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Original Article

Effect of Olive Oil on Hesperidin Nanovesicles in Treatment of Induced Corneal Ulcers in Rabbits, Morphological and Histopathologic Study

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Abstract

Hesperidin (HSP) and Olive oil possess many biological activities that are required for the safe and effective treatment of corneal ulcers. However, the poor aqueous solubility of HSP hinders its topical utilization. This work aims at enhancing the dissolution of HSP and combining the powerful effectiveness of Olive oil in treating corneal ulcers. HSP was isolated from orange peel and described by spectroscopic methods (^{1}H NMR and ^{13}C NMR), then HSP nanovesicles were prepared with and without Olive oil using the ethanol injection method. Nanovesicles were applied topically to rabbits' eyes in which alkali burn corneal ulcers were induced. After five weeks, histopathological studies were performed. No ulcers were determined after topical application of HSP, and the inclusion of Olive oil returned the eye to its normal conditions. Thus, this study clarified the potential role of the HSP - Olive oil combination in managing corneal ulcers.

Keywords: Corneal ulcer, Hesperidin, Histopathology, Olive oil, nanovesicles, rabbits, Topical application.

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1. Introduction

Microbial infection, trauma and chemical burns are the main causes beyond the deep corneal ulcers [1]. Chemical burns (acidic or alkaline) are absolute ophthalmic emergencies that provoke corneal impairment [2] and necessitate

rapid manipulation to reduce catastrophic consequences. Acidic and alkaline chemical burns accounts for about 11.5 and 22.1% of eye injuries respectively [3]. In general, about 66% of chemical burns arise out of an alkali, and the remaining are from alcohols and acids [4] as a result of the widespread utilization of alkali materials in manufacturing purposes and house cleaning products [5].

Sodium hydroxide and ammonium hydroxide are examples of alkalis that are widely utilized in our daily life. Sodium hydroxide (caustic soda) is employed for cleaning pipes and sinks, and ammonium hydroxide is utilized in the manufacture of fertilizers[6]. The ocular penetration of alkaline chemicals is deeper than that of acidic ones due to the presence of hydroxyl ions, which resulted in the saponification of fatty acids present in the cell membrane, inducing disruption of the cell [7]. As epithelium is impaired, alkaline material permeates quickly through the sub-adjacent tissues destructing collagen matrix and proteoglycan ground substance [7, 8]. Lysis of the cell membranes resulted in the liberation of inflammatory mediators such as leukotrienes and prostaglandins, resulting in an immediate immunological response [9, 10]. Consequently, this condition needs to be recognized promptly and treated immediately.

Conventional systems for ocular delivery frequently lead to low therapeutic effectiveness and bioavailability because of the fast elimination of the drug due to drain through the nasolacrimal duct and high turnover of lacrimal fluid. As a result, the frequent dosage is typically required, which is associated with patient discomfort [11, 12]. To successful ulcer management, various challenges must be addressed in a single formulation that maintains therapeutic concentrations for extended periods of time while balancing drug dosage [13]. Research efforts that have been conducted to overcome the major side effects of conventional ocular delivery systems led to the advent of novel approaches such as ocular inserts, polymeric or lipid-based nanoparticles[14], and *in-situ* gels [15].

HSP (3 hesperetin 7-rutinoside) is the main flavonoid abundantly present in citrus fruits. It is composed of an aglycone unit, hesperetin, and a disaccharide known as routines. It exerts variable biological activities such as anti-inflammatory, anti-apoptotic, antibacterial, and antioxidant effects, and so it was called bioflavonoids. Olive oil is a natural oil that contains abundant polyphenolic compounds [16, 17] and shows anti-inflammatory, angiogenetic, antioxidant, and antibacterial activities. Based on the biological activities, HSP and Olive oil are both utilized in the wound healing process.

Despite HSP's numerous advantages and biological activities, it has low solubility and permeability that may limit its topical application. Thus, utilizing new drug nanocarriers may offer promising drug delivery systems which improve its permeability and aqueous solubility, consequently, its therapeutic activity. Additionally, if this system can hold both Olive oil and HSP, so it is desirable as it combines the biological activities of both.

Nanovesicles (elastic niosomes) are novel analogs that belong to the vesicular delivery system; they are based on nonionic surfactant and edge activator (EA). Kakkar and Kaur were

the first ones to use nanovesicles as an ophthalmic system for delivery [18, 19]. Nanovesicles have numerous benefits in terms of being osmotically active, high elasticity, chemically stable, and can further enhance corneal permeability with prolonged residence time; these benefits may be because of the existence of an edge activator within the bilayer structure. Accordingly, nanovesicles were considered a hopeful ocular delivery system to maximize the therapeutic efficacy of different drug products [20, 21]. Therefore, a delivery method that can improve the permeability and sustains the HSP release as well as combining Olive oil in the same system, is considered an attractive device.

In view of mentioned points, this study aimed to prepare nanovesicular formulations of HSP with and without Olive oil and exploring the impact of these nanovesicles on the alkali burn-induced corneal ulcers in rabbits.

2. Materials and Methods

Extracted HSP, Olive oil brought from a local Olive press, Arish, Egypt. Dimethyl sulfoxide (DMSO) was purchased from Oxford Lab Chemicals Mumbai, India. Ethanol absolute and tween 80 were purchased from El-Nasr Pharmaceutical. Sorbitan tristearate (span 65) was Purchased from Sigma Chemical Company USA.

2.1. Isolation of HSP

HSP was isolated from the orange peel, as previously mentioned [22-24]. Orange peel was peeled out from *Citrus sinensis* fruits that were purchased from the local market, air-dried at 25°C, and crushed well to a fine powder. About 50 g of the powder were defeated by dichloromethane using a Soxhlet apparatus to eliminate oil, fats, and non-polar compounds. The defatted powder was further macerated in methanol (250 mL×3) and filtered. The filtrate (methanolic extract) was concentrated under a vacuum to get a sticky residue (56 g) of methanolic extract. Crude HSP (2.5 g) was precipitated from the methanolic extract using 6% aqueous acetic acid. Further, it was purified by crystallization using a mixture of DMSO and distilled water at (60-80 °C). Pure HSP was obtained as white crystals (HSP, 1.4 g). Spectroscopic methods were used to study and describe the result (^{1}H NMR and ^{13}C NMR).

HSP nanovesicles (HSPL) were drawn up using the method of ethanol injection formerly outlined by Ali et al. (2022) with some modifications [25] where 3 ml of ethanol was used to dissolve span 65, 10 mg of HSP were dissolved in DMSO and added to an ethanolic solution of span 65. The mix was then warmed up 60 °C and injected slowly into a 0.2% solution of tween 80 in water previously warmed to 60 °C. The formed emulsion is then stirred on a magnetic stirrer for 15 min, left to cool, and then maintained overnight in the refrigerator at 4 °C. The ratios of span 65: tween 80 were 1:1, 2:1, and 3:1, respectively. Another three formulae were prepared to have the same composition but with the addition of Olive oil. The formulaion of various HSP nanovesicular formulations is presented in (**Table. 1**).

Table 1. Drug content and % EE of HSP nanovesicular formulations.

Formulation code	Span 65 mg	Volume of Olive oil (ml)	Drug content%	%EE
HSPL-1	100	----	71.23±2.23	97.32±1.32
HSPL-2	200	----	82.60±1.62	97.69±1.28
HSPL-3	300	----	93.25±1.23	98.36±1.78
HSPL-4	100	0.3	62.24±2.14	96.08±1.64
HSPL-5	200	0.3	67.28±1.27	98.64±1.31
HSPL-6	300	0.3	89.34±2.32	97.76±1.28

2.2. Preparation characteristics HSP nanovesicles.

2.2.1. HSP content and (%EE) estimation.

After preparation of HSP nanovesicles, drug content was checked by dispersing 0.1 ml of made nanovesicular dispersions into 5 mL of absolute ethanol.

It was done for disruption of the vesicles, and then the content of HSP was detected spectrophotometrically using (Jenway 6320D, Staffordshire, UK) spectrophotometer at λ_{max} of 280 nm. The percent EE was detected by ultracentrifugation of nanovesicular dispersions at 15,000 rpm for 30 min using a centrifuge (Beckman, Fullerton, Canada) under cooling at 4 °C, the supernatant is then removed, and free drug concentration is estimated spectrophotometrically at λ_{max} of 280 nm, the %EE is determined based on this equation [26]:

$$\%EE = (\text{Total drug amount} - \text{Free drug amount}) / (\text{Total drug amount}) \; x \; 100$$

Equation (1).

2.2.2. In-vitro HSP release studies.

The *in- vitro* release of HSP from the developed nanovesicles and from the dispersion of free HSP were examined utilizing a semipermeable cellophane membrane. Rubber stripes sealed the membrane over a glass tube's open end. Tube opening surface area was 2.21 cm^2. 1 ml of each HSP rich nanovesicles preparation was dropped in the glass tubes and 1 mg of free drug was dispersed in 1 ml of distilled water, inserted into another tube, and considered as a control. The tubes were dipped in a 100 ml beaker containing 30 ml of (phosphate buffer pH 7.4+ ethanol) at a ratio of (1:1) and were kept in a thermostatically monitored shaker (shacked at 50 hits /min in a water bath kept at 37±1°C). Regularly over a period of up to twenty-four hours, five milliliters release media in the form of an aliquot were pulled out for the purpose of assay and substituted with an equivalent amount of freshly prepared medium to keep the volume constant. The absorbance of the gathered specimens was detected spectrophotometrically at λ_{max} of 280 nm using the new medium serving as a blank. Triplicate experiments were carried out and the averages were measured. Plain samples were subjected to the identical assays as the medicated ones.

2.2.3. Estimation of particle size, zeta potential, and polydispersity index.

Following the appropriate dilution with water that has been double-distilled in order to achieve the desired scattering intensity, the poly dispersity index (PDI), mean particle size, and zeta potential of some formulations were

estimated by Zetasizer (Malvern Instrument Ltd., Worcestershire, UK) at 25°C (Scognamiglio et al., 2013).

2.2.4. Differential scanning calorimetry (DSC)

To check the encapsulation of HSP into nanovesicular formulations, DSC studies were carried out using a calorimeter calibrated with indium (ShimadzuDTG-60/DTG-60A, Japan). 5 mg of HSP, span 65, physical mixture of both with and without olive oil and selected nanovesicles formulations were investigated using a differential scanning calorimeter. Samples were weighed and enclosed in standard aluminum pan. Thermo grams were achieved at a scanning rate of 10°C/min. Each sample was scanned between 20-300°C.

2.2.5. Fourier transform infrared spectroscopy (FT-IR)

To check any interaction between the components of nanovesicular preparations, the Hitachi 295 spectrophotometer (Hitachi, Tokyo, Japan) was utilized for performing Fourier transform Infrared spectroscopy (FT-IR) measurements employing the method of KBr disc [27, 28]. Samples were tested over the range of 4000 to 400 cm-1. Infrared spectroscopic analysis was run for HSP, span 65, tween 80, physical mixture of them (with and without Olive oil), and for the dried selected HSP nanovesicular formulations.

2.2.6. Scanning electron microscopy

The structure of HSP nanovesicular formulations was investigated utilizing the scanning electron microscope (JSM-4500 LV, JEOL Ltd., Japan) with a camera attachment operated at 20 kV. Chosen HSP nanovesicles formulations were installed on a glass slide and dewatered in a desiccant overnight. Samples were put on carbon tape after they dried and held on an aluminum stub, and sputter-coated with gold by a direct current sputter coater (Q-150RES, Quorem). The electron gun worked between 2 and 5 kV, working distance was kept 10 mm.

2.3. Morphological and histopathological studies

For estimating the effectiveness of HSP nanovesicles prepared with and without Olive oil on the alkali-induced ulcer in rabbits, animal experiments were performed.

24 New Zealand albino rabbits that are in good health (16 males and 8 females who are not pregnant and did not breastfeed) have been incorporated into this work. Their ages ranged from seven to eight months, and their body weight ranged from 1700-2500 g. The experiment and protocols were performed by virtue of the standards of the ethical committee of the care and use of laboratory animals and were in agreement with that of the National Ethical Committee of the Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt. The ethical approval number is aun/vet/1/0001.

All rabbits were examined cautiously to exclude any ophthalmic abnormalities. Eyes were inspected for their usual shape, ophthalmic secretions, movements and positions of the globe, corneal and palpebral reflexes (blinking upon contact with the cornea and eyelids, respectively, with the tip of a small blunt hemostat), and menace response (head movement in response to a sudden threat

by the hand's examiner toward the eyes). In a dim room, the light reaction in the pupil, the palpebral and bulbar conjunctiva, iris, lens, and cornea were studied [29]**.** All the animals received 200 µg/ kg of ivermectin by the subcutaneous route. They were vaccinated against the rabbit hemorrhagic viral disease through the subcutaneous injection of 0.5 ml/ animal of the vaccine.

Animals were injected intramuscularly with a mixture of (xylazine HCl 5 mg/kg of and ketamine HCl 25 mg/kg) for getting the surgical stage of anesthesia [30]. In every rabbit's right eye across all groups (except the negative control group), alkali burn-induced ulcer was generated using a sterile small swab of cotton immersed in 1% NaOH on the central cornea for 60 seconds. The surface of the eye was washed with 60 ml of physiological saline for 2 min. The test of fluorescein dye was utilized to verify and delineate the lesion induction [31].

The animals were categorized into 4 distinct groups, each one composed of six rabbits; Group I (negative control group) got topical dropping of normal saline solution without induction of corneal ulcer, Group II (+ve control group) received topical dropping of normal saline, and Group III (HD group) obtained topical application of 0.5 ml sterilized HSP-3 formulation containing 0.35 mg HSP only. Groups I and II were kept in the same cage. Group IV (HOD group) received topical application of a sterilized HSP-6 formulation containing HSP (0.7 mg/ml) and Olive oil. Groups III and IV were kept in the same cage. 0.9% normal saline, HSP-3, and HSP-6 were topically applied at the conjunctival sac (for Group I& II, III, and IV, respectively) twice daily at 12 hrs intervals for 5 weeks after corneal ulcer induction. The day after the last application was the day of slaughtering. Eyes were sent for histopathological and electron microscopical examinations.

2.3.1. Morphological evaluation

Eye examination was carried out daily before each treatment. Naked eye external and detailed ophthalmic investigations of each eye were carried out on days 1, 8, 15, 22, 29 and 36. Eyes were examined for the presence of perforation, vascularization, lacrimation, photophobia, eyelids adhesion, pus, and corneal opacity.

Magnitude of corneal neovascularization was marked from 0 to 3 [32] where 0 = no neovascularization, 1 = confined to the limbus of the cornea, 2 = extending up to the margin of the pupil, and 3 = extending beyond the margin of the pupil into the central cornea.

Corneal opacity marking was performed as follows: grade 0= clear or a trace haze; grade 1= mild opacity; grade 2= moderately dense opacity partially obscuring the details of the iris, and grade 3= severely dense opacity obscuring the details of the intraocular structure [33]**.**

The test of Fluorescein staining was performed following an ophthalmic inspection prior to the treatments as the morphological investigations on days 1, 8, 15, 22, 29 and 36. 2% fluorescein solution (One drop) was installed into the eye and left for 1 min. Sterile normal saline was used for rinsing to get rid of the extra stain [34]. The eye was

photographed at 8, 15, 22, 29 and 36 days after corneal ulcer initiation by a digitalized camera after rinsing with normal saline. The eye was captured in two photographs, once after completing an external ocular examination and once after applying fluorescein staining. The corneal ulcer surface area was quantified and examined by the ImageJ software (ImageJ 4.48v software, National Institutes of Health, USA) to track and measure how quickly an ulcer heals.

2.4. Statistical Analysis

Numerical data were examined for normality by testing the distribution of data and utilizing tests of normality (Kolmogorov-Smirnov and Shapiro-Wilk tests), and parameters were not normally distributed. Data were laid out as mean and standard deviation (SD). Mann Whitney U test was used to equate two groups in non-related samples. Wilcoxon t-test was used to compare between week1 & week5 in each group. The level of significance was set at ($P< 0.05$). Statistical analysis was carried out with IBM® SPSS® Statistics Version 26 for Windows.

2.5. Histopathology

Samples of corneal tissue were fixed in 10 % neutral buffered formalin then, dehydration by ascending grades of alcohol, clearing by xylene, and incorporating in paraffin. Tissue slicing with 4-5 µm thick and stained with hematoxylin and eosin stains (H&E) [35].

2.6 Scanning electron microscopy (SEM)

Representative corneal samples were promptly rinsed with normal saline. Thereafter, set in a mix of 5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M sodium phosphate buffer with a pH of 7.3 at 4°C for 24 hr. Subsequently washing with 0.1 M sodium phosphate buffer with a pH of 7.3, then dried utilizing ascending series of ethanol 30, 50, 70, and 90 for two hours, 100% for two days, and then to amyl acetate for two days. Critical point drying was applied to the samples by using liquid carbon dioxide. Each sample was stuck on metallic blocks using silver paint. Employing gold sputter coating apparatus, samples were evenly coated with gold at a thickness of 15nm. Specimens were investigated through the use of JEOL (JSM 5400 LV) scanning electron microscope 15- 25 kV and photographed within an electron microscope unit, Assiut University, Egypt [36]. Digital coloring scanning electron microscopic images. The scanning electron microscopic images were colorized digitally by way of the Photo Filter 6.3.2 program to recognize different types of cells and structures.

3. Results and Discussion

3.1. Isolation and identification of HSP

HSP was isolated and purified as white crystals from the orange peel as described under experimental parts. Its identity and purity were proven by ^{1}H and ^{13}C NMR spectroscopic analysis spectroscopic analysis and by comparison with the literature [23]. ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm) 11.87 (1H, br s, 5-OH), 9.36 (1H, br s, 3`-OH), 6.91-6.85 (3H, m, H-2`, H-5` and H-6`), 6.11 (1H, brs, H-6), 6.10 (1H, brs, H-8), 5.41 (1H, dd, J = 13.30, 3.20 Hz, H-2), 4.89 (1H, d, J = 7.40 Hz, H-1``), 4.52 (1H, s, H-1```), 3.72 (3H, s, 4-OCH_3), 3.41-3.52 (6H, m, H-2``- H-6``), 3.25 (1H, dd, J = 14.60, 8.76 Hz, H-3_{eq}), 3.12-3.23 (4H, m, H-2```- H-5```), 2.79 (1H, dd, J = 14.02 Hz & 3.6, H-3_{ax}), 1.04 (d, J

= 6.1, 3H, CH_3 of rhamnose).^{13}C NMR (100 MHz, DMSO) δ 197.46 (C-4), 165.56 (C-7), 163.47 (C-5), 162.92 (C-9), 148.42 (C-4`), 146.86 (C-3`), 131.29 (C-1`), 118.46 (C-6`), 114.55 (C-2`), 112.45 (C-5`), 103.77 (C-10), 101.00 (C-1```), 99.89 (C-1``), 96.84(C-6), 96.02 (C-8), 78.82 (C-2), 76.67 (C-3``), 75.95 (C-5``), 73.41 (C-2``), 72.52 (C-4```), 71.13 (C-3```), 70.71 (C-2```), 70.05 (C-4``), 68.74 (C-5```), 66.44 (C-6``), 56.12 (O-Me), 42.45 (C-3), 18.24 (C-6```).

3.2. Drug content and %EE

HSP was successfully entrapped into nanovesicular formulations, the %EE of all prepared nanovesicular formulations was about 98%, while the drug content of different HSP nanovesicles was ranged from 62.24±2.14 to 93.25±1.23% and the drug content of nanovesicles prepared with Olive oil was lower compared to that prepared without Olive oil, this may be attributed to the poor solubility of HSP in Olive oil as HSP (**Fig. 1** and **2**) is a polyphenolic compound has two sugar parts in its structure[37]. (**Table 1**) Shows the %EE and drug content of all developed nanovesicular formulations.

3.3. In vitro release analysis

For 48 hrs, *in-vitro* release analysis was performed. The %CDR of HSP from both HSP nanovesicular preparations was significantly higher *($p < 0.005$)* than what was derived from dispersion of free drug (**Fig. 3**). The % CDR of HSP from the free drug dispersion approached 9.23±1.13%.

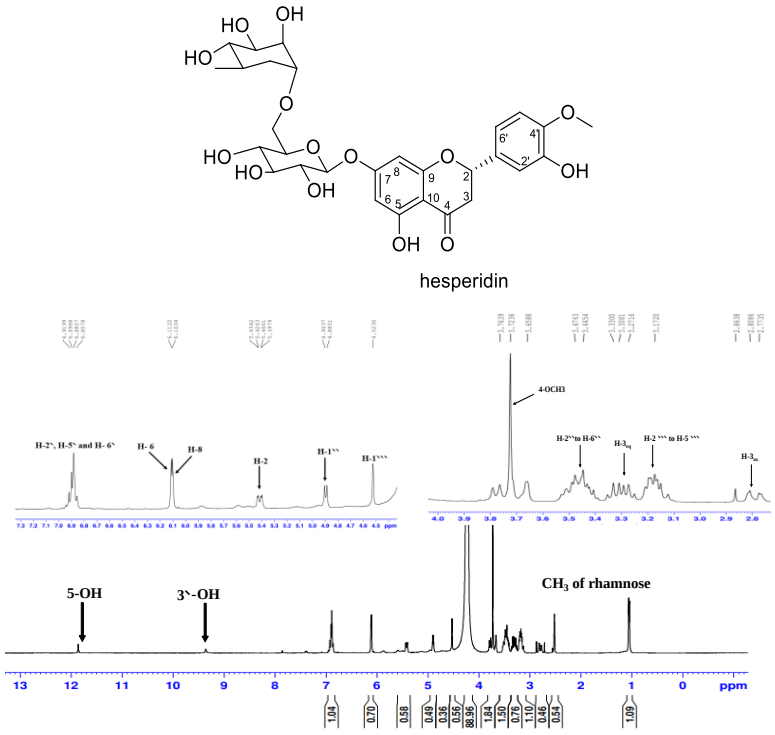

Figure 1. Structure of Hesperidin and 1H-NMR spectrum of hesperidin (DMSO-d6, 400 MHz)**.**

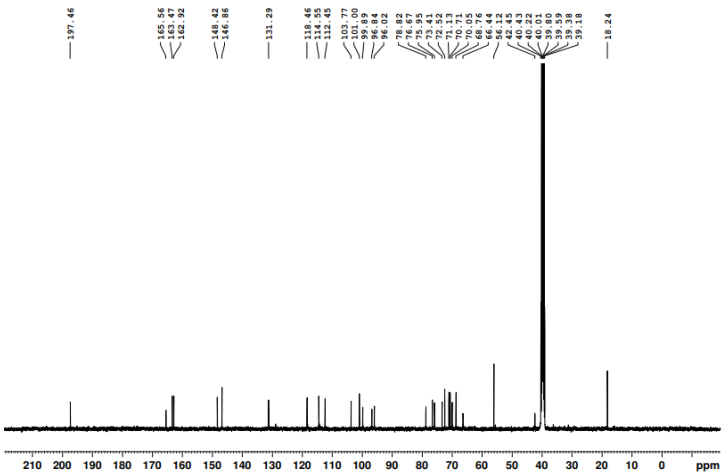

Figure 2. ^{13}C-NMR spectrum of hesperidin (DMSO-d_6, 100 MHz).

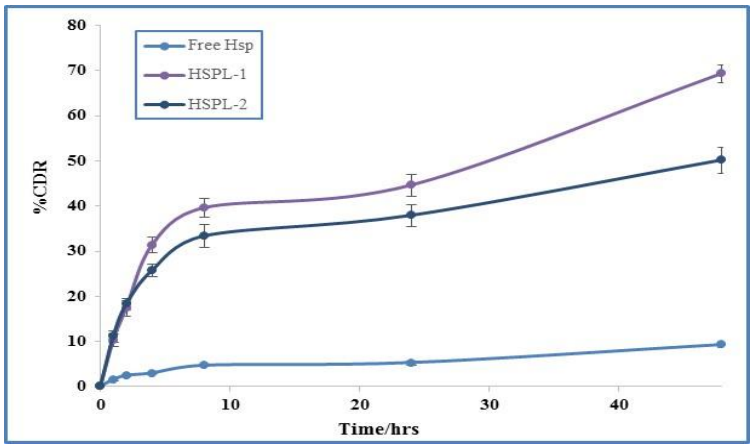

Figure 3. *In-vitro* release of HSP from different HSP nanovesicles and from free drug dispersion.

While that from HSPL-3 (prepared without Olive oil) came 69.289± 2.59 % while that from HSPL-6 (prepared with Olive oil) approached 50.15± 1.93 %. These results are attributed to the enhanced dissolution of HSP due to the effect of nanovesicles. These results are in concordance with our earlier research in which Quercetin nanovesicles had been developed, and the dissolution of Quercetin was significantly increased [25]. The %CDR of HSP from nanovesicular formulations prepared without Olive oil was higher than that prepared with Olive oil. The %CDR of HSP from HSPL-3 (62.28± 2.59) was significantly higher ($P<0.05$) than that from HSPL-6 (50.15± 1.93). Our results are in contrast with that obtained by Gul, U. et al., who found that Olive oil enhanced the permeability of terbinafine hydrochloride through its formulation into nanoemulsion [38], Olive oil concentration in their study was about 25- 30% v/v while in our study it is only 0.08% v/v additionally the solubility of terbinafine hydrochloride in Olive oil is about 82.42 ± 1.82 mg/ml. At the same time, in our case, HSP is a polyhydroxy flavonoid insoluble in Olive oil. Based on these results, HSPL-3 and HSPL-6 were selected for further

investigation. Kinetic analysis of release data was conducted to identify the drug release mechanism. Obtained data found out that the release of HSP from nanovesicles prepared with and without Olive oil exhibited a Higuchi diffusion pattern. The selected formulations' polydispersity index, zeta potential, and particle size are depicted in (**Table 2**).

Table 2. Zeta potential, Particle size, and polydispersity index of different nanovesicular formulas.

Formulae coding	**HSPL-3**	**HSPL-6**
Size of Particle (nm)	1549.66±128.72	642.66± 56.53
Polydispersity index	0.882± 0.203	0.829±0.206
Zeta potential	- 32.03± 2.12	- 17.76± 0.83

3.4. Differential scanning calorimetry (DSC)

HSP shows 2 endothermic peaks (**Fig. 4**), one at 88.77 ºC and the other at 263.4 ºC, in the physical mixture with span 65 (PM1) and in nanovesicular formulation prepared without olive oil (HSP-3), there is mild shift for both peaks while in the physical mixture of HSP with both span 65& olive oil (PM2), the peak at 263.4 ºC completely disappeared in addition to the appearance of an exothermic peak at 154 ºC in the thermogram of (HSPL-6) as a result of phase transition.

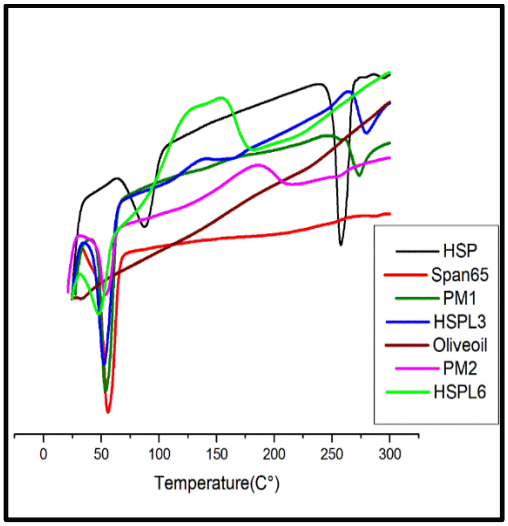

Figure 4. DSC thermograms of HSP, span 65, physical mixture of both (PM 1), HSPL-3 nanovesicles formulation (prepared without Olive oil), Olive oil, physical mixture of HSP, span 65, and Olive oil (PM 2) and HSPL 6 nanovesicles formulation (prepared with Olive oil).

3.5. FT-IR studies

For verifying the any chemical interaction between the ingredients, FT-IR studies were performed. FT-IR spectra of HSP, span 65, physical mixture of both (PM-1), nanovesicles formulation (HSPL-3), Olive oil, physical mixture of (HSP, span 65 and Olive oil (PM-2)) and nanovesicles formulation (HSPL-6) are depicted in (**Fig. 5**). HSP has characteristic (OH) peaks at 3547.23, 3473.72, and 3424.18 cm-1; however, in (PM-1, HSPL-3, PM-2, and HSP-6), these (OH) groups of HSP vanished, suggesting a type of interaction (hydrogen bonding) between HSP and span 65.

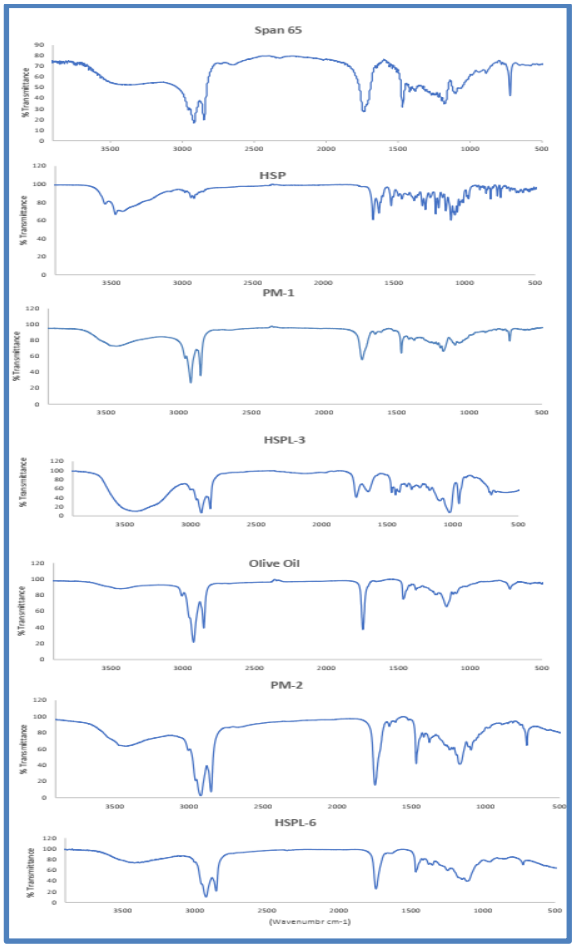

Figure 5. FTIR spectra of HSP, span 65, physical mixture of both (PM-1), nanovesicles formulation (HSPL-3), Olive oil, physical mixture of HSP, span 65 and Olive oil (PM-2) and nanovesicles formulation (HSPL-6).

3.6. Morphological studies

Photos obtained by SEM demonstrated that the prepared HSP nanovesicles prepared with and without Olive oil were spherical in shape as shown in (**Fig. 6**).

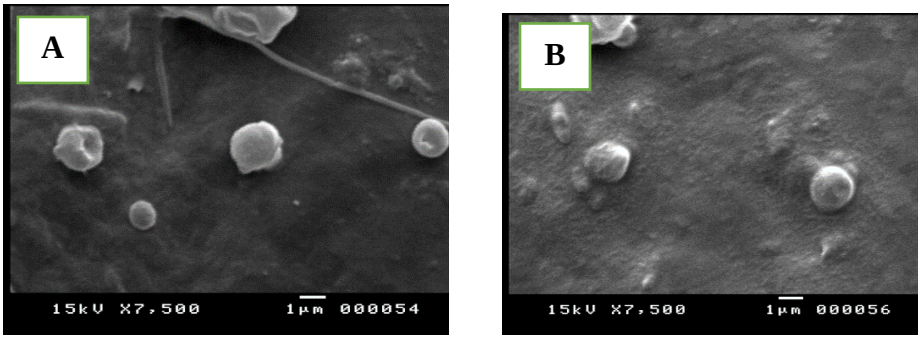

Figure 6. SEM photos of A) HSPL-3 (nanovesicular formulation) and B) HSPL-6 (nanovesicular formulation).

3.7. Clinical & histopathological studies

Table 3 shows the measurements of corneal opacity, corneal neovascularization, lacrimation, ulcer measurement, and presence of pus in all groups during the treatment period, as well as the statistical significance between groups during the treatment period. Wilcoxon t-test showed that there are insignificant differences between HD and HOD groups compared to the +ve control group in all items of comparison, while there is a significant decrease in ulcer measurement in both HD and HOD groups compared to the +ve control group in the period between week 1 and week 5 while Mann Whitney U test revealed that there is insignificant difference between HD and HOD groups in all items of comparison. Representative photos of rabbits' eyes during the treatment period with and without fluorescein staining for all groups are depicted in (**Fig. 7**).

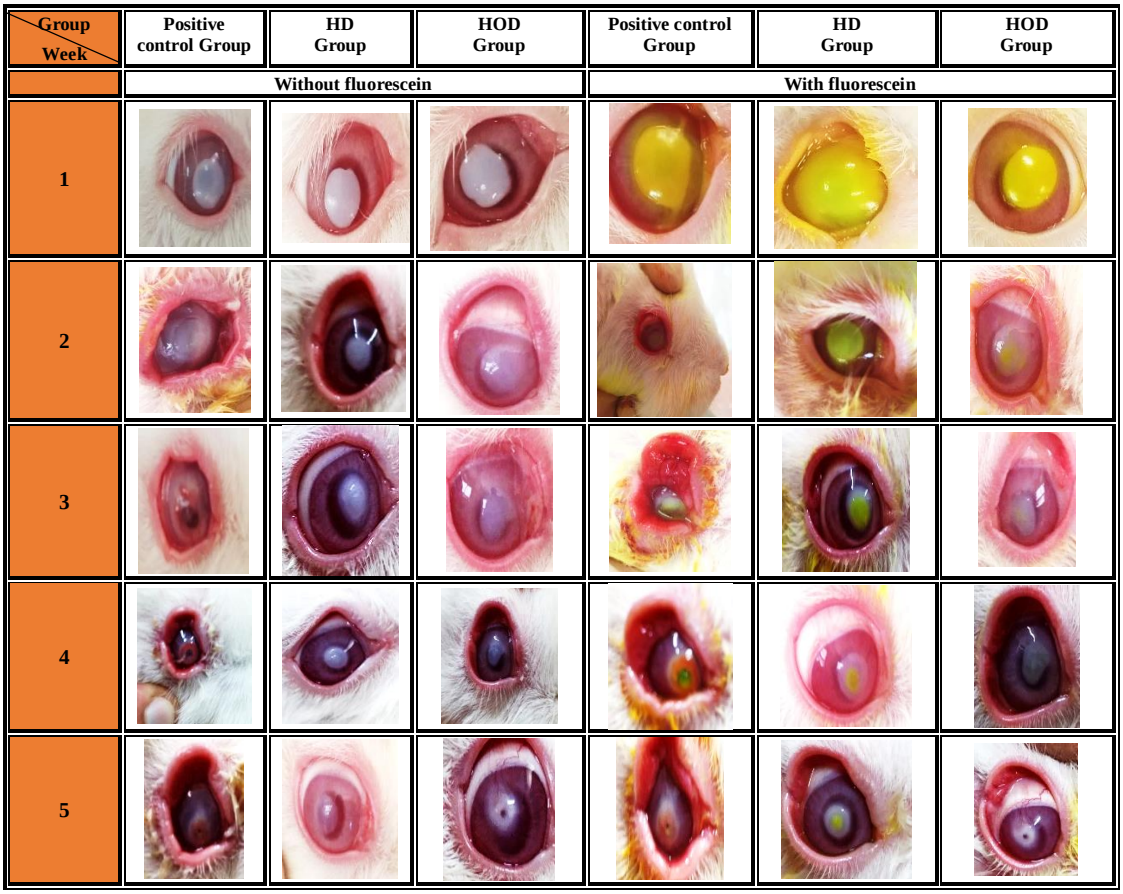

Figure 7. Eyes' photos of different groups during treatment period with and without fluorescein.

Table 3. Statistical analysis between different groups.

Item / Group	Control group Mean± SD	HD group Mean± SD	HOD group Mean± SD	P1	P2	P3
1- Corneal opacity						
Week 1	0.5±0.71	1.4±1.34	1.8±1.64	0.502	0.417	0.403
Week 2	2±1.41	0.6±1.34	1.2±0.84	0.217	0.130	0.417
Week 3	2.5±0.71	0.4±0.55	1.4±1.34	0.217	**0.042***	0.306
Week 4	2.5±0.71	0.2±0.45	1.4±1.34	0.125	**0.033***	0.306
Week 5	2.5±0.71	0.2±0.45	0.4±0.89	0.881	**0.033***	0.052
P. Value	0.180	0.109	0.102	--	--	--
2- Corneal neovascularization						
Week 1	0±0	0±0	0±0	1.000	1.000	1.000
Week 2	0±0	0±0	0.2±0.45	0.317	1.000	0.527
Week 3	2±1.41	0±0	0.6±1.34	0.317	**0.016***	0.130
Week 4	3±0	0±0	1±1.41	0.136	**0.014***	0.094
Week 5	3±0	0±0	1.4±0.89	**0.017***	**0.014***	**0.042***
P. Value	0.157	1.000	0.059	--	--	--
3- Lacrimation						
Week 1	0±0	0±0	0.8±1.1	0.134	**1.000**	**0.327**
Week 2	2±1.41	0±0	0.2±0.45	0.317	**0.016***	**0.052**
Week 3	3±0	0±0	0.4±0.89	0.317	**0.014***	**0.031***
Week 4	3±0	0±0	0.4±0.89	0.317	**0.014***	**0.031***
Week 5	3±0	0±0	0±0	1.000	**0.014***	**0.014***
P. Value	0.157	1.000	0.157	--	--	--

4- Ulcer measurement						
Week 1	5.55±2.76	7.82±3.55	9.5±6.07	0.465	**0.439**	**0.245**
Week 2	1.8±1.13	4.94±6.42	0.92±1.34	0.138	**0.558**	**0.324**
Week 3	5.9±0.57	6±5.44	0.76±0.87	0.071	**0.699**	**0.049***
Week 4	7.7±3.25	1.7±2.05	0±0	0.054	**0.051**	**0.016***
Week 5	6.95±0.64	1.1±1.62	1±1.41	1.000	**0.044***	**0.044***
P. Value	0.655	0.042*	0.043*	--	--	---
5- Presence of pus						
Week 1	0±0	0±0	0±0	1.000	**1.000**	**1.000**
Week 2	1.5±2.12	0±0	0±0	1.000	**0.114**	**0.114**
Week 3	2.5±0.71	0.2±0.45	0.2±0.45	1.000	**0.033***	**0.033***
Week 4	3±0	0±0	0±0	1.000	**0.014***	**0.014***
Week 5	3±0	0±0	0±0	1.000	**0.014***	**0.014***
P. Value	0.157	1.000	1.000	--	--	--

* Statistically significant difference ($p<0.05$), ** Highly statistically significant difference ($p<0.01$). P1: Comparison between HD group & HOD group. P2: Comparison between HD group & Control group. P3: Comparison between HOD group & Control group. P. Value: Comparison between week 1 & week 5 for each group.

Histopathological examination of the central part corneal stained with HE in the group of rabbits treated by dropping; the negative control group revealed normal corneal epithelium, stroma and descement layers (**Fig. 8-A**), positive control group revealed necrosis of the corneal epithelium, desquamation and multiple ulcerated areas with necrosis of the stroma (**Fig. 8-B**). HD group treated showed degenerated epithelium with no ulcers and normal stroma (**Fig. 8-C**).

HOD- treated group appeared to have slightly normal corneal epithelium with normal stroma descement layer (**Fig. 8-D**).

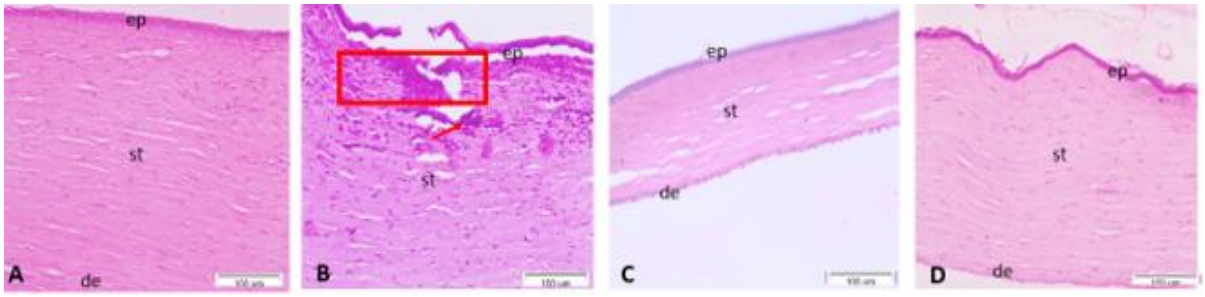

Figure 8. Representative micrograph of the cornea stained with HE stain. A) Negative control group showing normal epithelium (ep), normal stroma (st) and normal descement layer (de). B) Positive control group treated by dropping showing necrosed epithelium (ep), necrosed stroma (st), and large area of the ulcer (red rectangles). C) HD group showing degenerated epithelium with no ulcers (ep), normal stroma (st). D) HOD- treated group by dropping showing slightly normal epithelium (ep), normal stroma (st) and normal descement layer (de).

Scanning electron microscopical examination of the corneal samples in the + vie control group treated by dropping exhibited ulceration with the presence of leukocytes, red blood cells, colonies of bacteria and stroma showed of the collagenous fibers (**Fig.9-B**) which are not seen in the corneal samples of –ve control group (**Fig.9-A**).

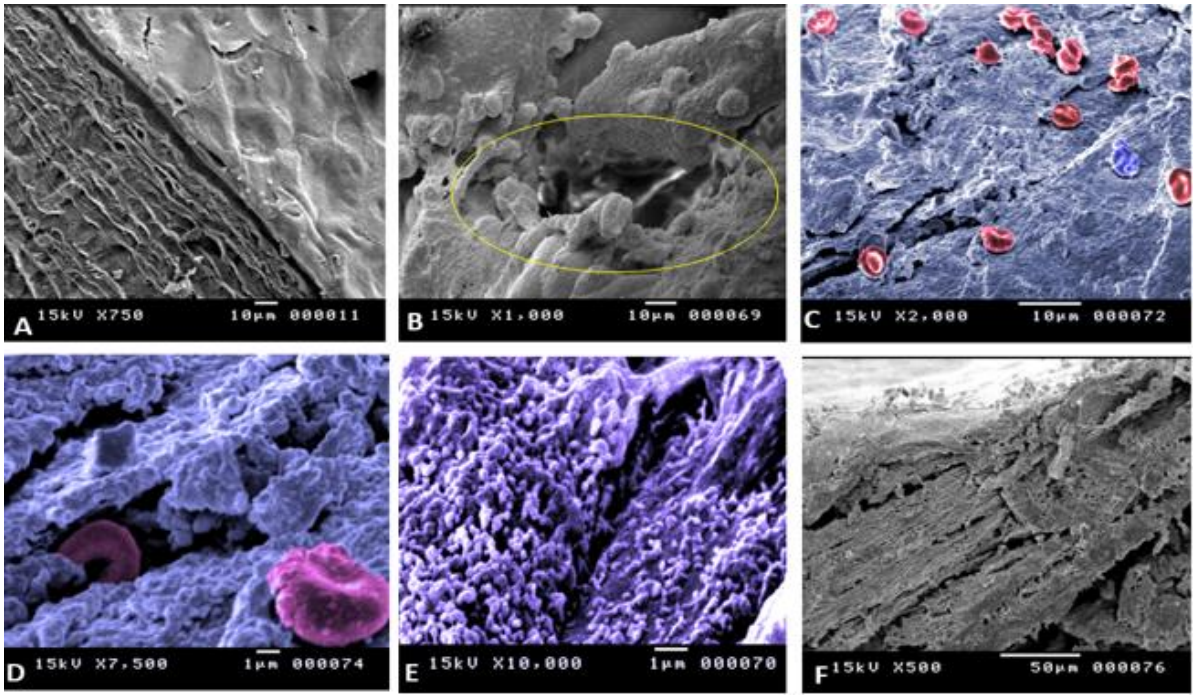

Figure 9. Scanning electron photos of the cornea in negative control group revealed normal surface of the corneal epithelium and normal stroma (A). Positive control group treated by dropping presented ulceration and denuded epithelium (B). Digitally colored Scanning electron micrograph showing ulcerated epithelium with presence of leukocytes (blue) and rbcs (red) (C). Digitally colored Scanning electron micrograph exhibiting red blood cells (red color) (D). Digitally colored Scanning electron micrograph showing colonies of bacteria (blue color) (E). Stroma with necrosis and destruction (F).

While HD treated group showed slightly normal appearance of the corneal surface with evidence of healed epithelium and stroma with no ulcers (**Figure 10; A, B**). The HOD treated group revealed a normal appearance of the corneal surface and stroma (**Figure 10; C, D**). These results pointed out the efficacy of HSP and the superiority of the combination between HSP and Olive oil in treating alkali burn-induced corneal ulcers. The effect of Olive oil addition was emphasized by the appearance of normal corneal epithelium with a normal stroma descement layer which was not observed in the case of treatment with HSP alone (HD group). These results are in accordance with that performed by Elwan and Kassab for eliciting the protective effect of HSP against Capecitabine Induced Corneal Toxicity in adult male albino Rat pointed out that the induced corneal changes by capecitabine administration could be improved by concurrent administration of HSP with Capecitabine. These findings could be attributed to the anti-apoptotic effect of HSP that could be due to the suppression of p53-proteins and also to its anti-inflammatory effect of HSP through its effect on the prostaglandin synthesis and the cyclooxygenase-2 (COX-2) pathway [39]. The appearance of normal corneal epithelium with normal stroma descement layer after the addition of Olive oil to HSP can be due to the

increased regeneration of epithelium as a result of the topical application of Olive oil [40]. Recent studies found that topical application of Olive oil resulted to angiogenesis by enhancing of intravascular endothelial growth factor (VEGF) levels. Much more, extra virgin Olive oil enhances the viability of cells by maximizing the capacity of antioxidants and providing higher MMP (mitochondrial membrane potential), which is vital for preserving the mitochondrial purpose of keratinocytes [41].

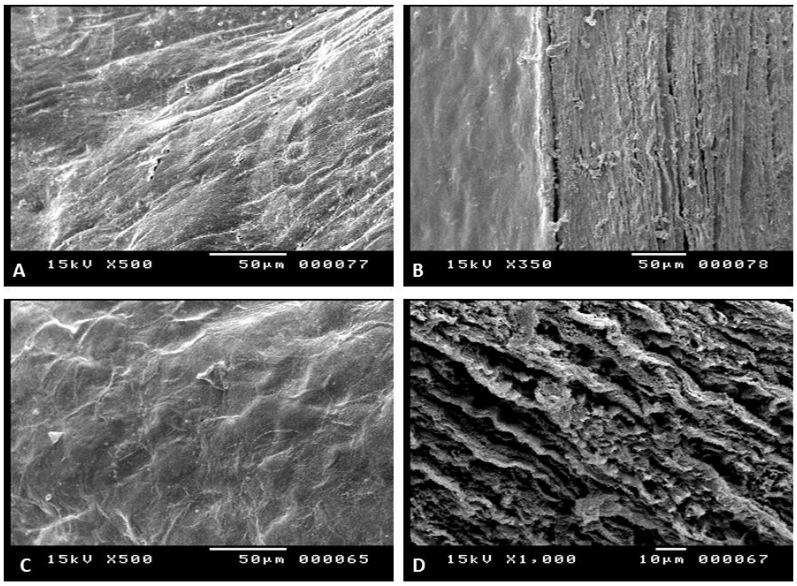

Figure 10. Scanning electron micrograph of the cornea in HSP and HSP Olive oil treated groups; HD treated group showing slightly normal appearance of the corneal surface and stroma with no ulcers (A, B). HOD treated group showing normal appearance of corneal surface and stroma (C, D).

The present work's aim is to evaluate the topical effect of HSP**,** and HSP olive oil on the healing of an experimentally induced corneal ulcer in rabbits. To our knowledge, this study is the first study in rabbits, that tried to investigate the healing process of the corneal ulcer after topical application of one of these treatments in different periods.

The results have shown that the topical application of HSP with olive oil gave the best results toward the management of corneal ulcers followed by the topical dropping of the HSP alone.

According the present findings of this study, it seemed that HSP had a role in the healing of the corneal ulcer. This suggestion is supported by da Silva et al., [42] (2019) who reported that the flavonoid HSP had a gastric healing activity in the ulcerated mucosa and they suggested that this effect is preferentially through the minimization of oxidative damage of the mucosa, due to decreasing of the migration of neutrophils and the strengthening of the protective barrier of mucus next to the mucosa. A similar result was obtained by Wang et al., [43] (2018) who recorded that HSP enhances healing in diabetic foot ulcers in rats. Our results revealed that corneal perforation occurred in one animal in the HSP group. It was on the second day of the study as a complication of ulcer induction. In this group presence of pus was not documented in all

animals from the third week till the end of the study. This effect may be attributed to the antibacterial effect of HSP. This explanation was supported in the study that was conducted by Köksal Karayıldırım [44] (2017). It had demonstrated that HSP possesses inhibitory effects against the tested bacterial strains, with MICs ranging from 128 to 8 µg/mL indicating that HSP microemulsion formulation significantly inhibits bacterial growth. Especially showed strong activity against E. Cole (8 µg/mL) and E. Facials (16 µg/mL) with a concentration equal to the standard antibiotic gentamicin. Better results were obtained when HSP was topically applied and dribbled in the eye.

Corneal neovascularization and lacrimation were low and disappeared in the last three weeks of the study. It may be the result of the synergistic anti-inflammatory effect between HSP and olive oil. This suggestion was supported by Puerta et al., (2000) [45] who stated that the topical application of specific minor compounds of virgin olive oil had anti-inflammatory effects in case of induced ear edema in rats. Wongwarawipat et al., (2017) [46] recorded that the consumption of extra virgin olive oil is associated with a reduction in inflammatory biomarkers and molecules implicated in atherosclerosis. As well as, Rosillo et al., (2014) [47] found that extra-virgin olive-oil polyphenol extract had anti-inflammatory effects which decreased joint edema and protected joints against cartilage alterations and bone erosion. In this group presence of pus was minimized and disappeared in the last week from all animals. It is ascribed to the anti-bacterial effect of HSP and olive oil. The antimicrobial effect of the latter was mentioned by Medina et al., (2007) [48]. The present work declared that the size of corneal ulcers decreased gradually to reach a minimum in the last week in the HO group. This finding could be attributed to the promoting effect of olive oil on epithelial regeneration. This regenerative potential effect of extra virgin olive oil phenolic compounds and their promotion of wound healing was achieved through the bio-stimulation of fibroblasts, augmenting their proliferative capacity and migration, increasing their expression of vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), insulin-like growth factor (IGF), Transforming growth factor beta 1 (TGFβ1), and exerting antimicrobial activity to reduce the bacterial load (Melguizo-Rodríguez et al., 2021) [49].

In the present study, the different ophthalmic signs (lacrimation, pus formation, opacity, ulcer size) appeared following induction of corneal ulcers increased in the 1st two weeks then gradually decreased. The inflammatory signs decreased gradually due to the anti-inflammatory effect of HSP, and the better regenerative effect is attributed to Olive oil. Martin et al., (1953) [50] and Salgado and Green (1956) [51] demonstrated that HSP decreases the inflammatory reaction when administered subcutaneously to rats. A similar result was obtained by Emim et al., [52] (2011) who found that HSP has an anti-inflammatory effect on dextran-induced paw edema in rats. On the other hand, Salgado & Green (1956) [51] found that HSP was ineffective after oral administration, but

effective after subcutaneous injection with no noticeable changes in rat behavior or apparent tissue damage, even after repeated administrations. When olive oil is added to HSP, normal corneal epithelium with normal stroma and Descemet's membrane can be seen due to increased epithelium regeneration [36]. This verified the synergistic effect that occurs when olive oil is added to HSP. Recent studies found that topical application of olive oil resulted in angiogenesis through the upregulation of intravascular endothelial growth factors (VEGF). Much more, extra virgin olive oil enhances the viability of cells by maximizing the capacity of antioxidants and providing higher MMP (mitochondrial membrane potential), that is vital for preserving the keratinocytes'mitochondrial function [38].

The histopathological findings in the topical HSP group showed degenerated epithelium with no ulcers and normal stroma. A slightly normal corneal epithelium with normal stroma and Descemet's membrane was observed in the use of HSP-olive oil combination. Microscopical examination of the cornea in the control positive group treated by dropping exhibited ulceration with the presence of leukocytes, red blood cells, and colonies of bacteria. These results pointed out the efficacy of HSP and the superiority of the combination between HSP and olive oil in the treating alkali burn-induced corneal ulcers. Olive oil's additive effect was emphasized by the appearance of normal corneal epithelium with a normal stroma but was not observed in the case of treatment with HSP alone. A previous study showed the protective effect of HSP in the corneal tissue against the detrimental effect of Capecitabine in adult male albino Rats. The results of that study revealed that the induced corneal changes by capecitabine administration could be improved by concurrent administration of HSP with Capecitabine. The authors of that study attributed these findings to the anti-apoptotic effect of HSP that may be due to the repression of p53-proteins and also to its anti-inflammatory effect through its influence on the prostaglandin synthesis and the cyclooxygenase-2 (COX-2) pathway [39].

Earlier in this year, we performed another study in which we have examined the subconjunctival injection of Oxytetracycline 5% effect on the rabbits induced alkali burn corneal ulcer [53] and found that, the subconjunctival injection of Oxytetracycline only prevented the bacterial colonization on the ulcerated corneal surface and failed to revert the ulcerated corneal surface to its normal condition while our present work represented a magic topical (no invasive procedures) solution for the treatment of corneal ulcer.

4. Conclusion

This work provides treatment of corneal ulcers from natural, safe, and easily accessible products. Olive oil-based Hesperidin nanovesicles are promising formulations for the treatment of corneal ulcers as a result of the anti-inflammatory, anti-apoptotic, antioxidant, angiogenetic, and antibacterial effects of the combination. Muchmore, our results explained the brilliant role of Olive oil in the treatment of Corneal ulcers. Future

studies are needed for clinical application in humans, much more this combination could be applied safely to wound healing processes in human.

Conflict of interest

The authors declare to have no conflict of interest.

References

[1] Ramani, C., et al., Surgical bacteriology and grading of corneal ulcers in dogs a retrospective study of 24 dogs. 2013. 5(1): p. 136-138.

[2] Singh, P., et al., Ocular chemical injuries and their management. 2013. 6(2): p. 83.

[3] Sharma, N., et al., Treatment of acute ocular chemical burns. Surv Ophthalmol, 2018. 63(2): p. 214-235.

[4] Said, D.G. and H.S.J.E. Dua, Chemical burns acid or alkali, what's the difference? 2020, Nature Publishing Group. p. 1299-1300.

[5] Pargament, J.M., J. Armenia, and J.A.J.C.i.d. Nerad, Physical and chemical injuries to eyes and eyelids. 2015. 33(2): p. 234-237.

[6] Dua, H.S., A.J. King, and A.J.B.J.o.O. Joseph, A new classification of ocular surface burns. 2001. 85(11): p. 1379-1383.

[7] Wagoner, M.D.J.S.o.o., Chemical injuries of the eye: current concepts in pathophysiology and therapy. 1997. 41(4): p. 275-313.

[8] Lin, A., et al., Management of ocular conditions in the burn unit: thermal and chemical burns and Stevens-Johnson syndrome/toxic epidermal necrolysis. 2011. 32(5): p. 547-560.

[9] Pfister, R., et al., Alkali-burned collagen produces a locomotory and metabolic stimulant to neutrophils. 1987. 28(2): p. 295-304.

[10] Pfister, R.R., et al., Alkali-degraded cornea generates a low molecular weight chemoattractant for polymorphonuclear leukocytes. 1993. 34(7): p. 2297-2304.

[11] Ahsan, S.M. and C.M.J.N. Rao, Condition responsive nanoparticles for managing infection and inflammation in keratitis. 2017. 9(28): p. 9946-9959.

[12] Le Bourlais, C., et al., Ophthalmic drug delivery systems—recent advances. 1998. 17(1): p. 33-58.

[13] Alharbi, W.S., K.M.J.J.o.D.D.S. Hosny, and Technology, Development and optimization of ocular in situ gels loaded with ciprofloxacin cubic liquid crystalline nanoparticles. 2020. 57: p. 101710.

[14] Yousry, C., et al., Nanoparticles as tool for enhanced ophthalmic delivery of vancomycin: a multidistrict-based microbiological study, solid lipid nanoparticles formulation and evaluation. 2016. 42(11): p. 1752-1762.

[15] Lou, J., et al., Optimization and evaluation of a thermoresponsive ophthalmic in situ gel containing curcumin-loaded albumin nanoparticles. 2014. 9: p. 2517.

[16] Bubonja-Sonje, M., J. Giacometti, and M.J.F.C. Abram, Antioxidant and antilisterial activity of olive oil, cocoa and rosemary extract polyphenols. 2011. 127(4): p. 1821-1827.

[17] Pannucci, E., et al., An hydroxytyrosol enriched extract from olive mill wastewaters exerts antioxidant activity and antimicrobial activity on Pseudomonas savastanoi pv. Savastanoi and Agrobacterium tumefaciens. 2021. 35(16): p. 2677-2684.

[18] Kakkar, S. And I.P.J.I.j.o.p. Kaur, Spanlastics—A novel nanovesicular carrier system for ocular delivery. 2011. 413(1-2): p. 202-210.

[19] Ali, A.U., et al., Serum levels of tumor necrosis factor (TNF) -α and vaginal gene expression of toll-like receptor 2 (TLR2) & microtubule-associated protein 1 light chain 3 (LC3) after treatment of vulvovaginal candidiasis with sertaconazole nitrate spanlastics. Journal of Drug Delivery Science and Technology, 2023: p. 105016.

[20] ElMeshad, A.N. and A.M.J.D.d. Mohsen, Enhanced corneal permeation and antimycotic activity of itraconazole against Candida albicans via a novel nanosystem vesicle. 2016. 23(7): p. 2115-2123.

[21] Liu, Y., et al., Cationized hyaluronic acid coated spanlastics for cyclosporine A ocular delivery: Prolonged ocular retention, enhanced corneal permeation and improved tear production. 2019. 565: p. 133-142.

[22] Khallaf, I., et al., IN VIVO EVALUATION OF ORANGE PEEL OIL AND ITS MAJOR COMPONENT HESPERIDIN AGAINST ENTERAL PHASE OF TRICHINELLA SPIRALIS.

[23] Selim, N.M., et al., Chrysophanol, physician, hesperidin and curcumin modulate the gene expression of pro-inflammatory mediators induced byaelps in hepg2: In silico and molecular studies. 2019. 8(9): p. 371.

[24] Ali, A.U., et al., Serum levels of tumor necrosis factor (TNF-α) and vaginal gene expression of toll-like receptor 2 (TLR2) & microtubule-associated protein 1 light chain 3 (LC3) after treatment of vulvovaginal candidiasis with sertaconazole nitrate spanlastics. Journal of Drug Delivery Science and Technology, 2023. 90: p. 105016.

[25] Ali, A.U., et al., Impact of quercetin spanlastics on living and caspase-9 expression in the treatment of psoriasis vulgaris. 2022: p. 103809.

[26] Sallam, N.M., et al., Impact of the mucoadhesive lyophilized wafer loaded with novel carvedilol nano-spanlastics on biochemical markers in the heart of spontaneously hypertensive rat models. 2021. 11(3): p. 1009-1036.

[27] Ali, A.U., et al., Impact of porous microsponges on minimizign theg myotoxic side effects of simvastatin. 2023. 13(1): p. 5790.

[28] Ali, A.U., et al., Performance of simvastatin microsponges as a local treatment for chronic periodontitis–Randomized clinical trial. 2021. 64: p. 102583.

[29] Stoppini, R. And B.C.J.E.o. Gilger, Equine ocular examination basic techniques. 2016: p. 1-39.

[30] Abdulrazaq, A.W., et al., Study the Effect of Ketamine, Xylazine and Their Combination on Liver and Kidney Features in Rabbits. 2021. 25(6): p. 6003-6008.

[31] Hosny, O.H., et al., Effect of Autologous Serum Derived from Advanced Platelet-rich Fibrin on the Healing of Experimentally-induced Corneal Ulcer in Donkeys (Equus asinus). 2022. 12(1): p. 73-85.

[32] Basu, S., et al., Simple limbal epithelial transplantation (SLET) in failed cultivated limbal epithelial transplantation (CLET) for unilateral chronic ocular burns. 2018. 102(12): p. 1640-1645.

[33] Fantes, F.E., et al., Wound healing after excimer laser keratomileusis (photorefractive keratectomy) in monkeys. 1990. 108(5): p. 665-675.

[34] Petroutsos, G. And Y.J.O.r. Pouliquen, Effect of ascorbic acid on ulceration in alkali-burned corneas. 1984. 16(4): p. 185-189.

[35] Banchroft, J., A. Stevens, and D. Turner, Theory and practice of histological techniques. 1996, Churchil Livingstone, New York, London, San Francisco, Tokyo.

[36] Bozzola, J.J. and L.D. Russell, Electron microscopy: principles and techniques for biologists. 1999: Jones & Bartlett Learning.

[37] Sansone, F., et al., Hesperidin gastroresistant microparticles by spray-drying: preparation, characterization, and dissolution profiles. 2009. 10(2): p. 391-401.

[38] Gul, U., et al., Olive oil and clove oil-based nanoemulsion for topical delivery of terbinafine hydrochloride: in vitro and ex vivo evaluation. 2022. 29(1): p. 600-612.

[39] Elwan, W.M. and A.A.J.E.J.o.H. Kassab, The potential protective role of hesperidin against capecitabine-induced corneal toxicity in adult male albino Rat. Light and electron microscopic study. 2017. 40(2): p. 201-215.

[40] Abooei Mehrizi, M., A. Eidi, and P.J.J.o.C.P. Mortazavi, Study of effect of olive oil on re-epithelialization of epithelial tissue in excision wound healing model in rats. 2016. 13(2): p. 1875-1884.

[41] Taheri, M., L.J.D.R. Amiri-Farahani, and Practice, Anti-Inflammatory and Restorative Effects of Olives in Topical Application. 2021. 2021.

[42] da Silva, L.M., et al., Hesperidin, a citrus flavanone glycoside, accelerates the gastric healing process of acetic acid-induced ulcer in rats. 2019. 308: p. 45-50.

[43] Wang, L., et al., Hesperidin enhances angiogenesis via modulating expression of growth and inflammatory factors in diabetic foot ulcer in rats. 2018. 16: p. 2058739218775255.

[44] Karayıldırım, Ç.K.J.C.B.U.J.o.S., Characterization, and in vitro evolution of antibacterial efficacy of novel hesperidin microemulsion. 2017. 13(4): p. 943-947.

[45] de la Puerta, R., E. Martínez-Domínguez, and V.J.Z.f.N.C. Ruíz-Gutiérrez, Effect of minor components of virgin olive oil on topical antiinflammatory assays. 2000. 55(9-10): p. 814-819.

[46] Wongwarawipat, T., et al., Olive oil-related anti-inflammatory effects on atherosclerosis: potential clinical implications. Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders), 2018. 18(1): p. 51-62.

[47] Rosillo, M.Á., et al., Anti-inflammatory and joint protective effects of extra-virgin olive-oil polyphenol extract in experimental arthritis. The Journal of nutritional biochemistry, 2014. 25(12): p. 1275-1281.

[48] Medina, E., et al., Antimicrobial activity of olive oil, vinegar, and various beverages against foodborne pathogens. 2007. 70(5): p. 1194-1199.

[49] Melguizo-Rodríguez, L., et al., Antimicrobial properties of olive oil phenolic compounds and their regenerative capacity towards fibroblast cells. 2021. 30(3): p. 372-378.

[50] Martin, G., R. Brendel, and J.J.A.i.d.p.e.d.t. Beiler, Effects of parenterally administered trypsin and phosphorylated hesperidin. 1953. 96(1): p. 124-129.

[51] Salgado, E. And D.J.J.o.A.P. Green, Action of bioflavonoids on inflammation. 1956. 8(6): p. 647-650.

[52] Emim, J.A.D.S., et al., Pharmacological evaluation of the anti-inflammatory activity of a citrus bioflavonoid, hesperidin, and the isoflavonoids, duartin and claussequinone, in rats and mice. 1994. 46(2): p. 118-122.

[53] Abdelhakiem, M.A., et al., Effect of the Subconjunctival Injection of Oxytetracycline 5% on the Induced Alkali Burn Corneal Ulcer in Rabbits. Journal of Advanced Veterinary Research, 2023. 13(1): p. 132-137.