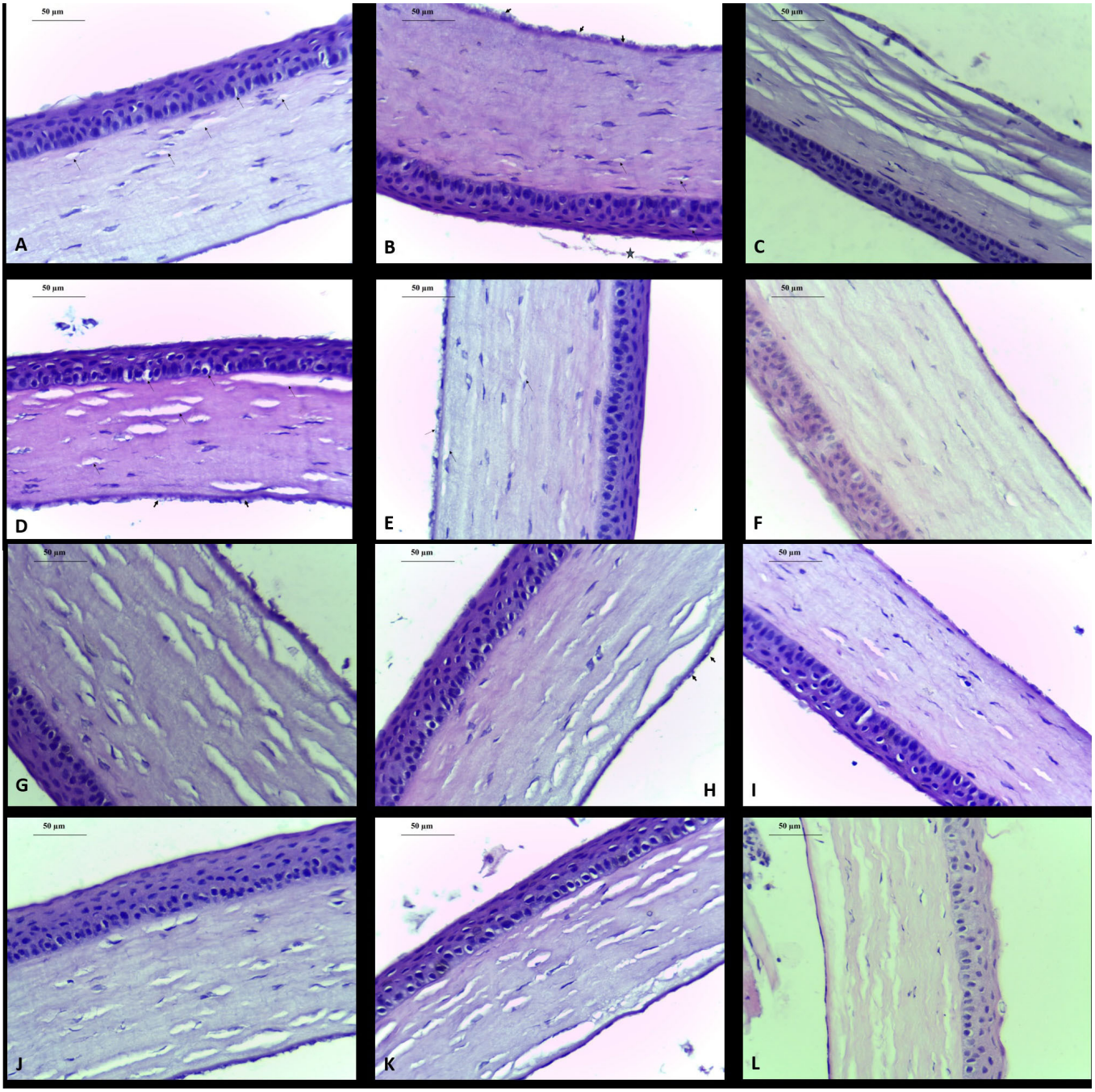

Figure 3. Histological changes (H&E x400, scale bar=50 µm) in the dry eye model created A. Control B. dry eye model group, C. BSS group, D. CYC group, E. CYC HA group, F. Fluorometalone 0.01% group. Histological changes (H&E x400, scale bar=50 µm) in the dry eye model created G. TQ solution group, H. TQ emulsion group, I. TQ hydrogel group, J. CO solution group, K. CO emulsion group, L. CO hydrogel group.

Table 4. Scores of histological changes of the groups.

Groups	Control	Untreated	BSS	CYC	CYC HA	FML	TQ Solution	TQ Emulsion	TQ Hydrogel	CO Solution	CO Emulsion	CO Hydrogel
	n=12	n=12	n=12	n=12	n=12	n=12	n=12	n=12	n=12	n=12	n=12	n=12
Epithelium	2	2	3	3	0	2	2	3	3	2	4	4
Stroma	2	3	5	5	2	0	4	4	3	5	4	5
Endothelium	0	1	1	1	1	0	1	2	0	1	1	1
Total Score	4	6	9	9	3	2	7	9	6	8	9	10
	(mild)	(moderate)	(severe)	(severe)	(mild)	(mild)	(moderate)	(severe)	(moderate)	(moderate)	(severe)	(severe)

n: number of eyes

In the dry eye model group, irregularities on the epithelial surface and separations in the squamous cells in places were noted. Vacuoles were observed in the epithelium and the upper half of the stroma. The irregular arrangement of collagen fibers and keratocytes in the stroma was noted. Although the endothelium did not separate from the stroma, prominent intracytoplasmic vacuoles were observed inside the cell. Dense inflammatory debris accumulation was seen in the anterior camera in all specimens.

Significant stromal edema was observed in all samples in the BSS group. Erosive areas in the epithelium and intracytoplasmic edema were observed in the endothelium. The Bowman's membrane was observed intact for all specimens and it was noted that the endothelium partly separated from the stroma.

In the CYC group, vacuoles were noted in the epithelium, stroma and endothelium. Stromal edema was observed to be located both in the intracytoplasmic and extracellular matrix and in all stromal layers. The appearance of collagen fibers and keratocytes was irregular due to diffuse edema. The anterior limiting membrane was observed as intact.

In the CYC HA group, stromal edema is prominently located in the lower 1/3 of the stroma. It was observed in endothelial cells. Regular appearance and the intact anterior limiting membrane were observed in the epithelial and upper stromal layers.

In the FML group, mild intracytoplasmic edema was observed in the lower half of the epithelium. No macroscopic changes were observed in the stroma and endothelium.

In the TQ solution group, edema was observed in the intracytoplasmic vacuoles and intercellular matrix, which was mild in the epithelium and widespread in all layers of the stroma. Although the endothelium appears intact, it is sometimes separated from the stroma due to edema in the lower stromal layers.

In the TQ emulsion group, erosive areas on the surface of the epithelium, intracytoplasmic vacuoles that cross half of the epithelium, intercellular and intracytoplasmic diffuse vacuoles in the stroma, and edema were observed. Local separations were observed in the endothelium due to lower stromal edema. In this group, intracytoplasmic vacuoles were observed in the endothelium.

In the TQ hydrogel group, full-layered intracytoplasmic edema and erosive changes were observed in the epithelium. Intracytoplasmic and intercellular edema was evident in the upper half of the stroma, and the endothelium was also considered intact.

Intracytoplasmic vacuoles and stromal edema were observed in the lower half of the epithelium and all stromal layers in the CO solution group. The endothelium was irregular and intracytoplasmic vacuoles were also seen in this layer.

In the CO emulsion group, full-thickness intracytoplasmic edema in the epithelium and irregular appearance on the surface (due to the shedding of squamous cells in the epithelium in places) were observed in all samples. Intracytoplasmic and intercellular matrix edema was observed in the stroma in all samples. The endothelium was found intact, but it was partially separated from the stroma due to lower stromal layer edema.

In the CO hydrogel group, irregularity in the epithelium and shedding in the squamous cell layer were observed to be increased compared to the previous group. Epithelial and stromal diffuse edema and endothelial detachment from the stroma were observed.

DISCUSSION

Black Cumin Seed Oil is traditionally used for the treatment of various diseases in many countries [9-11,16]. Thymoquinone (TQ) is the primary active ingredient found in seed's essential oil, playing a pivotal role in its therapeutic properties. It has been shown in various animal experiments that TQ suppresses both cyclooxygenase and lipoxygenase pathways of arachidonate metabolism in leukocytes [25]. These pathways are central components of inflammatory responses. Given that Dry Eye Disease (DED) is an inflammatory condition, this study aimed to explore the potential therapeutic associations and mechanisms of TQ and black cumin seed oil in its treatment. TFOS DEWS III supported the inflammatory etiology [4]. Furthermore, in allergic eye diseases, TQ treatment has demonstrated a reduction in symptoms through the eosinophil pathways. Notably, lipoxygenase and cyclooxygenase enzymes are key players in the inflammatory pathway. In the context of Dry Eye Disease (DED), TQ effectively suppresses this inflammatory process [25,26].

In this study, we found that the TQ solution and CO hydrogel groups exhibited a significant increase in tear measurements during the third week compared to the first week, accompanied by a substantial decrease in the degree of ocular surface staining. Moreover, subjects treated exclusively with the TQ emulsion demonstrated statistically significant improvements in ocular surface tests compared to the control group. In the CO emulsion group, full-thickness intracytoplasmic edema in the epithelium and irregular appearance on the surface were found. In the TQ solution group, edema was observed in the intracytoplasmic vacuoles and intercellular

matrix, which was mild in the epithelium and widespread in all layers of the stroma. These findings suggest that the TQ group may present fewer side effects compared to the CO group, indicating a potentially safer treatment option. Regarding the Polydispersity Index (PDI) of 0.481 and its relation to irritation, we would like to offer a formulation-based explanation centered on the safety-stability trade-off. It is well established in ocular drug delivery that surfactants, particularly at higher concentrations, can disrupt the corneal epithelial barrier and cause significant cytotoxicity. Therefore, our formulation strategy prioritized minimizing the surfactant-to-oil ratio to reduce the chemical toxicity potential, rather than aiming solely for a perfectly monodisperse system (low PDI). Reducing the surfactant concentration inherently limits the coverage of the oil-water interface, leading to a broader particle size distribution (higher PDI). While a PDI of 0.481 indicates polydispersity, we hypothesize that the irritation observed in our study was driven primarily by the chemical nature of the vehicle components (even at these reduced concentrations) rather than the physical polydispersity itself.

It is well established that cyclosporine emulsions and autologous serum have demonstrated efficacy in the treatment of DED [27,28]. Furthermore, hydrogel-based drugs have been under investigation for their potential in managing corneal epithelial erosions [29]. Notably, the TQ emulsion group exhibited a statistically significant clinical response, with Schirmer test results significantly higher than those of the control group and other drug formulations. While cyclosporine emulsions are used clinically for DED treatment [27], it's important to acknowledge that different product brands can yield varying side effects. Our study also revealed significant histopathological changes associated with the TQ emulsion group. These findings suggest the need for further investigation into potential side effects of emulsion-based drugs. TFOS DEW III reviewed the new treatment options. Antiinflammatory effects and new treatment steps are described with guidelines [30]. Also exosomes, which are extracellular vesicles, are investigated as new potential therapeutic agents in ophthalmology because they could modulate immune responses, facilitate cellular interaction, and promote tissue repair [31]. Exosomes are nanosized extracellular vesicles, typically 30 to 150 nm in diameter, that carry bioactive molecules—such as proteins, RNA, and microRNAs (miRNAs)—encapsulated within a protective lipid bilayer that shields them from degradation. Zhou T et al. showed mesenchymal stromal cells exosomes effective for the treatment of Graft versus host disease associated DED [32].

While our study shares similar objectives with previous research related to ocular surface diseases and the use of TQ (Thymoquinone), it's important to note that these studies fall outside the scope of our intended evaluation. In one study, which involved 40 eyes of 40 rats, TQ treatment demonstrated an inhibitory effect on neovascularization following corneal chemical cauterization. Notably, the effectiveness of TQ was found to be dose-dependent [18]. In another study conducted by Kocaturk T et al., TQ was investigated for its impact on inflammation in a dry eye model involving 36 rats. The results indicated that while TQ reduced inflammation, it did not lead to significant changes in tear cytokines [33]. The dry eye model employed in our study involved the application of a preservative substance solely to the ocular surface, aiming to disrupt surface properties. In our study, the dry eye model was created with environmental conditions. In our results, it was determined that the tear production was higher in the groups treated with TQ formulations and Black Cumin Seed Oil formulations, and the ocular staining was decreased compared to the control group. However, since there are very severe histopathological changes with the TQ emulsion, CO emulsion and hydrogel, CO hydrogel may not be preferred because it may create a side effect profile, despite a very significant increase in tear measurement in the third-week compared to the first-week and a decrease in staining compared to the other groups. In contrast, the TQ solution formulation emerged as the most promising treatment option for the future. This formulation resulted in a substantial increase in tear measurements during the third week compared to the first week, along with a decrease in ocular surface staining and moderate histopathological changes. Furthermore, our observations indicate that while the TQ (Thymoquinone) hydrogel led to a moderate histopathological change, the CO (Black Cumin Seed Oil) hydrogel resulted in a severe histopathological alteration. This discrepancy suggests that CO may play a more significant role in inducing these changes compared to TQ. As a result, TQ appears to be a more favorable option for consideration. Cytotoxicity was found to be high in the TQ emulsion, CO emulsion and hydrogel groups. The high cytotoxicity in the CO group is another aspect of CO activity that needs to be investigated with further studies. Ocular toxicity risk more critically and comparing with 0.05% cyclosporine emulsion tolerability [27]. The findings related to the effects of TQ and its cytotoxicity on the ocular surface have been presented in this study. However, to gain a more comprehensive understanding, it is imperative to support these findings with extensive clinical studies in the future, shedding further light on this subject.

Our results should be interpreted in the light of its potential limitations. The major limitation of the current study is

its animal study design; use of impression cytology would have probably been better to assess the epithelial stress and improvement post treatment; and collecting tear of rats to asses tear cytokine level. The current paper uses ocular surface staining and Schirmers test at 1 min. It would have been better to use tear film break up time as well to understand the tear film stability. Other limitations include the lack of long-term ocular surface toxicity, pharmacokinetic data, and ocular surface immunohistochemical markers for TQ and CO, no vehicle controls for complex formulations, including male rats only, single examiner for staining without inter-rater reliability, absence of intermediate histology time points or systemic safety, uncertainty about the suitability of the BCOP-derived grading in a living rat cornea.

In conclusion, a significant improvement in ocular surface parameters was observed in the treatment of DED due to the possible anti-inflammatory properties of TQ. DED is a prevalent clinical condition affecting a substantial portion of the population. Despite various treatment attempts, DED can lead to severe vision impairment due to corneal surface damage, negatively impacting quality of life and workforce productivity. TQ and CO formulations demonstrated potential efficacy in tear secretion and surface protection; however, cytotoxic changes warrant further refinement before clinical application. These formulations present an affordable alternative to the limited and expensive treatment agents currently available, potentially enhancing the management of DED.

TQ and CO formulations demonstrated potential efficacy in tear secretion and surface protection; however, cytotoxic changes warrant further refinement before clinical application. It is important to emphasize that the findings presented herein represent associations within a small animal model and should not be directly extrapolated as clinical efficacy in humans. Among the formulations tested, the TQ Solution demonstrated the most favorable balance between therapeutic efficacy (increased tear production) and ocular safety (minimal histopathological changes). In contrast, while the emulsion and hydrogel formulations showed functional improvements, their cytotoxicity profiles indicate that these delivery systems require significant reformulation and rigorous testing with vehicle-only controls to separate excipient effects from active drug effects before any potential clinical translation can be considered. The message is that several thymoquinone and oil formulations improved tear measures or staining in this desiccating-stress model. The solution showed the most favorable safety profile. Emulsions and hydrogels require additional safety work.

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