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Managing allergic conjunctivitis via ophthalmic microemulsions: Formulation, characterization, in vitro irritation studies based on EpiOcular™ eye irritation assay and in vivo studies in rabbit eye

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Abstract

The purpose of this work was the development of an alternative ocular tetrahydrozoline hydrochloride (THZ) microemulsion for the management of allergic conjunctivitis. Pseudo-ternary phase diagrams were used to produce the microemulsions. For the formulation of the microemulsions, isopropyl myristate was selected as oil. Furthermore, various surfactants were applied for the determination of their suitability; ME-1 and ME-4 were developed using Sorbitan monolaurate 80 and Polysorbate 80, for ME-2 and ME-5 Sorbitan monolaurate 80 and macrogolglycerol ricinoleate while for ME-3 and ME-6 Sorbitan monolaurate 80 and Polysorbate 20. For ME-1, ME-2, and ME-3 propylene glycol and ethanol, for ME-4, ME-5, and ME-6 ethanol and polyethylene glycol were used as co-surfactants. Various characterization parameters of microemulsions were evaluated such as droplet size, conductivity, zeta potential, viscosity, and pH. In addition, the drug release, stability, sterility, safety, ex vivo, and in vivo experiments were studied. The characterization showed that the formulations can act as suitable carriers for eye application. In addition, it was revealed that microemulsions were found stable and sterile. The formulations released the drug in a sustained manner. Ex vivo diffusion studies exhibited that the microemulsions can be used for topical delivery to the eye. Assessment of in vitro ocular irritation was conducted by an in vivo prediction model, EpiOcular™ eye irritation test. Based on the in vivo studies, the chosen ideal microemulsion showed longer contact time to the cornea than the commercial product. Moreover, the developed ocular carrier was found to be safe from the viewpoint of in vitro ocular irritancy and mutagenicity. In conclusion, according to characterization results, the THZ microemulsions can act as a hopeful approach for topical eye application.

KEYWORDS
EpiOcular™ eye irritation test, in vivo experiments, microemulsion, rabbit, tetrahydrozoline hydrochloride

INTRODUCTION

Successful topical ocular drug delivery would be quite challenging due to the inimitable structure of the eye (Mahboobian et al., 2020). Thus, for pharmaceutical scientists, the ocular drug delivery systems belong to the most interesting and demanding applications (Araújo et al., 2009; Gaikwad et al., 2012; Siafaka et al., 2015). Crossing of xenobiotics and toxins from the bloodstream to the ocular globe is mostly inhibited

by several cell-based anatomical and physiological barriers that are the main struggle of ocular drug delivery (Başaran & Yazan, 2012; Gaikwad et al., 2012; Yavuz et al., 2013).

Each ocular globe has anterior and posterior chambers. The cornea, conjunctiva, ciliary body, iris, aqueous humor, lens, and the lachrymal system can be listed as the essential components of the anterior chamber (Achouri et al., 2013). Ocular drug delivery is compelling with a physicochemical character of the drug that determines its elimination from lacrimal fluid, corneal barriers, and non-corneal absorption (Mainardes et al., 2005; Wadhwa et al., 2009). Even though numerous ways of application, the topical route of administration remains the preferred route due to its simplicity, non-invasiveness, and high patient compliance (Pai & Vavia, 2020). Anterior segments of the eye disorders are usually treated using topical application of drug solutions administered as eye drops (Siafaka et al., 2015). These commercial drugs are over 90% of the current ophthalmic formulations, owing to their ease of use and high patient acceptability (Achouri et al., 2013; Alany et al., 2006).

Microemulsions are thermodynamically steady water–oil dispersions that are often consolidated by a surfactant and a co-surfactant. The size of dispersed domains is typically at the nanoscale. They are optically transparent or translucent, and this form does not alter over time (Gasco et al., 2009; Spernath & Aserin, 2006). Low viscosity, easy formation, high surface area, thermodynamic stability, and very small droplet size are several standards of a liquid system. These standards could be seen in microemulsion systems that are optimal topical drug delivery systems to the eye (Ligório Fialho & Da Silva-Cunha, 2004). Furthermore, microemulsions are desirable for ophthalmic management since they can minimize the daily frequency of eye drops instillations. This is associated with the fact that the drugs can be prolonged released to the eye surface (Karasulu et al., 2007).

Several factors can promote dilatation or exposure of the conjunctival vascular network, resulting in eye redness. Dry eye syndrome, conjunctivitis, keratitis and corneal ulcers, corneal abrasions, blepharitis, episcleritis and scleritis, distichiasis, uveitis, endophthalmitis, subconjunctival hemorrhage, and corneal graft rejection represent the most prevalent redness-related issues (Tarff & Behrens, 2017).

The most common kind of acute and chronic conjunctivitis is allergic conjunctivitis, which can cause a variety of symptoms depending on how severe they are, including stinging, tears, periocular irritation, burning, photophobia, redness, and swollen or dry eyes. The pathognomonic symptoms linked to allergic conjunctivitis are redness and ocular irritation (Bielory et al., 2010; Tarff & Behrens, 2017). An alpha adrenoceptor agonist and decongestant, tetrahydrozoline hydrochloride (THZ) helps alleviate allergic conjunctivitis symptoms (Krogh, 1983; Mohamed et al., 2015). The narrowing of conjunctional blood vessels is the mechanism of action of THZ, and it can diminish eye redness produced by small irritants (Gumustas et al., 2016; Shadoul et al., 2016). Liquid form dosages of THZ are used as a conjunctival decongestant (Al-rimawi et al., 2012; El-bagary et al., 2016; Huang et al., 2002). THZ uses generally up to four times daily with 1–2 drops for allergic conjunctivitis treatment. Several negative effects were reported such as stinging, sleeplessness, vertigo, tremors, dryness tachycardia, ocular burning rebound erythema headache, and congestion which consist of due to absorption of ophthalmic applied THZ from the nasolacrimal duct and the nasal mucosa. THZ can result in rebound hypotension, bradycardia, hypertension, and conceivably arrhythmias when consumed in higher amounts than advised. Additionally, several effects such as rhinitis medicamentosa, rebound congestion, and vasodilation may emerge due to time-dependent usage of THZ (Cordes, 2008). Therefore, the standard treatment plans indicate the multiple application of the eye drop, which however might cause toxicity because of the nose-lachrymal drainage. This fact may be related to a patient's low level of compliance and potentially poor therapeutic management.

The novelty in the work is to develop a novel ocular formulation of THZ, which was not reported earlier with detailed in vitro and in vivo evaluation. The purpose of the research was to prepare THZ microemulsion systems for ocular delivery. The microemulsions are applied to improve the residence time of THZ, prolonged release of THZ, and decrease the dosing frequency of the drug.

MATERIALS AND METHODS

Materials

THZ was a generous donation from Bilim Pharmaceuticals, İstanbul, Turkey. Isopropyl myristate (IPM), Propylene glycol (PG), Polyethylene Glycol 400 (PEG 400), acetonitrile, Cremophor EL® (Cre), 4-nitro-o-phenylenediamine (NPD) and 2-aminofluorene were obtained from Sigma, Darmstadt, Germany. Labrafil M®, and Transcutol® were purchased from Gattefosse, Lyon, France. Span 80®, Tween 80®, Tween 20®, ethanol, potassium dihydrogen phosphate, and N-octanol were purchased from Merck, New Jersey, Germany. Orthophosphoric acid was purchased from Carlo Erba, Milan, Italy. Dialysis membrane (Spectro/por, 12–14 kDa molecular weight) was obtained from Spectrum, New Brunswick, USA. VISINE® is an eye drops solution by Johnson and Johnson. It contains 0.5 mg/mL THZ, and excipients are sodium chloride, boric acid, edetate disodium, benzalkonium chloride (17%), sodium borate, and pure water.

The solubility studies of THZ

Distilled water, ethanol, IPM, PG, Capryl 90®, Indian oil, oleic acid, almond oil, Span 80®, Tween 20®, Cremophor EL®, and Labrafil-M® media were used for solubility studies of THZ. THZ was added into Eppendorf tubes containing 1 mL media and mixed on a shaker for 24 h, for attaining saturation. After 24 h, it was examined whether the precipitated THZ was dissolved or not. A further amount of THZ was added to the medium and the medium was repeatedly mixed for 24 h on a shaker (25 ± 2°C) (Üstündag-Okur et al., 2014). The THZ concentration was detected using the HPLC method of THZ.

Detection of lipid/water partition coefficient of THZ

For the lipid/water partition coefficient assay of THZ, the n-octanol phase was saturated with water for 24 h before testing. THZ solution was prepared; 2 mL of this solution was combined with 2 mL of the n-octanol solution into tubes for 10 mL volume. The mouths of the tubes were sealed, and they were shaken for 24 h, at room temperature. After shaking, centrifugation followed by at 3500 U/min for 15 min. The concentration of THZ in water was determined using HPLC while its concentration in n-octanol (organic phase) was calculated by the difference between the water phase, initial and final (Üstündag-Okur et al., 2014).

Construction of ternary phase diagrams for optimized emulsion

A simple titration procedure was used to create the pseudo-ternary phase diagrams. The oil, surfactants, and co-surfactants were mixed in the appropriate ratios to make the mixes. During the formulation development study, the surfactants and co-surfactants were mixed (a/a) at 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, and 9:1 ratios. During the titration, water was applied drop by drop. All pre-formulation studies were accomplished at a room temperature of 25 ± 2°C. The center of gravity of the microemulsion obtained from phase diagrams was used to design the optimal ocular microemulsion (Üstündag-Okur et al., 2014).

Preparation of THZ microemulsions

The most ideal ocular microemulsions were loaded with 0.05% THZ. A mixture of IPM, surfactants, and co-surfactants was prepared by mixing the desired amount of each component. Further, the THZ was dissolved in the water and added to the oil-surfactant and co-surfactant mixture. The HPLC device was used to determine the content of THZ in the ideal microemulsion formulations (Okur et al., 2022).

Characterization of THZ ocular microemulsions

The detections of the physicochemical properties of the ideal ocular microemulsions, the physical appearance, pH, electrical conductivity, refractive index, droplet size, polydispersity index (PDI), active substance content, and stability of microemulsion formulations were investigated (Üstündag-Okur et al., 2014).

Droplet size, PDI, and zeta potential measurement

The droplet size, zeta potential, and PDI were detected using the dynamic light scattering technique (Malvern Instruments, Worcestershire, UK). The Helmholtz-Smoluchowski equation was used to calculate the zeta potential. The procedure was carried out using a software-based method. Six repeated measurements were practiced at 25 ± 1°C (Okur et al., 2022).

Viscosity measurement

The viscosity of the microemulsions was detected at 25 ± 1°C by a viscosimeter (AND, SV-10). The four repeated measurements were taken for each sample.

Determination of pH

A pH meter (Mettler Toledo, Greifensee, Switzerland) was used to determine the pH of microemulsions. The experiments were run at least four times.

Refractive index measurement

The refractive index was detected by a refractometer (Atago-Japan). The data were recorded four times.

Conductivity measurement

A conductometer was used to measure the electrical conductivity of the microemulsions. For each sample, five repeated measurements were performed at room temperature.

Drug content

To detect THZ content in the microemulsions, the THZ microemulsions were dissolved in the mobile phase and examined with HPLC.

HPLC analysis of THZ

The High-Performance Liquid Chromatography (HPLC) instrument was used for the determination of THZ amount throughout the experiments. The HPLC system consisted of a pump, a temperature adjustable column, and a UV detector. The used column was a GL Sciences, Japan C18 (5 μm, 150 × 4.6 mm). The flow rate was set at 1.2 mL/min at 25 ± 1°C. The injected volume was set at 20 μL, and the wavelength at which the maximum absorbance at the wavelength UV spectrophotometer was used was 212 nm. The mobile phase that was used in the assay was a combination of acetonitrile (35%) and phosphate buffer (65%) (Ali et al., 2002). The HPLC analytical method for THZ was developed and the validation of the analytical method was performed. Linearity, accuracy and reproducibility, precision, reproducibility, reproducibility, specificity and selectivity, detection limits, and stability parameters were evaluated for the validation of the HPLC method for THZ detection.

Sterilization and microbiological control of THZ microemulsions

For the sterilization of products, the aseptic fill procedure was chosen. While preparing the formulation, all the excipients were filtered through the membrane filter (0.22 μ) under laminar air flow. Glass vials were sterilized for 20 min in an autoclave at 121°C. Ideally defined microemulsions were prepared in aseptic condition (laminar air flow) into previously sterilized 10 mL vials. For the sterilization control of ideal microemulsions, 1 mL of formulations were placed in the Blood agar and Eosin Methylene Blue agar. In the case of the microbiological reproductive control of microemulsions, 100 colony forming unit suspensions from *Escherichia coli* ATCC 8739 standard strains were prepared and the same plaques were sown. All plates were incubated at 37°C. The reproduction results of the medium were examined.

Stability studies of THZ microemulsions

The ideal microemulsions were prepared and kept at 5 ± 3°C, 25 ± 2°C (relative humidity 60%), and 40 ± 2°C (relative humidity 75%) for 90 days to examine their stability. The properties of the formulations such as physical appearance, pH, conductivity, turbidity, phase separation and droplet size, zeta potential, and PDI were investigated initially in the first, second, and third months. The heating–cooling cycle test was also carried out by storing formulations between 4 ± 1°C and 40 ± 1°C for 24 h as one cycle. Six cycles were completed before investigating the formulations in terms of clarity, precipitation, and phase separation.

In-vitro release of microemulsions

In-vitro release experiments of THZ-containing formulations were performed using the dialysis membrane. 1 mL of microemulsions containing 0.05% THZ was placed on the dialyzed membrane while the ends of the membranes were sealed using closures. Afterward, the dialysis membranes were placed in 100 mL artificial tears on a multi-heater magnetic stirrer at 150 rpm and a temperature of 32 ± 1°C (eye surface temperature). 1 mL samples were withdrawn from the artificial tear media at 30 min, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, and 24 h in the in vitro release study and placed in HPLC vials. The samples were evaluated with HPLC to determine the THZ content. In vitro release experiments of microemulsions were performed in five parallels for each formulation (Üstündağ Okur et al., 2019).

Ex vivo study of THZ microemulsions

Sheep corneas were used for ex vivo diffusion examinations of microemulsions and the commercial preparation (VISINE®) containing THZ. Diffusion cells were used at 150 rpm. The corneas were placed on diffusion cells. 1 mL of formulations containing 0.05% THZ was placed on the corneas. A 10 mL artificial tear medium was used as the receptor phase. The tear environment temperature was set at 32–33°C (eye surface temperature). 0.5 μL samples were withdrawn from the artificial tears in which the corneas were placed, using a micropipette at 30 min, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, and 24 h. The samples were studied in HPLC. An ex vivo diffusion study was performed with four parallel corneas for each microemulsion formulation. After completion of the ex vivo diffusion study, the corneas were removed from the diffusion cells. The corneas were cleaned; the cleaned corneas were cut with a scalpel and left in 10 mL of methanol solution. They were left in a methanol solution for 24 h. At the end of 24 h, the methanol solution was filtered through a membrane filter of 0.4 μm diameter and diluted. Also, the amount of THZ contained in the cornea was determined using the HPLC method (Üstündağ Okur et al., 2019).

Ex vivo bioadhesion studies of THZ microemulsions

For bioadhesion studies, the TA-XT Plus (Texture Analyzer, Godalming, United Kingdom) was employed. The sheep cornea was fitted to the probe (P10 Delrin, θ: 10 mm). The probe with the cornea was lifted down to

the beaker's surface which contains 5 mL of the formulation with a constant speed of 0.1 mm s^{-1} and a contact force of 0.5 N applied. Allowed to contact for 2 min, the probe was thereafter pushed upward at a rate of 0.1 mm s^{-1}. The force-distance plot was used to calculate the area under the curve as the mucoadhesion process (Üstündağ-Okur et al., 2015). Equation (1) was applied to determine the work of mucoadhesion per cm^2 (mJ/cm^2). The experiments were performed in triplicate.

$$\text{Work of adhesion} = mJ \times \text{cm}^{-2} = \frac{\text{AUC}}{\pi r^2}, \quad (1)$$

where, πr^2 is the mucosal surface-formulation contact area.

Ocular irritation test (in-vitro)

To determine the ocular irritation capacity of a formulation, the EpiOcular™ Eye Irritation Test (EIT), developed by MatTek Corporation, was utilized (Corporation, 2015) by measuring the cytotoxic effect on the EpiOcular™ cornea epithelial model (OECD, 2019).

In this experiment, sterile deionized water (negative; NC) and methyl acetate (positive; PC) were used as controls for comparison. According to the liquid protocol, 50 μL of each group (PC, NC, and THZ loaded microemulsion (ME-1)) was directly applied for 30 min on the external part of the corneal epithelium. After the removal of the sample, the tissue is permitted to exhibit the consequent harm. For each group, two construct tissues were utilized. By measuring the decrease in the vital dye MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), relative tissue viability against NC-treated tissues was measured. The following equation was used to determine the percent viability of each of the two tissues to the negative control for:

$$\%\text{Viability} = \left[\text{text sample}_{\text{optical density}} / \text{negative control}_{\text{optical density}}\right] \times 100.$$

In accordance with the manufacturer's recommendations, if the sample treated tissue viability is equal to or more than %60, the experimental sample is classified as non-irritant (Corporation, 2015; OECD, 2019).

AMES test

The tester strains *Salmonella typhimurium* TA98 and TA100 were employed for routine mutagenicity assays. TA98 detected frameshift and TA100 detected base pair mutagens. The THZ loaded microemulsion (ME-1) was diluted with water and different solutions (10%, 25%, 50%, and 100%) were used for mutagenicity assay both in the presence and absence of metabolic activation (S9) according to the procedure of Maron and Ames (1983).

In vivo studies

Animals

Rabbits (New Zealand albino) weighing 2.5–3.5 kg and showing no symptoms of ophthalmic inflammation were selected for the animal experiments. Bezmialem University Ethical-Scientific Committee authorized the in vivo procedures (No. 2017/297). Rabbits housed at 55%–60% humidity and 22 ± 1°C with a repeating 12 h:12 h night-day cycle. Rabbits were allowed to intake ad libitum feeding and water at all times.

Determination of THZ in rabbit tear

Animals were treated with the formulations after being randomly split into two groups of six animals. The first group was treated with sterilized THZ loaded microemulsion (ME-1), whereas the other group was treated with the commercial formulation. A single 50 μL instillation was given to each rabbit's conjunctival sac. After the instillation of the microemulsions to the eye, their tear samples were taken at 0.5, 1, 2, 3, 4, 6, 8, and 24 h with a tear strip (Schirmer Tear Test (ERC®)). A standardized sterile strip was inserted into the conjunctival sac. Then tear absorbed strip was collected and THZ amounts were determined by HPLC (Byrro et al., 2012).

Ocular irritation test

The Draize assay (modified version) was used to assess the potential ocular irritant and/or damaging capacity of THZ microemulsions. ME-1 microemulsion (0.01 mL) was instilled onto the corneal surface of the right eye for 6 h. Sodium chloride (0.9%) was applied to the left eye as the control treatment. Each rabbit eye was graded for congestion (0–3), swelling (0–4), redness (0–3), discharge (0–3), irritation (0–4), and corneal opacity (0–4) (Üstündağ Okur et al., 2019).

Statistical data analysis

For statistical studies, the Student's *t*-test with $p < 0.05$ as the least level of significance was utilized. All data were presented as the mean of triplicates ± standard deviation. The Salmonella assay findings were evaluated with Dunnett's multiple comparison test.

RESULTS AND DISCUSSION

Preparation and characterization of THZ microemulsions

A water titration procedure was used for the preparation of the microemulsions. The use of the titration method which is a simple and suitable technique for the preparation of microemulsion is quite common. At first, the pseudoternary-phase diagrams were applied to acquire the desirable composition of the used compounds for the preparation of the microemulsions. Figure 1a shows the pseudoternary phase diagram of microemulsions. The areas painted in red in Figure 1 identify the microemulsion formulations. The developed ocular formulations can be criticized as clear and transparent. The microemulsions were produced in accordance with the area of microemulsion in the phase diagrams. The ratio with the highest area value was chosen. The area of ME-1, ME-2, and ME-4 was determined as 242.6, 370, and 279.2, respectively. The surfactant/co-surfactant ratio with the highest area was detected as 4:1 for ME-1, ME-3, and ME-4 microemulsions.

The area of phase diagrams, surfactant/co-surfactant ratio, and components of the ideal microemulsions are shown in Table 1. For the preparation of microemulsion, the isopropyl myristate was selected as the oil phase, Span 80®, Tween 80®, Cremophor EL®, and Tween 20® as surfactants, propylene glycol, ethanol, and polyethylene glycol as co-surfactants since they are common components for the preparation of ocular microemulsions and they are non-toxic for ocular tissues (Üstündag-Okur et al., 2014).

Microemulsions are optimum alternatives for ocular drug delivery since an ideal topical ocular system must be delivered on the ocular surface, avoiding blurred vision or irritability (Sahoo et al., 2008). Various literature studies on ocular microemulsions evaluate the use of IPM as the oil phase since it is suitable for ocular use (Gaudana et al., 2010). It has been shown that the microemulsions prepared using isopropyl myristate are in contact with the eye surface for a longer period. For example, interesting research evaluated the development of MEs containing diclofenac to act as topical ocular carriers for inflammatory ocular diseases. The authors suggested the use of IPM as the oil phase and Tween 80® and glycerin as surfactant co-surfactant, respectively. The prepared MEs showed desirable properties and sustained release of the drug for up to 24 h (Habib et al., 2011). In our study, the microemulsions composed of IPM as the oil, Span 80®, Cre EL®, Tween 80®, and Tween 20® as surfactants, and PG, Polyethylene Glycol 400, and Ethanol as co/surfactant while bidistilled water selected as the aqueous phase. The solubility results of THZ in the different mediums are shown in Table 2. It can be said that its water solubility was higher than other media. For this reason, THZ was dissolved in water and the prepared microemulsions type was planned as W/O. The maximum drug solubility was measured in distilled water, PG, ethanol, and Tween 80® calculated as 48.5 ± 0.6, 38.8 ± 2.2, 20.1 ± 1.1, and 1.4 ± 0.1 mg/mL, respectively. After THZ was dissolved in the formulations, a transparent system was identified.

The log *p*-value of THZ was around −1.691 ± 0.137 and its solubility in water was measured as 48.575 ± 0.691 mg/mL which is suitable for its addition into the water phase of the microemulsion. The Log *p*-value of the THZ, which has a high water solubility, is −1.6, which is an expected result and shows that the substance is not soluble in oil when compared to water. One of the reasons why we chose microemulsion as the drug form applied to the eye is to be more effective in the treatment of allergic conjunctivitis in the eye with a carrier that is very hydrophilic and has a lipophilic outer phase.

The physicochemical characterization of new carriers is a major issue during the formulation process, especially for those intended to be used for ocular administration. Besides, the appropriate physicochemical properties of newly developed carriers should ensure sufficient bioavailability of managed drugs and bio-compatibility with eye mucosa (Üstündag-Okur et al., 2014). The characterization results of blank and THZ loaded microemulsions are stated in Table 3.

A crucial characteristic of ocular formulations is their clarity. The clarity of the produced formulations, as determined by visual inspection, was adequate for the ocular application. The pH of the eye is normally neutral (7.0–7.3).

Tear flow could be stimulated by a formulation applied to the eye. Tear fluid can swiftly dilute and buffer small amounts of additional substances, allowing the eye to withstand a wide pH range (Gonnering et al., 1979). Thus, the pH of ocular formulations should be near these limits to avoid patients' compliance. Herein, the pH of the blank microemulsions ranged between 6.26 ± 0.07 and 6.88 ± 0.02. Also, the pH of THZ loaded microemulsions was found between 6.37 ± 0.01 and 7.44 ± 0.05 as can be seen in Table 3. Thus, the pH of the formulations was suitable for ophthalmic application because they were iso-hydric.

The morphological properties such as droplet size and PDI as well as the physical stability which is affected by zeta potential are important values for the fabrication of ocular carriers to avoid being irritant and to improve the resident time on the ocular surfaces (Kalam et al., 2016). The droplet sizes of prepared microemulsions were in the colloidal size range between 5.25 ± 0.14–31.3 ± 3.42 nm, which was far below the droplet size of 10 μm that could irritate the eye. The droplet sizes of THZ loaded formulations ranged between 2.26 ± 0.30 and 30.68 ± 1.36 nm. This small droplet size could be associated with the presence of a co-surfactant. The co-surfactant can penetrate the interfacial film of the nearby

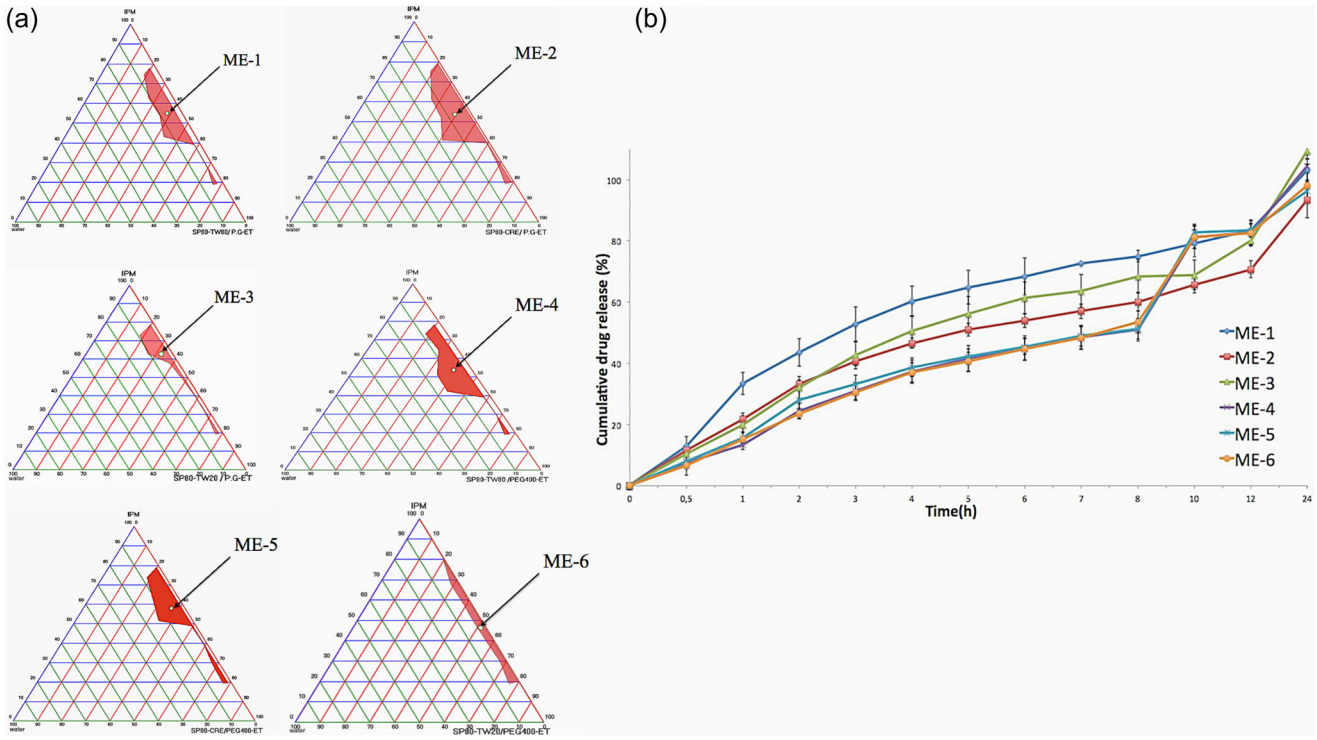

FIGURE 1 Pseudo-ternary phase diagrams of microemulsions (a) in vitro release results of THZ microemulsions (b).

TABLE 1 The composition percentages of oil, surfactants, co-surfactants, and water and area of phase diagrams values of the ideal microemulsions.

Formulation code	Rate of surfactant/ co-surfactant	Oil (%)	Surfactant (%)	Co-surfactant (%)	Water (%)	Area of phase diagrams
ME-1	4:1	IPM	Span80® (3) Tween80® (1)	PG (1)–Ethanol(8)	Distilled water	242.69
		54.93	30.831	7.707	6.53	
ME-2	5:1	IPM	Span80® (3)–CreEL® (1)	PG(1)–Ethanol(8)	Distilled water	370
		53.82	32.976	6.595	6.61	
ME-3	4:1	IPM	Span80® (4) Tween20® (1)	PG(1)–Ethanol(8)	Distilled water	100.79
		59.92	29.24	7.31	3.55	
ME-4	4:1	IPM	Span80® (3) Tween80® (1)	PEG 400(1)–Ethanol(8)	Distilled water	279.29
		53.84	31.208	7.802	7.15	
ME-5	3:1	IPM	Span80® (3)–CreEL® (1)	PEG 400(1)–Ethanol (8)	Distilled water	237.63
		58.02	27.24	9.08	5.65	
ME-6	9:1	IPM	Span80® (4) Tween20® (1)	PEG 400(1)–Ethanol (8)	Distilled water	126.31
		46.58	46.35	5.15	1.92	

Note: The numbers in parentheses show the ratios of surfactants to each other. In the same way, the numbers in parentheses show the ratios of co-surfactants to each other.

oil droplets reducing the interfacial film fluidity and surface viscosity leading to the lower of the nanodroplets radius of curvature (Mahboobian et al., 2020).

A low PDI value suggests a good homogeneity of droplets in microemulsions (Bharti & Kesavan, 2017; Gupta et al., 2019). Herein, a narrow PDI was determined; the PDI of THZ loaded and unloaded formulations, was below 0.46 indicating uniformity (Table 3). In further, the electrical charge, in terms of zeta potential of THZ loaded and unloaded formulations, was found between -0.24 ± 0.01 and 0.61 ± 0.02 mV (Table 3). The results were almost neutral indicating their possible

use for ocular drug administration. Besides, zeta potential near zero would be non-irritant for ocular use (Gupta et al., 2019).

Viscosity can also impact the characteristics of the ocular formulations (Gupta et al., 2019). For example, the viscosity can affect the drug residence time on the eye surface. High viscosity could prolong the resistance time but could lead to troubled drug instillation, blinking, pain, and others (Radomska-Soukharev & Wojciechowska, 2005). Hence, this value must be within acceptable limits for ophthalmic application (Zignani et al., 1995).

The ocular retention time of a formulation is highly associated with a viscosity level of its own. Low viscosity can lead to the rapid expiration of ophthalmic formulations from the corneal region. As a result, a balance between extended retention time and easy application is ensured by ideal viscosity (Soliman et al., 2019). Ophthalmic drops are cleared away from the tear fluid in a short time when the viscosity value of drops possessed that almost the same as the human tears (1.5 mPa.s (cP)). (Zhu & Chauhan, 2008). In our study, the viscosity of the blank microemulsions ranged between 23.533 ± 0.115 cP and 39.633 ± 1.069 cP (Table 3). Also, the viscosity of THZ loaded microemulsions was found between 21.767 ± 0.057 cP and 37.7 ± 0.2 cP. Because of this reason, an increase in the residence time of ophthalmic formulations will likely enhance therapeutic benefits.

One of the main indicators to detect the external phase of the emulsion is the conductivity level of the emulsion. The assessment of the electrolytic conductivity is essential to reveal the emulsion character because the oil phase mostly does not contain electrolytic. The conductivity of formulations ranged from 0.13 ± 0.05 to 0.43 ± 0.05 μs/cm. The phase systems of the formulations were defined by calculating the conductivity. Consistent with the results, all microemulsions were identified as w/o phase systems. The refractive index of the developed microemulsions in the presence and absence of THZ ranged between 1.43 ± 0.01 and 1.45 ± 0.01.

It can be concluded that the ME1-ME6 microemulsions could be determined as appropriate carriers for ocular application. Besides, the microemulsions present great properties since they could be easily prepared and sterilized. Moreover, microemulsions possess stability and a great capacity to dissolve drugs. Finally, the THZ content of all formulations were found to be in the range of 95.00%–100.045% which was desirable.

TABLE 2 The solubility results of THZ in different solvents.

Medium	Solubility (mg/mL)
Distilled water	48.57 ± 0.69
Ethanol	20.12 ± 1.10
IPM	0.007 ± 0.01
Capryl 90®	1.02 ± 0.01
PG	38.88 ± 2.22
Oleic acid	0.05 ± 0.01
Almond oil	0.07 ± 0.01
Span 80®	0.39 ± 0.08
Tween 80®	1.47 ± 0.14
Tween 20®	1.40 ± 0.12
Cre EL®	0.49 ± 0.04
Labrafil M®	0.15 ± 0.01

Sterilization and microbiological control of THZ microemulsions

In addition to being stable, ophthalmic formulations should be sterile to prevent further contamination of the

TABLE 3 Characterization findings of microemulsion formulations.

Formulation		pH	Electrical conductivity (μs)	PDI	Droplet size (nm)	Zeta potential (mV)	Viscosity (cP)	Drug content (%)
Blank formulations	ME-1	6.69 ± 0.10	0.43 ± 0.05	0.17 ± 0.055	10.48 ± 0.74	0.13 ± 0.01	37.06 ± 0.60	–
	ME-2	6.473 ± 0.02	0.1 ± 0.004	0.314 ± 0.021	7.76 ± 0.52	0.07 ± 0.01	37.03 ± 0.96	–
	ME-3	6.67 ± 0.07	0.3 ± 0.058	0.22 ± 0.01	31.30 ± 3.42	0.25 ± 0.01	23.53 ± 0.11	–
	ME-4	6.69 ± 0.08	0.2 ± 0.004	0.20 ± 0.02	9.96 ± 0.76	0.60 ± 0.04	35.53 ± 0.11	–
	ME-5	6.88 ± 0.02	0.3 ± 0.058	0.16 ± 0.05	12.6 ± 0.72	0.32 ± 0.01	30.70 ± 0.10	–
	ME-6	6.26 ± 0.07	0.3 ± 0.058	0.33 ± 0.04	5.25 ± 0.14	0.45 ± 0.01	39.63 ± 1.06	–
Drug loaded formulations	ME-1	7.34 ± 0.04	0.43 ± 0.05	0.46 ± 0.02	2.26 ± 0.30	0.39 ± 0.33	31.60 ± 0.79	98.70 ± 2.50
	ME-2	7.22 ± 0.05	0.13 ± 0.05	0.27 ± 0.02	8.49 ± 0.99	0.02 ± 0.01	37.70 ± 0.20	100.05 ± 1.54
	ME-3	6.37 ± 0.01	0.4 ± 0.004	0.13 ± 0.01	30.6 ± 1.36	−0.24 ± 0.01	21.76 ± 0.05	99.30 ± 3.20
	ME-4	7.06 ± 0.08	0.2 ± 0.004	0.15 ± 0.03	20.0 ± 0.782	0.61 ± 0.02	32.63 ± 0.40	95.01 ± 1.18
	ME-5	7.44 ± 0.05	0.36 ± 0.05	0.11 ± 0.01	20.89 ± 0.34	0.02 ± 0.01	28.60 ± 0.26	96.90 ± 7.67
	ME-6	7.42 ± 0.40	0.23 ± 0.05	0.36 ± 0.09	7.86 ± 0.86	−0.20 ± 0.01	35.16 ± 0.45	100.64 ± 2.5

eyes (Üstündağ Okur et al., 2019). It is a required necessity for all ophthalmic products to be sterile and free of microorganisms to confirm their safety for patients (Tighsazzadeh et al., 2019). All developed formulations were sterile, and no contamination was observed. During sterilization inspection, droplet size, PDI, and pH values were not changed.

Stability studies of THZ microemulsions

The developed THZ loaded microemulsions were kept for 90 days to examine their stability. The stability results of physical appearance, pH, conductivity, phase separation, droplet size, zeta potential, and PDI were investigated initially in the first, second, and third months and no significant difference was observed after 3 months. In addition, THZ microemulsions showed no changes in physical appearance after freeze–thaw cycles.

Besides, microemulsions compared to other micellar systems are superior in terms of solubilization potential and thermodynamic stability and quite advantageous in comparison to other unstable dispersions, such as emulsions and suspensions. This property is related to their manufacture process given that they are developed using less energy input (heat and mixing) presenting long shelf life. Various researchers have investigated the stability of microemulsions; most of these studies revealed that, except for surface pressure-induced interfacial tension depression and microemulsions are formed by a complicated interaction between thermodynamic stability and zero interfacial tension. Chemical potentials, solubility parameters, enthalpy, stress gradients, interfacial compressibility, entropy, bending, and tensional components of interfacial free energy, osmotic pressure, and species concentrations in the bulk and interphase are examples of thermodynamic variables (Mehta & Kaur, 2011). The contributions of co-surfactants in reducing the interfacial tension of the surfactant layer in microemulsions generated the spontaneous development of a microemulsion and promoted its thermodynamic stability (Kogan & Garti, 2006).

In-vitro drug release study of THZ microemulsions

THZ is a water-soluble drug; consequently, its release rate should be prolonged and controlled to achieve the greatest therapeutic outcome. Besides, if the drug is dissolved immediately, it's in vivo performance would be decreased. More specifically, most of the ocular drugs are immediately cleared from the corneal surface due to tears flow leading to the short residence time on ocular mucus (Siafaka et al., 2015). Figure 1b exhibited the in vitro THZ release experiment of the microemulsions ME-1, ME-2, ME-3, ME-4, ME-5, and ME-6 at 32 ± 1°C in simulated tear fluid. The amount of THZ in the medium was determined using an HPLC at 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, and 24 h. The microemulsions depicted sustained drug release for 12 h with the absence of a burst phenomenon. Only 5%–10% of the THZ drug was released within 1st hour of the experiment. After 1 h, a progressive release rate was demonstrated reaching the maximum release between 97% and 100% at 24 h. M5 and M6 display a secondary phase of a rapid release and are excluded from the ex vivo permeation studies, since this rapid dissolution may result in patients' compliance.

In the past, w/o moxifloxacin loaded microemulsions were developed for the management of bacterial keratitis. It was revealed that the fabricated w/o microemulsions exhibited sustained release revealing greater therapeutic efficacy than the marketed product (Bharti & Kesavan, 2017). Consequently, the w/o microemulsion whereby the oil plays the role of the external phase while the water droplets form a nano-reservoir for the hydrophilic drug help the transportation of the drug from the aqueous phase. Thus, the instillation of the formulation will not take place frequently improving patient compliance (Bharti & Kesavan, 2017).

Ex vivo permeation/penetration study of THZ microemulsions

Sheep corneas were used for ex vivo experiments. The permeation/penetration experiments of the optimum formulations (ME-1, ME-2, ME-3, and ME-4) and commercial product were performed with diffusion cells in artificial tear fluid using the excised sheep corneas at 32 ± 1°C. The results of the permeation and penetration of selected formulations and commercial products are shown in Table 4. At the end of 24 h, the permeation of THZ from the cornea for ME-1, ME-2, ME-3, and ME-4 was demonstrated between 40.213% and 57.752% and for commercial products 69.469%. Also, at the end of 24 h, the penetration of THZ to the cornea for ME-1, ME-2, ME-3, and ME-4 was between 12.088% and 4.161%, and for commercial product 5.631%. Similarly, Kumar et al. showed significant drug accumulation from the microemulsions in corneal tissue compared to voriconazole suspension (Kumar & Sinha, 2014).

Ex vivo bioadhesion studies of THZ microemulsions

The ex vivo bioadhesion studies were performed to detect the adhesive strength of microemulsions to the cornea. Ex vivo bioadhesion studies of selected formulations (ME-1, ME-2, ME-3, and ME-4) were performed using rabbit corneas. The results of the ex vivo bioadhesion study of THZ loaded formulations are shown in Table 4 and bioadhesion results of

formulations determined between 0.19 and 0.23 mJ/cm^2. The mucoadhesion test results matched the adhesiveness values collected from the textural profile study. Also, it was observed that the formulations were suitable for application to the eye. Tuğcu-Demiröz et al. prepared gel formulations using Poloxamer 407 and Chitosan; the formulations were evaluated for their bioadhesion. They exhibited that the work of mucoadhesion values for the gels were between 0.18 ± 0.04 and 0.31 ± 0.03mj/cm^2 (Tuğcu-Demiröz, 2017).

In vitro ocular irritation test

ME-1 was chosen for further studies due to its better penetration ability and small droplet size. The reconstructed human cornea-like Epithelium (RhCE) analysis procedure has been practiced for many years by the industry as a non-animal, in vitro alternative in vitro method for detecting compounds that do not require categorization and labeling for serious ocular damage or ocular irritation (OECD Test No. 492). A cytotoxic capacity of a chemical could be tested with RhCE tissue which resembles the physiological, morphological, and biochemical characteristics of the human corneal epithelium (OECD, 2019).

In accordance with the manufacturer's recommended procedure, the test passed the requirement for acceptance as far as the negative control OD >0.8 and <2.5, the mean relative viability of the positive control was below 50% of control viability after 30-minute exposure as well as the difference of viability between the two relating tissues of a single chemical was <20% in the same run for PC, NC, and tissues of THZ loaded microemulsion. The examined formulation was determined to be non-irritating (Figure 2) because the tissue viability after treatment with THZ microemulsion exceeded 60% of the mean vitality of the NCs.

Ames test

Testing for genotoxicity is a crucial part of evaluating the safety of xenobiotics. Tumorigenic or heritable mutation capacity of the chemicals can be detected by genotoxicity testing which is aimed to identify genetic impairments such as chromosomal aberration and gene mutations (Wu et al., 2010). Since there is no data available in the literature regarding THZ, the Ames test was performed to assess the mutagenic potential of THZ loaded microemulsion (ME-1). Table 5 represents the ME-1-induced mutagenicity test findings in the TA98 and TA100 strains with/without metabolic activation.

When compared to the negative control, the positive control had a higher frequency of revertant colonies ($p < 0.01$) in both investigated strains with/without S9 activation. None of the concentrations examined resulted in a substantial increase in the revertant number of TA98 and TA100 strains with/without S9 activation, mutagenicity to the tested strains whether or not incubated with S9, indicating no mutagenicity to the tested strains whether or not incubated with S9.

TABLE 4 Results of permeation-penetration and bioadhesion of THZ microemulsions and commercial product.

Formulation code	Permeation of THZ from the cornea (%)	Penetration of THZ to the cornea (%)	Ex vivo bioadhesion results (mJ/cm^2)
ME-1	52.84 ± 13.45	12.08 ± 4.91	0.19 ± 0.008
ME-2	57.75 ± 5.91	8.55 ± 0.83	0.20 ± 0.01
ME-4	44.10 ± 10.74	4.41 ± 1.54	0.23 ± 0.003
ME-5	40.21 ± 10.76	4.16 ± 2.35	0.22 ± 0.001
Commercial product	69.46 ± 16.83	5.631 ± 0.63	

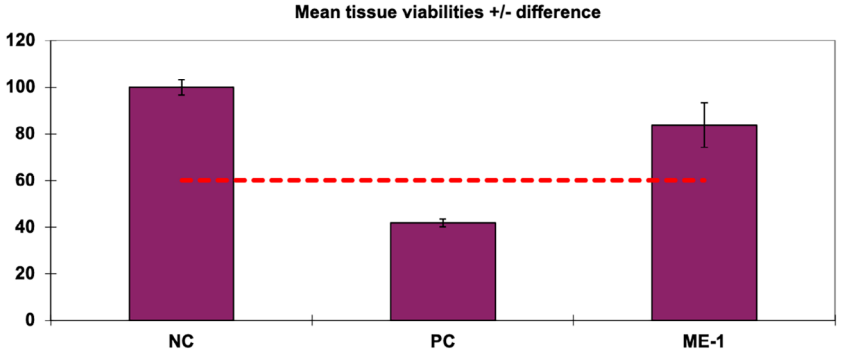

FIGURE 2 The percentage of relative cell viability after EpiOcular SIT in vitro eye irritation test on ME-1 loaded THZ. Results represent the mean + SD of two individual tissues. NC, negative control (Sterile deionized water); PC, positive control (Neat methyl acetate); ME-1 (THZ loaded microemulsion).

Although the tested compound was not mutagenic in *Salmonella* stains TA98 and TA100, according to OECD guidelines some more studies are required using other *Salmonella* stains such as TA97a, TA102, and TA1535 and also in vitro chromosomal aberration or micronucleus assay before concluding about the mutagenic and genotoxic potential of this compound.

In vivo studies of THZ microemulsions

The rabbits were used for in vivo studies of ME-1 and commercial product. THZ concentration in the tear was depicted in Figure 3. The maximum concentration of THZ was obtained from the ME-1 formulation which was quite improved in comparison to the commercial formulation. The THZ concentration for ME-1, measured within 1 min after the application was observed at 7.409 μg/mL. For the commercial product (VISINE®) THZ concentration was found as 3.403 μg/mL, a result, which is similar to our previous study (Üstündağ Okur et al., 2020). Besides, a 2.17-fold increase in tear→1 min was obtained for ME-1 relative to the commercial. The viscosity of the ophthalmic formulation could figure the retention time and drainage rates of eye drops. ME-1 microemulsion viscosity value was found 31.6 ± 0.7 cP which is higher than commercial eye drops solution (10 cP).

The microemulsion systems have advantages such as reduced dose application which result in fewer side effects as compared to conventional dosage forms. Aside from this, the use of microemulsions can lead to sustaining ocular drug delivery and prolong drug residence time on the eye surface. Furthermore, the potential for sustained drug release from microemulsions makes these delivery systems highly appealing for ophthalmic application and can significantly reduce the frequency of eye drop application.

Additionally, studies have revealed that pilocarpine loaded microemulsions prolong the drug's effect to the point that two time/day instillations of microemulsion are comparable to four time/day instillations of conventional dosage forms (Sahoo et al., 2008).

TABLE 5 Mutagenicity results of THZ loaded microemulsion (ME-1) in *Salmonella typhimurium* TA98 and TA100.

Without S9 number of revertant/plate			
		TA98	TA100
Negative control		23.5 ± 6.4	168.7 ± 18.6
Positive control		682.0 ± 62.3	975.0 ± 72.8
Concentrations	100%	22.5 ± 3.5	179.0 ± 22.6
	50%	21.0 ± 1.4	170.3 ± 22.2
	25%	22.5 ± 0.7	167.7 ± 14.3
	10%	21.5 ± 7.8	162.0 ± 10.4
With S9			
Negative control		27.0 ± 4.2	154.7 ± 11.0
Positive control		992.5 ± 3.5	697.6 ± 29.3
Concentrations	100%	26.0 ± 1.4	160.5 ± 9.2
	50%	26.5 ± 2.1	141.7 ± 15.3
	25%	24.0 ± 2.8	151.5 ± 16.3
	10%	25.5 ± 3.5	145.5 ± 10.6

Note: Dunnett's multiple comparison test was carried out for statistical analysis. For TA98, NPD (20 μg/plate) and 2-aminofluorene (10 μg/plate) were used as positive mutagen without and with S9 experiment, respectively. For TA100, SA (1 μg/plate) and 2-aminofluorene (10 μg/plate) were used as positive mutagen without and with S9 experiment, respectively. *$p < 0.05$ versus negative control.

Ocular irritation experiment

The ocular irritation of the ideal microemulsion was tested using the Draize Rabbit Eye Test. Figure 4 shows the obtained data of ocular irritation tests following 6 h of instillation. In accordance with the modified

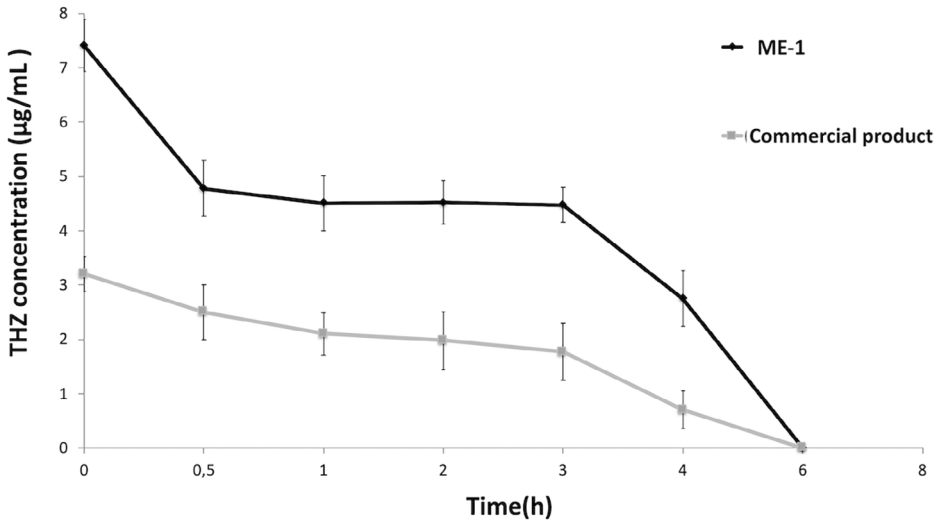

FIGURE 3 THZ concentration (μg/mL) in the tear.

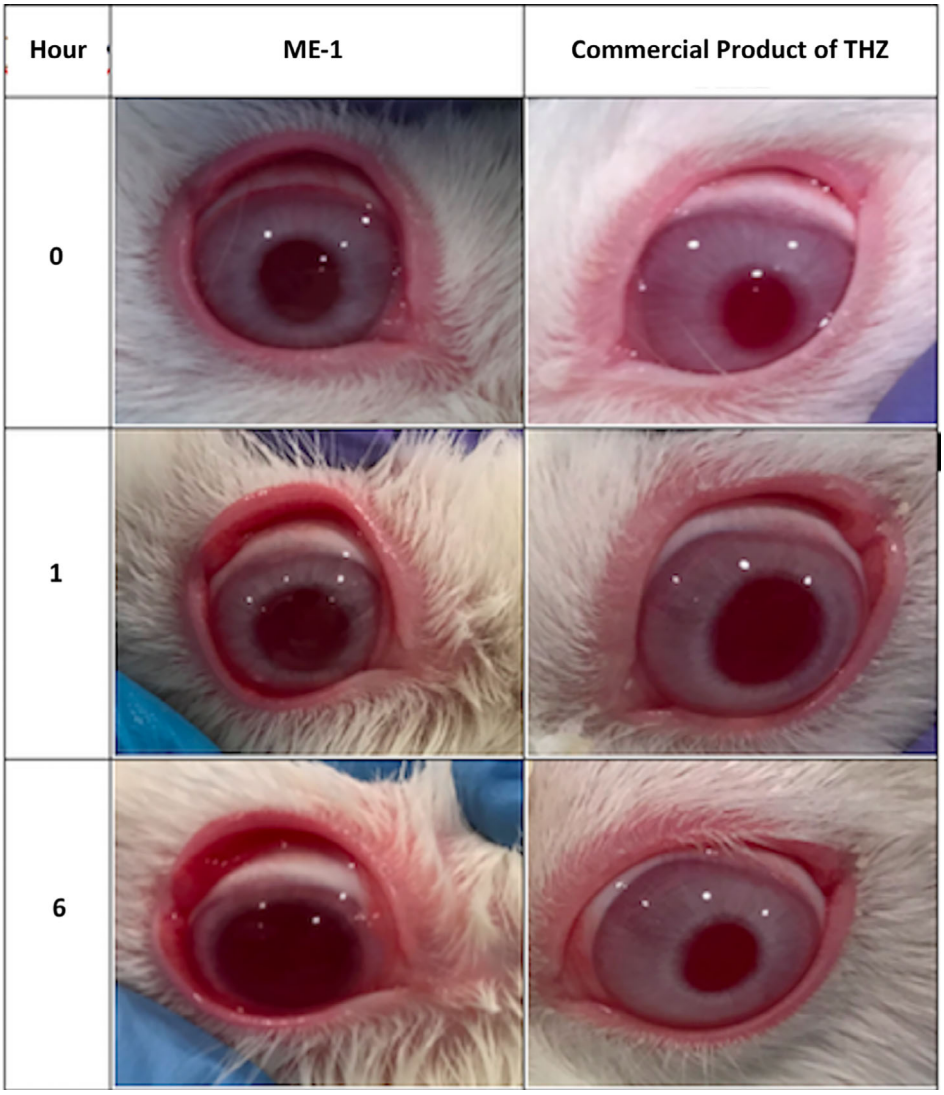

FIGURE 4 Results of ocular irritation tests after the instillation of ME-1 and commercial product of THZ.

Draize test, solely grades 0 and occasionally 1 were reported. There was also no difference between the control and microemulsion-treated eyes. The results obtained from in vitro EpiOcular™ Eye Irritation Test and in vivo ocular irritation test were compatible expressing non irritancy. These results confirmed that the developed microemulsions are suitable for ocular administration and offer a promising alternative for the management of allergic conjunctivitis.

CONCLUSIONS

The developed microemulsions were prepared and sterilized simply whereas the characterization results of the formulations showed that the microemulsions were appropriate for eye treatment. In further, the prepared formulations demonstrated sustained in vitro release while ex vivo permeation, penetration, and bioadhesion results were criticized as satisfactory for ophthalmic drug delivery. Also, from the results of in vivo studies, the formulation ME-1 showed a remarkably long contact time to the eye surface than the commercial product. The developed system exhibited longer contact time of THZ to the cornea. Thus, the dosing frequency of the formulation containing THZ could be reduced using the developed ocular microemulsion. To conclude, this research indicated that the microemulsions could be alternatively applied as topical ophthalmic drug delivery systems of THZ.

AUTHOR CONTRIBUTIONS

Vildan Yozgatlı and Neslihan Üstündağ Okur conceived and designed the study, and wrote the first draft of the manuscript. Vildan Yozgatlı, Neslihan Üstündağ Okur, Mehmet Evren Okur, Hande Sipahi, and Mohammad Charehsaz carried out the research. Neslihan Üstündağ Okur, Mehmet Evren Okur, Ahmet Aydın, and Timuçin Uğurlu analyzed the data. All authors contributed to and approved the final draft of the manuscript.

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CONFLICT OF INTEREST STATEMENT

Authors declare no conflict of interest.

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