Journal of
Clinical & Translational Ophthalmology

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In Vitro Anti-Inflammatory Potential of Pomegranate Extract (Pomanox®) in a Reconstituted Human Corneal Epithelium Model

Marcos Peñalver-Mellado [1], Fredy Silva-Fuentes [2,*], Agustín Villar [2], Anna Mula [2] and Andrea Zangara [2,*]

[1] Probelte Pharma S.L.U., S/Antonio Belmonte Abellán, 3-7, 30100 Murcia, Spain
[2] Euromed S.A., C/Rec de Dalt, 21-23, 08100 Mollet del Valles, Spain
* Correspondence: fsilvafuentes@euromed.es (F.S.-F.); azangara@euromed.es (A.Z.); Tel.: +34-93-5541442 (ext. 167) (F.S.-F.)

Abstract: An in vitro study was conducted using a model of reconstituted human corneal epithelial (SkinEthic™ HCE/Human Corneal Epithelium) to test the modulation of cytokines secretion activity of Pomanox® (PMX), a standardized commercial extract of pomegranate fruit characterized by high punicalagin α + β content. Cell viability and inhibition of the release of interleukin-8 (IL-8) was evaluated in four conditions: negative control, positive inflammatory control with lipopolysaccharide (LPS) from *Escherichia coli*, positive anti-inflammatory control (LPS plus dexamethasone), and LPS plus PMX after 24 h of culture. The mean (±standard error of mean (SEM)) IL-8 level was 48.7 ± 5.1 pg/mL in the PMX condition vs. 172.7 ± 19.3 and 26.6 ± 1.2 in the LPS from E. coli and negative control, respectively ($p < 0.05$) and 93.8 ± 8.7 pg/mL in LPS plus dexamethasone ($p = 0.165$). The percentages of inhibition of IL-8 release were 45.7% for LPS plus dexamethasone and 63.8% for LPS plus PMX. The percentage of cell viability (86%) was also higher for the LPS plus PMX condition. The present findings add evidence to the anti-inflammatory effect of a PMX in an in vitro model of reconstituted corneal epithelial cells.

Keywords: pomegranate; anti-inflammatory; interleukin-8; corneal epithelium; in vitro study; cytokines

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Citation: Peñalver-Mellado, M.; Silva-Fuentes, F.; Villar, A.; Mula, A.; Zangara, A. In Vitro Anti-Inflammatory Potential of Pomegranate Extract (Pomanox®) in a Reconstituted Human Corneal Epithelium Model. *J. Clin. Transl. Ophthalmol.* **2023**, *1*, 52–60. https://doi.org/10.3390/jcto1020007

Academic Editors: Gary Peh and Georgios Labiris

Received: 9 February 2023
Revised: 27 March 2023
Accepted: 12 April 2023
Published: 19 April 2023

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1. Introduction

The corneal epithelium is the outermost layer of the cornea, whose main functions include maintaining transparency and protecting the eye from the external environment. Exogenous stimuli can lead to the activation of proinflammatory signaling pathways, resulting in corneal neovascularization, scarring, loss of transparency, and decreased visual acuity. In addition to serving as a physical barrier against injury and infection, the corneal epithelium also contributes to the ocular immune response by activating signaling defense pathways mechanisms with subsequent production of proinflammatory cytokines [1,2].

Interleukin-8 (IL-8) is a well-characterized soluble neutrophil and lymphocyte chemotaxin secreted by a number of tissue-based cells exposed to proinflammatory cytokines, such as IL-1 and tumor necrosis factor (TNF) [3,4]. The induction of IL-8 facilitates an early innate immune response to infection in the corneal stroma and represents an important mechanism in corneal wound healing. IL-8 enhances healing by rapidly chemoattracting leukocytes and fibroblasts into the wound site, stimulating the latter to differentiate into myofibroblasts [5]. Both human corneal keratinocytes and epithelial cells have been shown to synthesize and release IL-8 following cytokine stimulation and/or infection [6].

Experimental studies have also shown that corneas may be stimulated to produce high levels of IL-8 by inflammatory cytokines, such as IL-1β, which may be released by corneal stromal cells, vascular endothelial cells at the corneoscleral limbus, and infiltrating leukocytes during the initial host responses to inflammatory stimuli [7]. In cultures of human corneal keratocytes and epithelial cells, it was found that treatment with various concentrations of human recombinant IL-1α or TNF-α, stimulated IL-8 mRNA synthesis

and IL-8 production in a dose–response fashion [6]. Furthermore, it has been shown that IL-8 overexpression in corneal tissue causes ulcer formation in the cornea through chemoattraction of neutrophils, suggesting the causative role of IL-8 in some type of ulcers [8]. In cultures of epithelial cells and keratinocytes established from human corneas, infection with herpes simplex virus type 1 (HSV-1) led to the synthesis of IL-8-specific mRNA, suggesting that these corneal cells can contribute to the induction of the acute inflammatory response seen in herpes stromal keratitis [9]. Therefore, targeting IL-8 production by cultured human corneal epithelial cells may be a useful model to assess the anti-inflammatory potential of natural products.

Fruits rich in polyphenols, such as pomegranates, have shown to have health benefits related to their antioxidant and anti-inflammatory properties. Pomegranate (*Punica granatum* L.) has been used for centuries in traditional and folk medicine for its pleiotropic effects affecting various cellular pathways, especially those triggered by oxidative stress and mediators of the inflammatory cascade [10–14]. Although pomegranate contains a broad array of active phytochemicals, most of pomegranate health benefits have been attributed to hydrolyzable tannins (ellagitannins), in particular the phenolic compounds punicalagin and its isomers a and b, punicalin, and ellagic acid derivatives [15–17]. Upon digestion, ellagitannins are hydrolyzed to ellagic acid, further metabolized by gut microbiota into urolithins, highly bioavailable phenolics currently considered responsible for ellagitannins health effects [18]. Clinical human studies with pomegranate juice and extracts (Pomanox®, PMX) have investigated several therapeutic activities, including against inflammation, cardiovascular diseases (e.g., hyperlipidemia and hypertension), diabetes and cancer [19]. PMX clinical data indicate the extract attenuated cardiovascular risk factors, with anti-hypertensive, anti-atherogenic, and cardioprotective effects [17–21], as well as reduced food intake by promoting satiety [22], improved cognitive functions [23], and sports performance [24].

There is limited data on the effect of pomegranate in eye diseases. In a rabbit model of chronic and moderately-elevated intraocular pressure (IOP), a significant decrease in IOP was observed in glaucoma rabbits treated with diet containing 20% pomegranate, which appeared to occur by maintaining the antioxidant defense system possibly by preventing depletion of antioxidant enzymes and inhibition of lipid peroxidation [25]. In a model of diabetic rats, those treated with 500 mg/kg body weight of pomegranate peel extract orally once per day with the drinking water for 40 weeks showed marked amelioration of pathological changes of the retina and lens compared with untreated rats [26]. Pomegranate treatment as an antioxidant also attenuated retinal structural and functional ischemia/reperfusion injury in rats [27].

These experimental studies have mostly examined the antioxidant activity of PMX, and no previous data from in vitro studies of have been reported regarding the anti-inflammatory potential of this product in corneal tissue samples. Therefore, the present in vitro study was designed to evaluate the anti-inflammatory potential of PMX by using a commercial model of reconstituted human corneal epithelium (SkinEthic™ HCE), activated with lipopolysaccharide (LPS) from *Escherichia coli* and analyzing the production of IL-8. The SkinEthic™ HCE model has been used in previous studies as an in vitro tool for testing eye damage/irritation of different products including chemicals and cosmetics, with satisfactory specificity and reproducibility [28–33].

2. Materials and Methods

2.1. Reconstituted Human Corneal Epithelium

The study was performed in samples of SkinEthic™ HCE/Human Corneal Epithelium (EPISKIN Laboratories, Lyon, France). Technical and safety data provided by the laboratory for the batch number 10-HCE-017 used in the study included the following: (a) description: 0.5 cm^2 and 0.33 cm^2 epithelium reconstituted by airlifted culture of transformed human corneal keratinocytes for 5 days in chemically defined medium on inert polycarbonate filters (60 μm thickness at day 5); (b) storage: the product was prepared and packaged

using aseptic techniques and stored in an incubator at 37 °C, 5% CO_2 with saturated humidity; (c) origin: immortalized human corneal epithelium cells (HCE); (d) handling: human corneal epithelium reconstructed using transformed human cells of class 2; and (e) biological safety on the cell strain: absence of HIV integrated pro-viral DNA, hepatitis C viral RNA, hepatitis B viral DNA, and viral contamination of the virus used for the cell line immortalization; and (f) biological safety on culture supernatant: absence of mycoplasma. The reconstructed tissue forms a stratified and well organized epithelium which is structurally, morphologically, and functionally similar to the human cornea with presence of basal, wing and mucus production cells (https://www.episkin.com/HCE-Corneal-Epithelium, accessed on 6 March 2023).

2.2. In Vitro Assay

After its reception, each reconstituted HCE was placed into a 24-well microplate with 600 µL of the maintenance medium, and stored in an incubator at 37 °C, 5% CO_2, and 95% humidity. The test products included the following: phosphate-buffered saline (PBS) (negative control); LPS from *Escherichia coli* O55:B5 (Sigma/L2880) (100 µg/mL) (positive inflammatory control); LPS plus water-soluble dexamethasone (Sigma/D2915) (10 µM) (anti-inflammatory positive control); and test sample (LPS plus PMX) ($\leq$200 µM). The pomegranate fruit extract was Pomanox®, obtained through a water-based extraction process (Pure Hydro Process®) and provided by Euromed® (Barcelona, Spain). According to the manufacturer, the test product Pomanox® P50 (more concentrated than the clinically studied Pomanox® P30) is standardized for punicalagins α + β 50% (*w*/*w* as db) and ellagic acid 5% (*w*/*w* as db) as detailed in patent EP1967079B1 [34]. The test products were diluted in the maintenance medium and were tested at least in duplicate. Reconstituted HCE samples were treated with the test products for 24 h.

2.3. Study Variables and Assessment of Response

Study variables included cell viability, release of IL-8, and extrapolation of the inhibition of IL-8 release.

Cell viability was measured in relation to a negative control (untreated tissue sample) and a positive control (sodium dodecyl sulfate [SDS] 0.5%) using an MTT assay. Each reconstituted HCE sample was incubated in MTT solution (1 mg/mL [p/V] in PBS) diluted at 1:2 (*v*/*v*) in the maintenance medium for approximately 1 h in a CO_2 incubator heater. Then, 1 mL of isopropanol was added to each tissue sample and agitated at room temperature for approximately 1 h. The optical density at 542 nm against isopropanol (blank) was measured. Results were expressed as percentage of cell viability.

Determination of the release of IL-8 in the supernatant was performed using a human IL-8 ELISA kit (ab46032, Abcam plc, Cambridge, UK). Results are expressed in pg/mL. The extrapolation of the inhibition of IL-8 release was estimated by the percentage of reduction in the amount of IL-8 released in response to LPS in the tissue sample treated with Pomanox® as compared to the positive control.

2.4. Statistical Analysis

Results of continuous variables are expressed as mean (±standard error of mean [SEM] and 95% confidence interval (CI). Differences in the distribution of the study variables in the groups of negative control, positive inflammatory control, positive anti-inflammatory control, and test product were compared with the Mann–Whitney *U* test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Cell Viability

As shown in Figure 1, there were differences in the percentage of viable cells after 24 h of treatment with the test products. In reconstituted HCE samples treated with the negative control, cell viability was 100%. In the other groups treated with LPS (posi-

tive inflammatory control), LPS plus PMX (test sample), and LPS plus dexamethasone (positive anti-inflammatory control), the percentages of cell viability were 76%, 86%, and 85%, respectively.

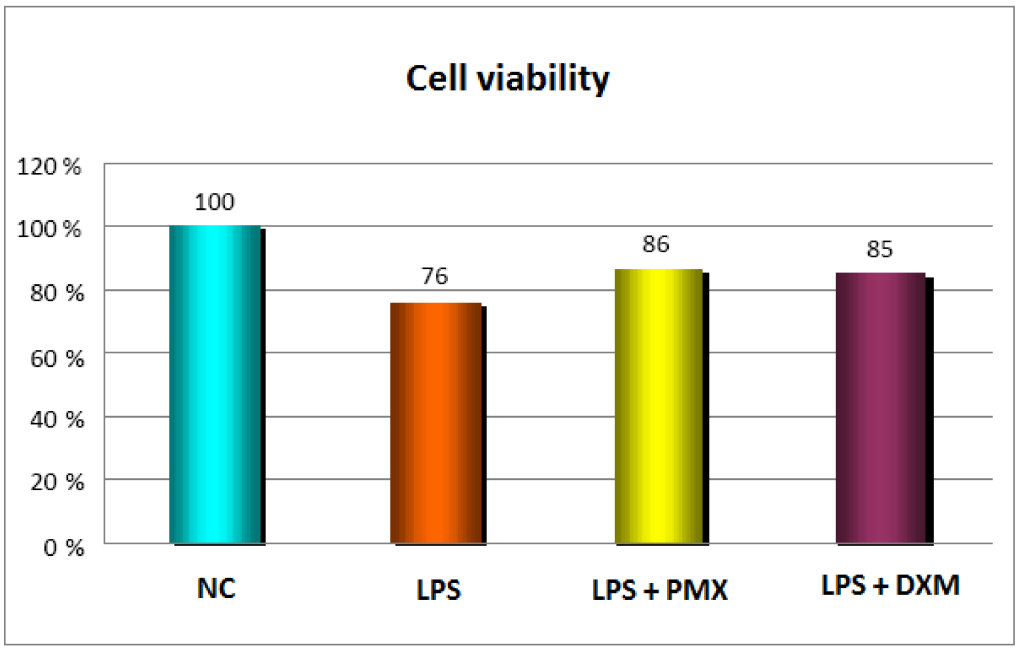

Figure 1. Percentage of cell viability in the different study groups: phosphate-buffered saline (PBS), negative control (NC); lipopolysaccharide from *E. coli* (LPS), positive inflammatory control; LPS plus PMX, test sample; and LPS plus dexamethasone (DXM), positive anti-inflammatory control. See number of runs in Table 1.

Table 1. IL-8 levels in the four experimental conditions.

Test Condition	IL-8 Levels, pg/mL	
	Mean ± SEM	95% CI
Negative control (PBS), n = 3	26.6 ± 1.2	21.6 to 31.7
Positive inflammatory control (LPS *E. coli*), n = 4	172.7 ± 19.3	111.2 to 234.2
Positive anti-inflammatory control (LPS + DXM), n = 2	93.8 ± 8.7	−17.1 to 204.7
Test sample (LPS + PMX), n = 4	48.7 ± 5.1	32.6 to 64.8

IL-8: interleukin-8, SEM: standard error of mean; CI: confidence interval; PBS: phosphate-buffered saline; LPS: lipopolysaccharide; DXM: dexamethasone; PMX: Pomanox®.

3.2. Release of IL-8

The results obtained in the levels of IL-8 in the different test conditions are shown in Table 1. The lowest level of IL-8 (mean ± SEM) was 48.7 ± 5.1 pg/mL and was observed in the LPS plus PMX test condition.

There were statistically significant differences in the release of IL-8 between the negative control (PBS) and the positive inflammatory control (LPS *E. coli*) ($p < 0.05$) as well as between the test sample (LPS + PMX) and the negative and positive control groups ($p < 0.05$). Differences between positive inflammatory and anti-inflammatory control groups were not statistically significant ($p = 0.248$) as well as between the test sample and the positive anti-inflammatory control ($p = 0.165$) (Figure 2).

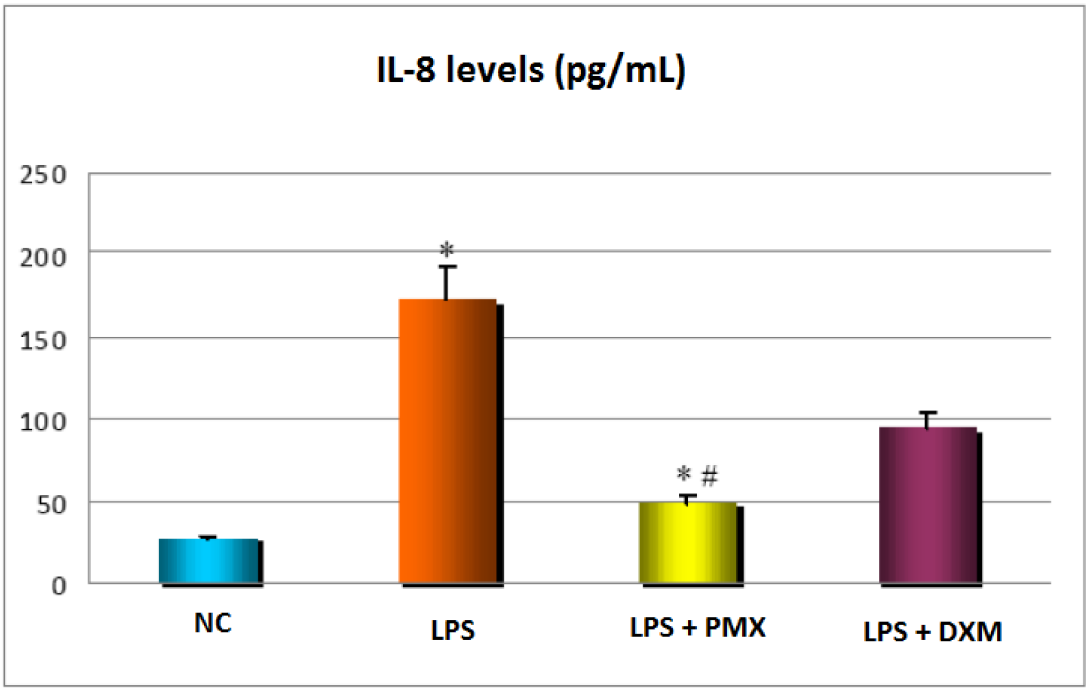

Figure 2. Release of IL-8 in the different study groups. * $p < 0.05$ of LPS and LPS + PMX vs. negative control; #: $p < 0.05$ vs. LPS. (NC) (LPS: lipopolysaccharide, PMX: Pomanox®; DXM: dexamethasone). Data expressed as mean ± SEM. See number of runs in Table 1.

3.3. Extrapolation of the Inhibition of IL-8 Release

The average percentage of inhibition of IL-8 release was calculated taking as 100% the average of IL-8 release from the culture treated with LPS. The percentages of inhibition of IL-8 release were 45.7% for LPS plus dexamethasone (IL-8 release 54.3%) and 71.8% for LPS plus PMX (IL-8 release 28.2%).

4. Discussion

The present findings of an in vitro study in reconstituted HCE confirm the anti-inflammatory potential of a natural extract of pomegranate as demonstrated by a remarkable inhibition of the release of IL-8 after induction of an inflammatory status with LPS from *E. coli*. The percentage of inhibition of IL-8 release was also higher than that obtained with LPS plus dexamethasone.

We used the SkinEthic™ HCE/Human Corneal Epithelium, a reconstructed human cornea-like epithelium consisting of a multilayered epithelium prepared from immortalized human corneal epithelial cell. It constitutes a three dimensional structure which reproduces the multi-layer organization of the human cornea. This model has been shown to be a valid alternative to the in vivo Draize rabbit eye procedure for the assessment of eye irritation potential of chemical compounds and cosmetic products [33,35] as well as for eye toxicity studies [36]. However, assessment of interleukins release in different testing conditions using the SkinEthic™ HCE/Human Corneal Epithelium model has not been previously reported.

Punicalagin is a polyphenol extracted from pomegranate with multiple functions, including anti-inflammatory properties, mainly ascribed to its antioxidant activity. In a human retinal pigment epithelial cell line (ARPE-19) exposed to hydrogen peroxide (H_2O_2) for 24 h, pre-treatment with punicalagin significantly increased cell viability and preserved mitochondrial functions, reducing oxidative stress [37]. In a model of bovine endometrial epithelial cells, punicalagin pre-treatment significantly decreased the production of IL-1β, IL-6, and IL-8 [38]. Punicalagin regulates signaling pathways in inflammation-associated disorders, and numerous experimental studies in inflammatory models have shown that

treatment with punicalagin significantly reduced inflammatory markers and TNFα-induced expressions of pro-inflammatory cytokines, including IL-8 [39–42].

The ellagic acid, a minor component of the study product, has also shown anti-inflammatory potential. In an in vitro study of LPS-induced RAW267.4 macrophages, ellagic acid, gallic acid and punicalagins α + β isolated from *P. granatum* inhibited the production of nitric oxide, prostaglandin E2 (PGE-2), and IL-6 [43].

The study product Pomanox® (PMX) demonstrated anti-inflammatory properties in few research studies. In a mouse model of inflammation induced by an acute injection of TNF-α, prior supplementation with PMX prevented the loss of muscle mass induced by TNF-α [44]. In skeletal muscle, reduced the activation of the NF-κB signaling and the induction of cytokines mRNA. Additionally, the punicalagin metabolite urolithin A was identified as the active compound protecting skeletal muscle against TNF-α-induced inflammation [44]. In a pre-clinical study PMX supplementation significantly decreased, by about 50%, the expression of the inflammatory chemokine MCP-1 in dyslipidemic animals [45]. Recently published an in vitro study found PMX significantly modulated collagen and hyaluronic acid metabolisms and showed a marked inhibitory capacity of tyrosinase activity. In UV-exposed human fibroblasts Hs68 cells, both preventive and regenerative treatments with PMX positively modulated hyaluronic acid metabolism, showed a drastic decrease in ROS levels and proinflammatory MMP-1 levels in the cell culture medium in comparison with the untreated cells decreased [46]. Proprietary data support anti-inflammatory effects in human studies as well.

Overall, these observations in previous studies are consistent with the anti-inflammatory effect of the test product composed of LPS plus PMX found in the present model of reconstituted human corneal epithelium. The LPS plus PMX test condition was associated with a significant decrease in the production of IL-8 in reconstituted human corneal epithelial cells in which inflammation had been induced by means of LPS from *E. coli*. In addition, in the analysis of cell viability, the condition of LPS plus PMX showed the highest percentage of viable cells as compared with LPS and LPS plus dexamethasone. However, a limitation of the study is the reduced number of experiments especially in the test condition of LPS plus dexamethasone, and further studies are needed for replication of the present results with more than 5 runs. Finally, the present results could be expanded and validated by including the assessment of other markers of inflammation affected by punicalagin in other models, such as IL-6 and IL-1β. Moreover, an additional study of PMX use after inflammatory induction (LPS) would be of interest for the assessment of a potential therapeutic effect.

5. Conclusions

In an in vitro study based on a reconstituted human corneal epithelial model, culture for 24 h with LPS plus a natural extract of pomegranate composed of punicalagin and ellagic acid (PMX) was associated with a high percentage of cell viability. In the present conditions, with a limited number of samples for positive anti-inflammatory control (n = 2), we found a tendency for experiment of LPS plus PMX to show a greater inhibition of the release of IL-8 as compared with a positive anti-inflammatory control with dexamethasone. Further research is warranted as these results suggest a promising ocular anti-inflammatory application of PMX, which should be of interest for eye health.

Author Contributions: Conceptualization, M.P.-M.; methodology, M.P.-M.; formal analysis, M.P.-M. and F.S.-F.; investigation, M.P.-M.; data curation, M.P.-M. and F.S.-F.; writing—original draft preparation, M.P.-M. and A.Z.; writing—review and editing, M.P.-M., F.S.-F., A.Z., A.V. and A.M.; visualization, M.P.-M.; supervision, A.Z.; project administration, M.P.-M., F.S.-F. and A.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available from the corresponding author upon request.

Acknowledgments: The authors thank Marta Pulido, for editing the manuscript and editorial assistance.

Conflicts of Interest: F.S.-F., A.V., A.M. and A.Z. are full employees of Euromed.

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