Journal of Photochemistry & Photobiology, B: Biology 173 (2017) 618–625

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Pharmacological activities of an eye drop containing *Matricaria chamomilla* and *Euphrasia officinalis* extracts in UVB-induced oxidative stress and inflammation of human corneal cells

Elisabetta Bigagli, Lorenzo Cinci, Mario D'Ambrosio, Cristina Luceri*

Department of Neuroscience, Psychology, Drug Research and Child Health, NEUROFARBA, Section of Pharmacology and Toxicology, University of Florence, Viale G. Pieraccini 6, Florence, Italy

ARTICLE INFO

Keywords:
UVB
Cornea
Oxidative stress
Inflammation
Matricaria chamomilla
Euphrasia officinalis

ABSTRACT

Ultraviolet B (UVB) exposure is a risk factor for corneal damage resulting in oxidative stress, inflammation and cell death. The aim of this study was to investigate the potential protective effects of a commercial eye drop (Dacriovis™) containing *Matricaria chamomilla* and *Euphrasia officinalis* extracts on human corneal epithelial cells (HCEC-12) against UVB radiation-induced oxidative stress and inflammation as well as the underlying mechanisms. The antioxidant potential of the eye drops was evaluated by measuring the ferric reducing antioxidant power and the total phenolic content by Folin-Ciocalteu reagent. HCEC-12 cells were exposed to UVB radiation and treated with the eye drops at various concentrations. Cell viability, wound healing assay, reactive oxygen species (ROS) levels, protein and lipid oxidative damage and COX-2, IL-1β, iNOS, SOD-2, HO-1 and GSS gene expression, were assessed. Eye drops were able to protect corneal epithelial cells from UVB-induced cell death and ameliorated the wound healing; the eye drops exhibited a strong antioxidant activity, decreasing ROS levels and protein and lipid oxidative damage. Eye drops also exerted anti-inflammatory activities by decreasing COX-2, IL-1β, iNOS expression, counteracted UVB-induced GSS and SOD-2 expression and restored HO-1 expression to control levels. These findings suggest that an eye drop containing *Matricaria chamomilla* and *Euphrasia officinalis* extracts exerts positive effects against UVB induced oxidative stress and inflammation and may be useful in protecting corneal epithelial cells from UVB exposure.

1. Introduction

Ultraviolet (UV) light is the most common cause of radiation injury to the eye [1]. The cornea is a transparent avascular tissue that under normal conditions, absorbs the majority of UVB rays, protecting the inner eye against UVB-induced damaging effects [2].

UVB exposure is a well-documented environmental stressor that leads to the generation of free radicals. Cellular redox homeostasis is normally maintained by a delicate balance between reactive oxygen species (ROS) generation and antioxidant defenses but when this balance is disrupted, the overproduction of ROS elicits DNA, protein and lipid oxidative damage and induces pro-inflammatory cytokines, leading to UVB-mediated corneal inflammation [2–4].

Excessive exposure to UVB radiation is associated to numerous eye diseases, including photo-keratitis, pterygium, damage to the epithelium, edema and apoptosis of corneal cells [5,6]. ROS also play a role in the pathogenesis of glaucoma, stimulating apoptotic and inflammatory pathways [4].

Chamomile, *Matricaria chamomilla* L., a member of the Asteraceae family, is one of the most common herbs used worldwide for a variety of medicinal purposes. Traditional uses include the treatment of inflammation, ulcers, wounds, gastrointestinal disorders, stomachache, pharyngitis, skin irritation and rheumatic pain [7]. Chamomile has moderate antioxidant and antimicrobial activities and significant antiplatelet activity in vitro [8]. Animal model studies indicate potent anti-inflammatory actions, some anti-mutagenic and cholesterol-lowering activities, as well as anti-spasmodic and anxiolytic effects [8]. On the basis of its long-standing use, the Committee on Herbal Medicinal Products indicated that *Matricaria chamomilla* L. medicines can be used in the management of various inflammatory diseases of the gastro-

* Corresponding author.
E-mail address: cristina.luceri@unifi.it (C. Luceri).

http://dx.doi.org/10.1016/j.jphotobiol.2017.06.031
Received 28 March 2017; Received in revised form 19 June 2017; Accepted 23 June 2017
Available online 24 June 2017

intestinal tract or of the oral cavity (http://www.ema.europa.eu/).

The best known bioactive constituents of chamomile include apigenin, luteolin, quercetin, α-bisabolol, α-bisabolol oxides A and B and matricin (usually converted to chamazulene). Chamomile also contains high levels of coumarins, hydroxycoumarins, terpenoids and is one of the richest sources of dietary antioxidants [7–11].

Eyebright, *Euphrasia officinalis* L., is a medicinal plant traditionally used as eyewash to treat and prevent eye disorders such as conjunctivitis, blepharitis, eye fatigue, purulent ocular inflammation, and sties [12]. Eye drops made from *Euphrasia officinalis* L. are also useful and safe in the treatment of ocular allergy symptoms [13].

Pharmacological investigations of eyebright extracts revealed various biological activities, including anti-inflammatory [14] and antimicrobial [15]. *Euphrasia officinalis* L. extracts decreased pro-inflammatory cytokine expression (IL-1β, IL-6 and TNF-α) in human corneal cells [14] and improved inflammatory conjunctivitis in a clinical trial [16]. Its biological activity is mainly due to the presence of highly active compounds such as iridoids, flavonoids, phenolic acids or etheric oils [14]. Collectively, the use of *Euphrasia officinalis* and *Matricaria chamomilla* preparations for the treatment of eye disorders is mainly based on a long standing tradition but to date, the claimed ocular effects needs further investigation.

The aim of the present study was therefore to explore whether a commercial eye drop containing *Matricaria chamomilla* and *Euphrasia officinalis* extracts, exerts protective effects against UVB induced oxidative damage and inflammation in human corneal epithelial cells and the involved mechanisms.

2. Materials and Methods

2.1. Eye Drops

Eye drops (Dacriovis™, Steve Jones, Sesto Fiorentino, Italy) contains 0.1% of *Matricaria chamomilla* fluid extract, 0.1% of *Euphrasia officinalis* herbal extract and disodium edetate isotonic solution at pH 7.2. Eye drops were tested at concentrations ranging from 0.5% to 5%, corresponding to herbal extract final concentrations between 0.005 and 0.0005%. Reagents, if not otherwise stated, were purchased from Sigma-Aldrich, Milan, Italy).

2.2. In Vitro Antioxidant Assay

The ferric-reducing antioxidant power (FRAP) of the eye drops was performed according to Lodovici et al. [17] with few modifications. Briefly, a FRAP reagent solution was freshly prepared by mixing 300 mM acetate buffer, pH 3.6, TPTZ solution (10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl), and 20 mM $FeCl_3 \cdot 6H_2O$ in a ratio of 10v:1v:1v. To perform the assay, 0.9 mL of FRAP reagent, 90 μL of distilled water, and 30 μL of sample were mixed and incubated at 37 °C for 30 min. The absorbance was measured at 595 nm. The antioxidant potential of samples was determined from a standard curve plotted using the $FeSO_4 \cdot 7H_2O$ linear regression.

2.3. Total Phenolic Content

Total phenolic content in the extracted samples was determined as described elsewhere [18] with minor modifications. Briefly, sample extracts (0.5 mg/mL, 60 μL) and Folin-Ciocalteu's reagent (60 μL) were mixed and incubated at room temperature for 5 min. After incubation, 60 μL of Na_2CO_3 (10% w/v) was added and the reaction mixture was further incubated for 60 min at room temperature in the dark. After incubation, the absorbance was measured at 760 nm. Gallic acid was used as standard and phenolic content expressed as mg gallic acid equivalents (GAE)/mL of eye drop.

2.4. Cells, UVB Light and Treatments

Human corneal epithelial cells (HCEC-12, kindly gifted by Prof. Chiarugi, University of Florence, [19]) were cultured under standard conditions. Briefly, the cells were maintained at 37 °C in a humidified incubator in an atmosphere of 5% CO_2 to 95% air and passaged at a 1:3 ratio with trypsin, every 5 to 7 days. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 10% fetal bovine serum, and antibiotics. In indicated experiments, cells (2×10^5) were seeded into 6-well culture dishes and allowed to grow 3–4 days until approximately 90% confluent. Preliminary experiments were performed to identify the suitable UVB dose able to induce a significant ROS production with no cytotoxic effects, within 2 h post irradiation. Cells were thus exposed to UVB (5–20 mJ/cm^2) in a Bio-link BLX apparatus (Vilber Lourmat, Cedex, France) covered with a thin layer (100 μL) of phosphate-buffered saline (PBS); PBS was then removed and the cells were refed with medium. Where indicated, the cells were treated with eye drops or vehicle alone. In preliminary experiments, eye drops were also tested for cytotoxicity and applied in four different modalities: 1) pre-treatment for 1 h before UVB irradiation; 2) treatment in continuum, starting 1 h before UVB irradiation, during irradiation and 1 h post irradiation; 3) treatment only during UVB irradiation and 4) treatment only post-irradiation for 1 h.

2.5. Cell Viability

In preliminary experiments, cell viability after UV-irradiation or eye drops treatments was assessed as described in Cinci et al. [20], by the colorimetric method based on [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (Promega Corporation, WI). The optical density of the chromogenic product was measured at 490 nm.

To determine the effect of eye drops on cell viability in UVB exposed HCEC-12 cells, we used the method described by Black et al. [21] by measuring protein content in cells exposed to eye drops at concentrations ranging from 0.5 and 5%, irradiated with UVB 20 mJ/cm^2.

Protein content was estimated by using the Bio-Rad DC protein assay kit (Bio-Rad, Segrate, Milan, Italy).

2.6. Measurement of ROS Production

Intracellular hydrogen peroxide production was measured using 2′,7′-dichlorofluorescein diacetate DCFH-DA (Calbiochem, San Diego, CA, USA). Briefly, the cells were exposed to UVB light (5, 10 and 20 mJ/cm^2) alone, or to 20 mJ/cm^2 and treated with eye drops in the four modalities described above. 100 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was then added to the culture medium at 37 °C for 1 h in the darkness. After washing with PBS for three times, ROS levels were determined by measuring the fluorescent intensity at excitation wavelength 485 nm and emission wavelength 535 nm using a Perkin Elmer Wallac 1420 Victor3 Multilabel Counter.

2.7. Protein Oxidative Damage (Carbonyl Residue Assay)

Carbonyl residues were determined according to Manni et al. [22] following the method of Correa-Salde and Albesa [23] with few

modifications. Cell lysates (150 μL) were treated for 1 h with 800 μL of 0.1% dinitrophenylhydrazine in 2 M HCl and precipitated with 1 mL of 20% trichloroacetic acid before being centrifuged for 10 min at 4 °C at 10,000 × *g*. The pellets were extracted with 500 μL of ethanol:ethyl acetate mixture (1:1) three times, centrifuged for 10 min at 4 °C at 10,000 × *g* and then dissolved in 100 μL of 6 M guanidine HCl in 20 mM potassium phosphate buffer (PBS) pH 7.5. The solutions were incubated at 37 °C for 30 min and insoluble debris was removed by centrifugation. The absorbance was measured at 370 nm. Carbonyl content was calculated using a molar absorption coefficient of 22,000 M^{-1} cm^{-1}. Carbonyl residue assay was performed in cell lysates collected after 2 and 24 h post irradiation.

2.8. Lipid Oxidative Damage (Thiobarbituric Acid Reactive Substances Assay)

Thiobarbituric acid reactive substances (TBARS) were evaluated as an index of lipid peroxidation. Cells were lysate in 300 μL RIPA buffer and then 150 μL were added to 800 μL reaction mixture consisting of 36 mM thiobarbituric acid, 20% CH_3COOH, and 20 μL 8% sodium dodecyl sulfate, and pH was adjusted to 4.0 with NaOH. The mixture was heated for 60 min at 100 °C, and the reaction was stopped by placing the vials in an ice bath for 10 min. After centrifugation (at 1600 × g at 4 °C for 10 min), the absorbance of the supernatant was measured at 532 nm (Perkin Elmer Wallac 1420 Victor3 Multilabel Counter). TBARS were quantified in nmol/mg of proteins using 1-methoxypropane as standard for calibration curve. TBARS were performed in cell lysates collected after 2 and 24 h post irradiation.

2.9. Wound Healing Assay

HCEC-12 cells were grown to reach confluence in 6-well tissue culture plates and were wounded with a sterile 20 μL pipet tip to remove cells by a linear scrape. The debris of damaged cells were removed by washing, and the cells were refed with DMEM in the presence. The progression of migration was photographed immediately and 24–48 h after wounding with an inverted microscope equipped with a digital camera (Nikon digital Sight DS-5M). The extent of healing is defined as the ratio of the area difference between the original wound and the remaining wound 24 h after injury compared with that of the original wound. The wound area was determined by the number of pixels in histogram (freely available ImageJ NIH software).

2.10. RT-PCR

Total RNA was extracted from cell lysates using the Nucleo Spin® RNA kit (Macherey-Nagel) according to manufacturer's instructions. For first-strand cDNA synthesis, 1 μg of total RNA from each sample was reverse-transcribed. Primers were designed on the basis of the human GenBank sequences for cyclooxygenase-2 (COX-2), inducible NO synthase (iNOS), IL-1β, SOD-2, HMOX, GSS. GAPDH was co-amplified as the reference [24].

2.11. Immunocytofluorescence for COX-2 Protein Expression

HCEC-12 cells were grown in poly-D-lysine-coated glass dishes for 24 h, then irradiated with UVB (20 mJ/cm^2) and treated with eye drops in continuum at 0.5–1–3–5% for 24 h. Cells were then fixed with cold 4% (w/v) paraformaldehyde for 20 min, rehydrated in PBS for 15 min, and permeabilized in 0.1% (w/v) TritonX-100 at RT for 10 min. After being washed with PBS, the cells were blocked for unspecific fluorescence with 3% BSA for 1 h and then incubated with Rabbit anti-COX2 polyclonal antibody (1:200) (Cayman, Ann Arbor, MI, USA) at 4 °C overnight followed by the fluorescent secondary antibodies: AlexaFluor 488 goat anti-rabbit (1:333) (Invitrogen, Carlsbad, CA, USA). Cells were also counterstained with DAPI dye to show the nuclear morphology.

2.12. Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Newman-Keuls post hoc test when normally distributed or using the non-parametric Kruskal–Wallis with Dunn's post hoc test and expressed as means ± standard error (SEM) of three independent experiments. All analyses were carried out using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). p value of 0.05 was considered significant.

3. Results

3.1. Antioxidant Power and Total Phenolic Content of eye Drops Containing Matricaria chamomilla and Euphrasia officinalis Extracts

FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine [Fe^{3+}-TPTZ] complex, producing a colored ferrous tripyridyltriazine [Fe^{2+}-TPTZ]. The FRAP value of eye drops was 3 mM. The total phenolic content of the eye drops was 1.1 mg/mL, gallic acid equivalent.

3.2. Effect of the eye Drops on Cell Viability in UVB Exposed Human Corneal Cells

Preliminary experiments were conducted to select the most suitable experimental conditions; UVB 5–10–20 mJ/cm^2 were firstly tested for HCEC-12 viability after very short term irradiation (2 h). None of the tested UVB doses affected cell viability (Fig. 1A). Short term potential intrinsic cytotoxic effect of the eye drops were also evaluated in non-irradiated HCEC-12 cells. As it is shown in Fig. 1B, none of the tested concentration, affected cell viability after 2 h of treatment and concentrations of 1 and 3% even showed a trend toward increased cell viability. The potential protective effects of the eye drops in UVB treated cells were also assessed by measuring total protein content of the cultures at 24 h post irradiation, an indirect method to assess cell viability [21]. The results in Fig. 1C show that UVB significantly reduced cell viability after 24 h post irradiation and treating cells with the eye drops exerted a significant, concentration-dependent protective effect.

Gene	Primer forward	Primer reverse	Base pair
GAPDH	CCCTCAAGGGCATCCTGGGCT	GCAGGGACTCCCCAGCAGTGA	275
SOD2	GCATCAGCGGTAGCACCA	CCGTTAGGGCTGAGGTTTGT	355
INOS	CAGACAGTGCGCCTGGAGGC	GGACCCTGGCTATCTCGGGT	419
COX2	TTGCCCGACTCCCTTGGGTGT	CCTCCTGCCCCACAGCAAACC	398
IL1β	GGACAAGCTGAGGAAGATGC	TCTTTCAACACGCAGGACAG	360
GSS	GCTGTGCAGATGGACTTCAA	CATAGAGCTCCCAGGCTTTG	400
HO-1	GGCTGCCCTGGAGCAGGAC	AGGTCACCCAGGTAGCGGGT	165

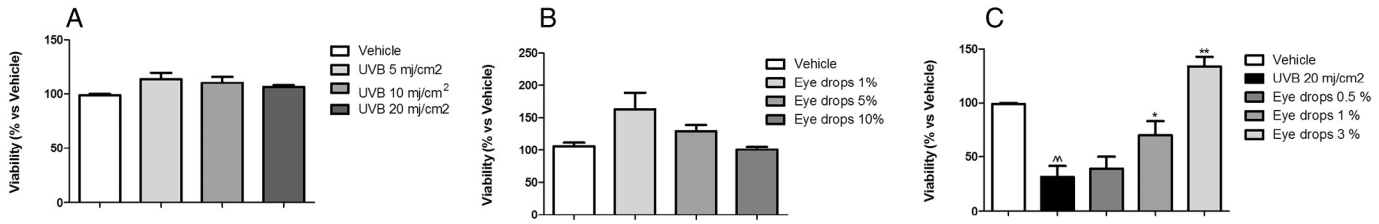

Fig. 1. Panel A: cell viability measure by MTS assay, of HCEC12 cells exposed to UVB 5–10–20 mJ/cm^2 after 2 h from exposure. Panel B: cell viability, measures by MTS assay, of HCEC12 cells exposed to different eye drops concentrations (1–5–10%) after 2 h from exposure. Panel C: cell viability, measured as the percentage of total protein content vs that of the vehicle, of HCEC12 cells exposed to UVB 20 mJ/cm^2 and exposed to different eye drops concentrations (0.5–1–3%) after 24 h. ˆˆ($p < 0.01$ vs vehicle); *($p < 0.05$ vs UVB-irradiated cells; **($p < 0.01$ vs UVB-irradiated cells). Data are expressed as mean ± SE of three independent experiments.

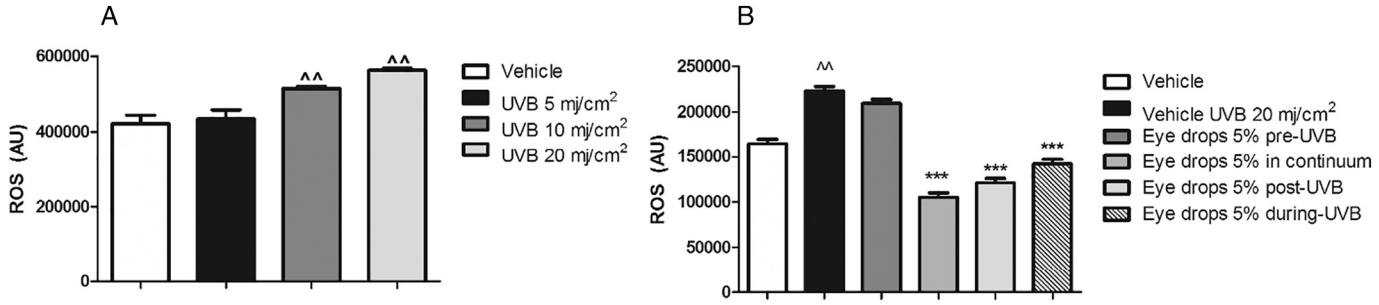

Fig. 2. Panel A: levels of intracellular ROS generated in UVB-irradiated HCEC12 cells compared with control after 2 h from irradiation. Panel B: eye drops-treated UVB-irradiated cells in four different modalities: 1) pre-treatment for 1 h before UVB irradiation; 2) treatment in continuum starting 1 h before UVB irradiation, during irradiation and 1 h post irradiation; 3) treatment only during UVB irradiation and 4) treatment only post-irradiation for 1 h. ˆˆ($p < 0.01$ vs vehicle); ***($p < 0.001$ vs UVB-irradiated cells). Data are expressed as mean ± SE of three independent experiments.

3.3. Effect of the Eye Drops on ROS Levels in UVB Exposed Human Corneal Cells

We firstly determined the UVB dose able to cause oxidative stress in corneal epithelial cells by quantifying its ability to generate intracellular ROS. UVB (5–20 mJ/cm^2) treatment resulted in a dose-dependent increase in ROS production (Fig. 2A); a significant reduction was observed when eye drops 5% was applied as follows: 1) during UVB irradiation ($p < 0.001$), 2) in continuum ($p < 0.001$) and 3) post UVB treatment ($p < 0.001$). No effect was observed when eye drops were applied only before UVB treatment. A 2.1 fold decrease in ROS production was evident after treatment with eye drops 5% in continuum and for this reason, this modality of treatment was chosen for further experiments Fig. 2B.

3.4. Effect of the Eye Drops on Wound Healing

Fig. 3 shows cell migration toward the center of a scratch wound in cultured HCEC-12 cells after 24 h. Eye drops 1% enhanced wound closure (150% of wound coverage) compared to the vehicle. At 48 h, no differences were observed (data not shown).

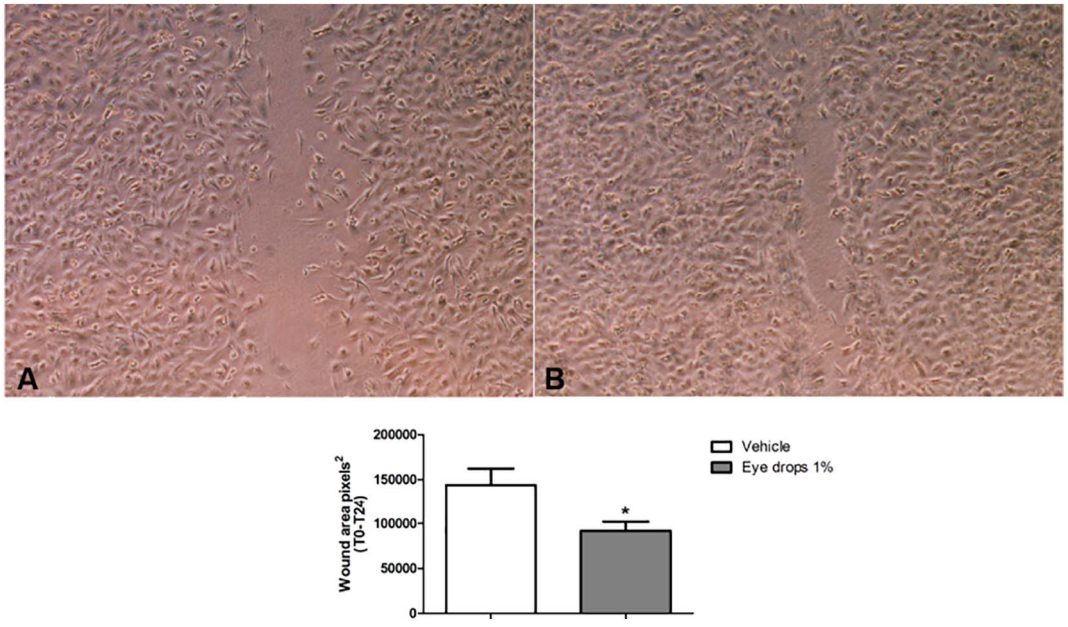

Fig. 3. Representative photomicrographs showing the healing of scratch wound in cultured human corneal epithelial cells. Panel A wound in HCEC-12 cells monolayer in the presence of vehicle and, panel B, in the presence of eye drops 1% for 24 h (day 1). Panel below, quantitative results are shown as wound area ± SE of three samples (*$p < 0.05$).

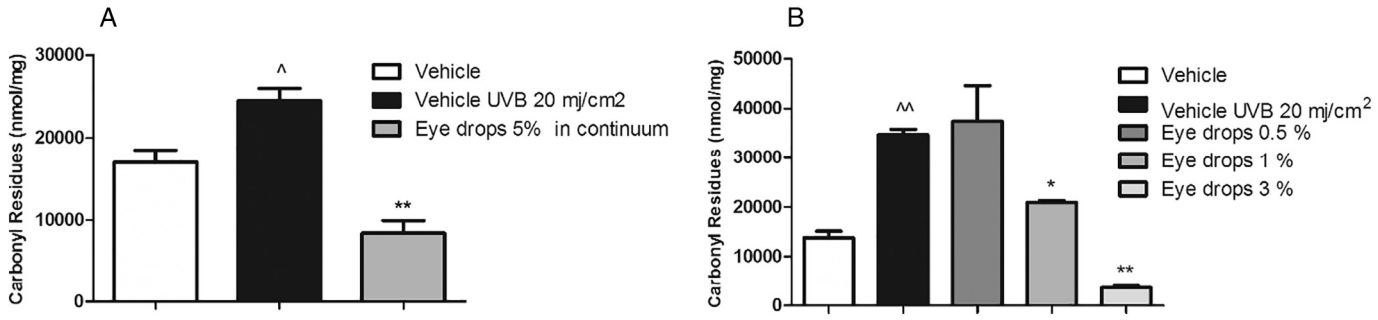

Fig. 4. Panel A: effect of eye drops on corneal cells protein carbonyl residues after 2 h from UVB. Panel B: effect of eye drops at different concentrations (0.5–3%) on corneal cells protein carbonyl levels after 24 h from UVB. ˆ($p < 0.05$ vs vehicle); ¨($p < 0.01$ vs vehicle); *$p < 0.05$ vs UVB-irradiated cells **$p < 0.01$ vs UVB-irradiated cells. Data are expressed as mean ± SE of three independent experiments.

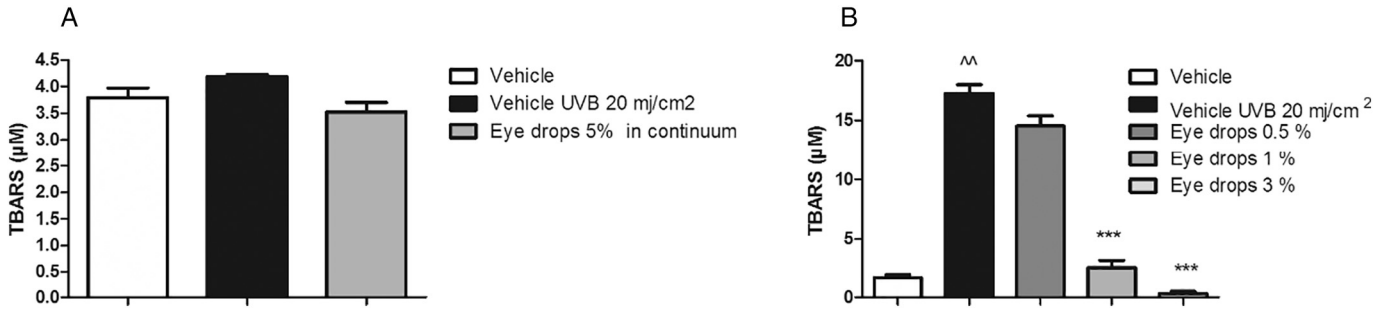

Fig. 5. Panel A: effect of eye drops on corneal cells thiobarbituric acid-reactive substances (TBARS) after 2 h from UVB. Panel B: Effect of eye drops in different concentrations (0.5–3%) on corneal cells TBARS after 24 h from UVB. ¨($p < 0.01$ vs vehicle); ***($p < 0.001$ vs UVB-irradiated cells). Data are expressed as mean ± SE of three independent experiments.

3.5. Effect of the Eye Drops on Carbonyl Residues in UVB Exposed Human Corneal Cells

The protein carbonyl levels were significantly higher in HCEC-12 cells treated with UVB 20 mJ/cm^2 than in the control group ($p < 0.05$) already after 2 h. In contrast, eye drops 5% treated cells showed a significantly decreased the percentage of UVB-induced oxidative damage of the proteins, by 66%, compared to the UVB-treated cells ($p < 0.01$), (Fig. 4A). Carbonyl residues were significantly diminished in cells treated with eye drops 1 and 3% after 24 h post UVB irradiation Fig. 4B.

3.6. Effect of the Eye Drops on Thiobarbituric Acid-Reactive Substances (TBARS) in UVB Exposed Human Corneal Cells

After 2 h post UVB (20 mJ/cm^2) irradiation we did not detect any significant increase in the TBARS levels (Fig. 5A); however, after 24 h, TBARS levels in UVB-treated cells were significantly higher (17.25 ± 0.75 nmol/mg protein) than those in the control group (1.75 ± 0.25 nmol/mg protein), ($p < 0.01$), (Fig. 5B). TBARS in the eye drops treated cells were 2.6 ± 0.6 and 0.35 ± 0.15 nmol/mg protein at 1% and 3%, respectively, significantly lower than those in the UVB-treated group ($p < 0.001$) and even below those of the control cells. Eye drops 0.5% was not effective (Fig. 5B).

3.7. Effect of the Eye Drops on Inflammatory and Antioxidant Gene Expression in UVB Exposed Human Corneal Cells

UVB light did not significantly induced the expression of COX-2 compared to not irradiated cells, however, eye drops 3% halved its expression compared to UVB treated cells ($p < 0.05$) (Fig. 6A). UVB caused a significant increase in IL-1β mRNA and eye drops 3% significantly abolished this effect (Fig. 6B). The slight increase of iNOS observed in UVB treated cells was counteracted by eye drops 3% ($p < 0.05$) (Fig. 6C). The expression for HO-1 in UVB treated corneal epithelial cells was reduced by 50% and eye drops 3% completely restored it at levels similar to those of control cells (Fig. 6E). On the contrary, SOD-2 and GSS expression was significantly induced by UVB compared to not irradiated cells and restored to control levels by eye drops 3% (Fig. 6D, F, Fig. 7). The effect of eye drops on COX-2 protein expression was also confirmed by immunocytochemistry. As shown in Fig. 7, eye drops 0.5, 1 and 3% were able to significantly and concentration dependently attenuate COX-2 protein expression compared to UVB irradiated cells ($p < 0.001$).

4. Discussion

Excessive exposure to UVB radiation is a risk factor for corneal diseases including photo-keratitis, epithelial damage and edema [3].

One of the major causes of ocular damage induced by UVB irradiation is the generation of ROS which mediate oxidative damage in the form of DNA modifications, lipid peroxidation and protein oxidation [25].

We found that UVB light rapidly and dose-dependently, stimulates the formation of intracellular ROS in human corneal epithelial cells, consistently with the results reported by Black et al. [21] and by Pauloin et al. [2] in the same in vitro model as well as in the mouse cornea [26]. ROS levels significantly decreased when UVB-exposed cells were treated with the eye drops containing *Matricaria chamomilla* and *Euphrasia officinalis* extracts (Dacriovis™). The lack of efficacy in reducing ROS levels when eye drops were applied before UVB treatment suggests a protective, rather than a preventive effect; a significant control of ROS levels was in fact observed when eye drops were incubated either during UVB irradiation and post UVB irradiation. Interestingly, an improved efficacy was found when eye drops treatment was prolonged even after the end of irradiation. These results suggest that *Matricaria chamomilla* and *Euphrasia officinalis* extracts containing eye drops exert both a “sunglass effect” due to UV interception and a direct cyto-protective effect.

Total phenolic content and eye drops antioxidant power

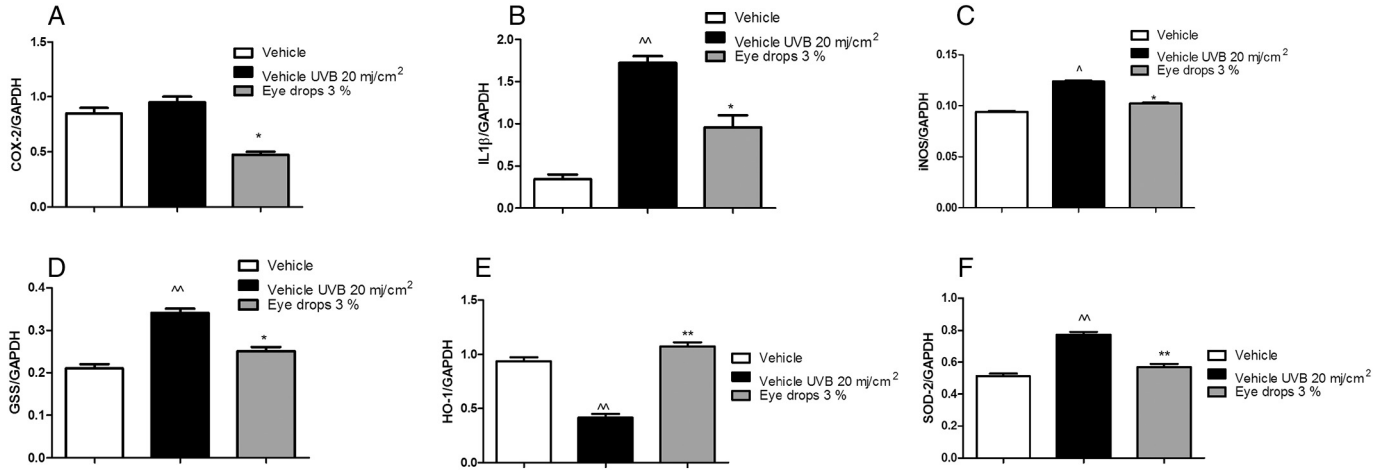

Fig. 6. Effect of eye drops 3% on COX-2 (Panel A), iNOS (Panel B), IL-1β (Panel C), GSS (Panel D), SOD-2 (Panel E) and HO-1 (Panel F) expression after 24 h from UVB. ^^($p < 0.01$ vs vehicle); **($p < 0.01$ vs UVB-irradiated cells); *($p < 0.05$ vs UVB-irradiated cells). Data are expressed as mean ± SE of three independent experiments.

measurements suggested that phenolic compounds can be responsible for the observed protective effects. Phenolic compounds are in fact well known as antioxidant and radical scavengers, able to counteract ROS induced cellular damage; this is particularly important for the ocular tissues because of their direct exposure to light, which causes production of ROS in situ.

Traditionally, *Euphrasia officinalis* is used for nasal catarrh, sinusitis and specifically for conjunctivitis when applied locally as an eye lotion [27], whether *Matricaria chamomilla* is mainly used to treat various inflammatory diseases of the gastrointestinal tract and oral cavity, however, little scientific information are available to support their use in the management of ocular diseases.

Eyebright contains iridoids, flavonoids, phenolic acids or etheric oils [14]. Camomile contains polyphenolic compounds such as caffeic acid, apigenin, luteolin and chamaemeloside [9]. Flavonoids from chamomile are able to protect cells from excessive superoxide radicals and hydrogen peroxide [28]. Moreover, radical scavenging and antioxidant effects of *Matricaria chamomilla* polyphenolic–polysaccharide conjugates were previously reported in cell free systems [11] and in a rat model of diabetes-induced oxidative stress [29]. Similarly, the antioxidant activity of the *Euphrasia rostkoviana* methanolic extract was previously reported [30,31] in cell free systems.

Oxidative damage is implicated in the pathogenesis of numerous traumatic, metabolic, inflammatory and iatrogenic diseases of the cornea [32] and strategies to treat oxidative stress related eye diseases are needed.

To evaluate the effects of eye drops on UVB-induced oxidative damage in the cornea, protein carbonyl levels, the most frequently used indicators of oxidative protein damage, were determined and the results showed that eye drops, as early as two hours, were able to protect corneal cells from ROS induced oxidative protein damage and the effect was evident also after 24 h from UVB exposure. During oxidative stress,

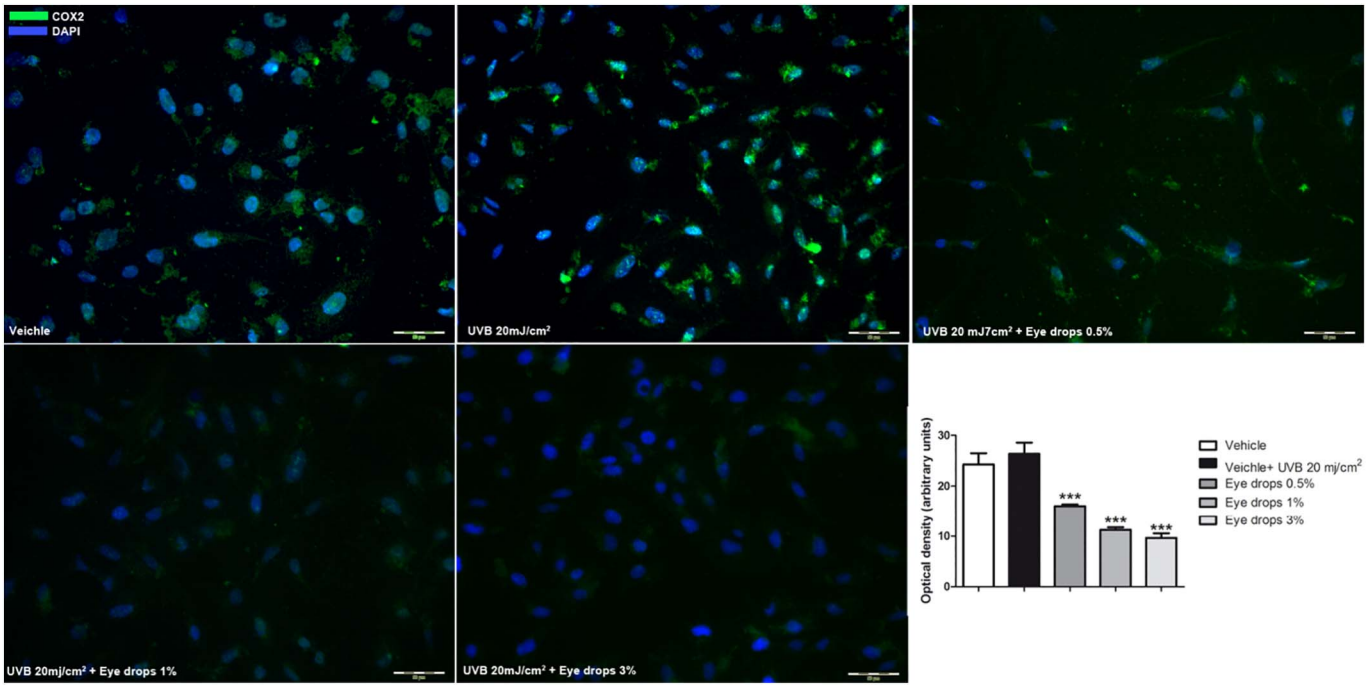

Fig. 7. Effect of eye drops 0.5, 1 and 3% on COX-2 protein expression by immunocytochemistry. Graph represents the densitometric analysis and reported the mean ± SE of four microscopic fields. ***($p < 0.001$ vs UVB-irradiated cells).

ROS can also initiate lipid peroxidation, another important mechanism mediating corneal injury induced by UVB [44]. After 24 h from UVB exposure, eye drops significantly reduced UVB-induced phototoxic effects in the cornea, as evidenced by the reduced levels of lipid peroxidation.

Exceeded levels of UVB light also cause cell death in corneal epithelial cells [33]. Our results showed that eye drops containing *Matricaria chamomilla* and *Euphrasia officinalis* extracts significantly reduced the UVB-induced cell death in corneal cells and increased wound-healing ability. The positive effects on wound healing might be ascribed to increased cells migration but other molecular mechanism involved in the extracellular matrix remodeling could be at work. *Chamomilla recutita* exerted beneficial effects on the healing of ulcers in rats [34] and *Matricaria chamomilla* extract had wound healing activity in rats [35,36], whereas, no data are currently available for *Euphrasia officinalis*.

Excessive UVB light absorption by the cornea also induces an inflammatory response, which typically appears 6–12 h after exposure [37]. Eye drops containing *Matricaria chamomilla* and *Euphrasia officinalis* extracts were effective also in reducing UVB-induced expression of the expression of COX-2, IL-1β and iNOS. In cell culture, chamomile has been demonstrated to modulate the production of many pro-inflammatory mediators such as superoxide anion, tumor necrosis factor-alpha, interleukins and prostaglandins by selectively targeting COX-2 [38,39] and iNOS [45]. Moreover, *Euphrasia officinalis* extracts decreased pro-inflammatory cytokine (IL-1β, IL-6 and TNF-α) and anti-inflammatory IL-10 expression by human corneal cells [14]. The efficacy of *Euphrasia officinalis* eye drops was also demonstrated in a human trial for the treatment of inflammatory conjunctivitis [16].

Interestingly, we also found the UVB insult increases the expression of SOD2 and GSS suggesting that upon oxidative stress, cellular antioxidant enzymes are induced to counteract ROS mediated-damage [22,40].

The heme oxygenase-1 (HO-1) protein exhibits cyto-protective, anti-inflammatory, anti-proliferative, antioxidant and anti-apoptotic activities and appears as a promising therapeutic target for the treatment of inflammatory diseases [46]. In macrophages, activation of HO-1 inhibits cytokine secretion [41] and suppresses COX-2 expression [42]. Increased expression of HO-1 in human corneal epithelial cells following UVB treatment was reported among the antioxidant/anti-inflammatory defense mechanisms [21]. Interestingly, in our experiments, eye drops were shown to induce HO-1 gene expression, an effect also reported in murine RAW 264.7 macrophages stimulated with H_2O_2 and exposed to an aqueous chamomile extract [43].

Overall, our results indicate that *Matricaria chamomilla* and *Euphrasia officinalis* containing eye drops have significant beneficial effects against UVB induced oxidative stress and inflammation supporting their use for protecting corneal epithelial cells from UVB exposure.

Conflict of Interest

Nothing to declare.

Funding

A liberal financial grant was provided by Steve Jones Srl.

References

[1] L. Kolozsvári, A. Nógrádi, B. Hopp, Z. Bor, UV absorbance of the human cornea in the 240- to 400-nm range, Invest. Ophthalmol. Vis. Sci. 43 (7) (2002 Jul) 2165–2168.
[2] T. Pauloin, M. Dutot, F. Joly, J.M. Warnet, P. Rat, High molecular weight hyaluronan decreases UVB-induced apoptosis and inflammation in human epithelial corneal cells, Mol. Vis. 15 (2009) 577–583.
[3] M.H. Chen, C.F. Tsai, Y.W. Hsu, F.J. Lu, Epigallocatechin gallate eye drops protect against ultraviolet B-induced corneal oxidative damage in mice, Mol. Vis. 20 (2014 Feb 7) 153–162 (eCollection 2014).
[4] M. Nita, A. Grzybowski, The role of the reactive oxygen species and oxidative stress in the pathomechanism of the age-related ocular diseases and other pathologies of the anterior and posterior eye segments in adults, Oxidative Med. Cell. Longev. 2016 (2016) 3164734.
[5] J.E. Downes, P.G. Swann, R.S. Holmes, Differential corneal sensitivity to ultraviolet light among inbred strains of mice. Correlation of ultraviolet B sensitivity with aldehyde dehydrogenase deficiency, Cornea 13 (1994) 67–72.
[6] L.W. Hirst, R.A. Axelsen, I. Schwab, Pterygium and associated ocular surface squamous neoplasia, Arch. Ophthalmol. 127 (1) (2009 Jan) 31–32.
[7] J.K. Srivastava, E. Shankar, S. Gupta, Chamomile: a herbal medicine of the past with bright future, Mol. Med. Rep. 3 (6) (2010 Nov 1) 895–901.
[8] D.L. McKay, J.B. Blumberg, A review of the bioactivity and potential health benefits of chamomile tea (*Matricaria recutita* L.), Phytother. Res. 20 (7) (2006 Jul) 519–530.
[9] A. Carnat, A.P. Carnat, D. Fraisse, L. Ricoux, J.L. Lamaison, The aromatic and polyphenolic composition of Roman camomile tea, Fitoterapia 75 (1) (2004 Jan) 32–38.
[10] A. Kato, Y. Minoshima, J. Yamamoto, I. Adachi, A.A. Watson, R.J. Nash, Protective effects of dietary chamomile tea on diabetic complications, J. Agric. Food Chem. 56 (17) (2008 Sep 10) 8206–8211.
[11] J. Kolodziejczyk-Czepas, M. Bijak, J. Saluk, M.B. Ponczek, H.M. Zbikowska, P. Nowak, M. Tsirigotis-Maniecka, I. Pawlaczyk, Radical scavenging and antioxidant effects of *Matricaria chamomilla* polyphenolic-polysaccharide conjugates, Int. J. Biol. Macromol. 72 (2015) 1152–1158.
[12] European Medicines Agency, Assessment report on *Euphrasia officinalis* L. and *Euphrasia rostkoviana* Hayne, Herba, a report of Committee on Herbal Medicinal Products, European Medicines Agency, London, UK. http://www.ema.europa.eu/docs/en_GB/document_library/Herbal_HMPC_assessment_report/2011/01/WC500100385.pdf, (2010).
[13] L. Bielory, J. Heimall, Review of complementary and alternative medicine in treatment of ocular allergies, Curr. Opin. Allergy Clin. Immunol. 5 (2003) 395–399.
[14] R. Paduch, A. Woźniak, P. Niedziela, R. Rejdak, Assessment of eyebright (*Euphrasia officinalis* L.) extract activity in relation to human corneal cells using in vitro tests, Balkan Med. J. 31 (1) (2014 Mar) 29–36.
[15] P. Novy, H. Davidova, C.S. Serrano-Rojero, J. Rondevaldova, J. Pulkrabek, L. Kokoska, Composition and antimicrobial activity of *Euphrasia rostkoviana* Hayne essential oil, Evid. Based Complement. Alternat. Med. 2015 (2015) 734101.
[16] M. Stoss, C. Michels, E. Peter, R. Beutke, R.W. Gorter, Prospective cohort trial of Euphrasia single-dose eye drops in conjunctivitis, J. Altern. Complement. Med. 6 (6) (2000 Dec) 499–508.
[17] M. Lodovici, E. Bigagli, F. Tarantini, C. Di Serio, L. Raimondi, Losartan reduces oxidative damage to renal DNA and conserves plasma antioxidant capacity in diabetic rats, Exp. Biol. Med. (Maywood) 240 (11) (2015) 1500–1504 (Nov).
[18] E.A. Ainsworth, K.M. Gillespie, Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent, Nat. Protoc. 2 (4) (2007) 875–877.
[19] R. Mencucci, E. Favuzza, C. Boccalini, A. Lapucci, R. Felici, F. Resta, A. Chiarugi, L. Cavone, CoQ10-containing eye drops prevent UVB-induced cornea cell damage and increase cornea wound healing by preserving mitochondrial function, Invest. Ophthalmol. Vis. Sci. 55 (11) (2014 Oct 9) 7266–7271.
[20] L. Cinci, C. Luceri, E. Bigagli, I. Carboni, S. Paccosi, A. Parenti, D. Guasti, M. Coronnello, Development and characterization of an in vitro model of colorectal adenocarcinoma with MDR phenotype, Cancer Med. 5 (6) (2016 Jun) 1279–1291.
[21] A.T. Black, M.K. Gordon, D.E. Heck, M.A. Gallo, D.L. Laskin, J.D. Laskin, UVB light regulates expression of antioxidants and inflammatory mediators in human corneal epithelial cells, Biochem. Pharmacol. 81 (7) (2011 Apr 1) 873–880.
[22] M.E. Manni, E. Bigagli, M. Lodovici, M. Zazzeri, L. Raimondi, The protective effect of losartan in the nephropathy of the diabetic rat includes the control of monoamine oxidase type A activity, Pharmacol. Res. 65 (4) (2012 Apr) 465–471.
[23] V. Correa-Salde, I. Albesa, Reactive oxidant species and oxidation of protein and haemoglobin as biomarkers of susceptibility to stress caused by chloramphenicol, Biomed Pharmacother 63 (2) (2009 Feb) 100–104.
[24] E. Bigagli, L. Cinci, S. Paccosi, A. Parenti, M. D'Ambrosio, C. Luceri, Nutritionally relevant concentrations of resveratrol and hydroxytyrosol mitigate oxidative burst of human granulocytes and monocytes and the production of pro-inflammatory mediators in LPS-stimulated RAW 264.7 macrophages, Int. Immunopharmacol. 43 (2017 Feb) 147–155.
[25] L. Raimondi, M. Lodovici, F. Guglielmi, G. Banchelli, M. Ciuffi, E. Boldrini, R. Pirisino, The polysaccharide from *Tamarindus indica* (TS-polysaccharide) protects cultured corneal-derived cells (SIRC cells) from ultraviolet rays, J. Pharm. Pharmacol. 55 (3) (2003 Mar) 333–338.
[26] A. Lennikov, N. Kitaichi, R. Fukase, M. Murata, K. Noda, R. Ando, T. Ohguchi, T. Kawakita, S. Ohno, S. Ishida, Amelioration of ultraviolet-induced photokeratitis in mice treated with astaxanthin eye drops, Mol. Vis. 18 (2012) 455–464.
[27] J. Barnes, L.A. Anderson, J.D. Phillipson, Herbal Medicines. 3rd, Pharmaceutical Press, London, UK, 2007.
[28] K.M. Yoo, I.K. Hwang, B. Moon, Comparative flavonoids contents of selected herbs and associations of their radical scavenging activity with antiproliferative actions in V79-4 cells, J. Food Sci. 74 (6) (2009 Aug) C419–C425.
[29] M. Cemek, S. Kağa, N. Simşek, M.E. Büyükokuroğlu, M. Konuk, Antihyperglycemic and antioxidative potential of *Matricaria chamomilla* L. in streptozotocin-induced diabetic rats, J. Nat. Med. 62 (3) (2008 Jul) 284–293.
[30] B. Blazics, A. Alberti, A. Kéry, Antioxidant activity of different phenolic fractions separated from *Euphrasia rostkoviana* Hayne, Acta Pharm. Hung. 79 (1) (2009) 11–16.
[31] R. Teixeira, L.R. Silva, Bioactive compounds and in vitro biological activity of *Euphrasia rostkoviana* Hayne extracts, Ind. Crop. Prod. 50 (2013) 680–689.
[32] A. Shoham, M. Hadziahmetovic, J.L. Dunaief, M.B. Mydlarski, H.M. Schipper, Oxidative stress in diseases of the human cornea, Free Radic. Biol. Med. 45 (8) (2008 Oct 15) 1047–1055.
[33] L. Tong, Z. Chen, C.S. De Paiva, R. Beuerman, D.Q. Li, S.C. Pflugfelder, Transglutaminase participates in UVB-induced cell death pathways in human corneal epithelial cells, Invest. Ophthalmol. Vis. Sci. 47 (10) (2006 Oct) 4295–4301.
[34] C.M. Duarte, M.R. Quirino, M.C. Patrocínio, A.L. Anbinder, Effects of *Chamomilla recutita* (L.) on oral wound healing in rats, Med. Oral Patol. Oral Cir. Bucal. 16 (6) (2011 Sep 1) e716–e721.

[35] M. Jarrahi, A.A. Vafaei, A.A. Taherian, H. Miladi, Pour A. Rashidi, Evaluation of topical *Matricaria chamomilla* extract activity on linear incisional wound healing in albino rats, Nat. Prod. Res. 24 (8) (2010 May) 697–702.
[36] M.D. Martins, M.M. Marques, S.K. Bussadori, M.A. Martins, V.C. Pavesi, R.A. Mesquita-Ferrari, K.P. Fernandes, Comparative analysis between *Chamomilla recutita* and corticosteroids on wound healing. An in vitro and in vivo study, Phytother. Res. 23 (2009) 274–278.
[37] A.R. Young, Acute effects of UVR on human eyes and skin, Prog. Biophys. Mol. Biol. 92 (2006) 80–85.
[38] A.T. Smolinski, J.J. Pestka, Modulation of lipopolysaccharide-induced proinflammatory cytokine production in vitro and in vivo by the herbal constituents apigenin (chamomile), ginsenoside Rb(1) (ginseng) and parthenolide (feverfew), Food Chem. Toxicol. 41 (10) (2003 Oct) 1381–1390.
[39] J.K. Srivastava, M. Pandey, S. Gupta, Chamomile, a novel and selective COX-2 inhibitor with anti-inflammatory activity, Life Sci. 85 (19–20) (2009 Nov 4) 663–669.
[40] M. Lodovici, E. Bigagli, G. Bardini, C.M. Rotella, Lipoperoxidation and antioxidant capacity in patients with poorly controlled type 2 diabetes, Toxicol. Ind. Health 25 (4–5) (2009) 337–341 (May–Jun).
[41] T.S. Lee, H.L. Tsai, L.Y. Chau, Induction of heme oxygenase-1 expression in murine macrophages is essential for the anti-inflammatory effect of low dose 15-deoxy-Delta 12,14-prostaglandin J2, J. Biol. Chem. 278 (21) (2003) 19325–19330.
[42] G.Y. Suh, Y. Jin, A.K. Yi, X.M. Wang, Choi AM CCAAT/enhancer-binding protein mediates carbon monoxide-induced suppression of cyclooxygenase-2, Am. J. Respir. Cell Mol. Biol. 35 (2) (2006 Aug) 220–226.
[43] N. Bhaskaran, S. Shukla, R. Kanwal, J.K. Srivastava, S. Gupta, Induction of heme oxygenase-1 by chamomile protects murine macrophages against oxidative stress, Life Sci. 90 (25–26) (2012 Jun 27) 1027–1033.
[44] R. Zheng, I. Po, V. Mishin, A.T. Black, D.E. Heck, D.L. Laskin, P.J. Sinko, D.R. Gerecke, M.K. Gordon, J.D. Laskin, The generation of 4-hydroxynonenal, an electrophilic lipid peroxidation end product, in rabbit cornea organ cultures treated with UVB light and nitrogen mustard, Toxicol Appl Pharmacol 272 (2) (2013 Oct) 345–355.
[45] N. Bhaskaran, S. Shukla, J.K. Srivastava, S. Gupta, Chamomile: an anti-inflammatory agent inhibits inducible nitric oxide synthase expression by blocking RelA/p65 activity, Int J Mol Med. 26 (6) (2010 Dec) 935–940.
[46] A. Paine, B. Eiz-Vesper, R. Blasczyk, S. Immenschuh, Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential, Biochem Pharmacol. 80 (12) (2010 Dec 15) 1895–1903.