


Original Research

# Protective Role of *Cynara cardunculus* L. var. *atilis* Against Oxidative Stress and Corneal Dysfunction in High Glucose-Damaged Human Corneal Epithelial Cells

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## Abstract

**Background:** The global increase in diabetes mellitus has been accompanied by a significant rise in related complications. Diabetic patients frequently experience ocular surface disorders and multiple studies have demonstrated that the diabetic corneal epithelium is characterized by increased cellular fragility and compromised barrier integrity. It has been demonstrated that the processes of oxidative stress and inflammation are pivotal in causing ocular tissue damage in diabetic patients. Numerous studies have explored the protective effects of various antioxidants, especially those sourced from plants. *Cynara cardunculus* L. var. *atilis* (DC.), a species widely integrated into the Mediterranean diet and commonly known as cultivated cardoon (CC), is particularly rich in bioactive phenolic compounds, recognized for their antioxidant effects. **Methods:** The current work focuses on assessing the effect of CC leaf extracts on high glucose-treated human corneal epithelial cells (HCEpiCs). HCEpiCs were cultured for 24 h in a medium supplemented with glucose up to a concentration of 25 mM. Mannitol treatment was included to distinguish whether the observed effects were due to glucose metabolism or solely osmotic stress. To evaluate the effect of CC extracts, corneal cells were pre-incubated with the CC extract 10–20 µg/mL for 24 h before high glucose (HG) treatment. Cell viability, transepithelial electrical resistance, wound healing assay and reactive oxygen species (ROS) measurements were performed after HG treatment. To evaluate the levels of oxidative stress, the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), along with glutathione (GSH) levels were assayed. The mitogen-activated protein kinase ERK1/2/cytosolic phospholipases A<sub>2</sub> (cPLA<sub>2</sub>)/cyclooxygenase-2 (COX-2) signaling pathway, triggering the inflammatory process, was evaluated by western blot analyses. **Results:** Our findings indicate that CC extract (i) improved viability, reducing oxidative stress by lowering ROS production and enhancing SOD, CAT activities and GSH content in human corneal epithelial cells exposed to high glucose concentrations; (ii) maintained a high TEER and promoted corneal epithelial wound healing; and (iii) induced down-regulation of the ERK 1/2/cPLA<sub>2</sub>/COX-2 signaling pathway involved in the inflammatory process and ROS production. **Conclusions:** CC leaf extract could improve human corneal epithelial cell function suggesting its potential as a preventive agent against the development of chronic diabetic complications.

**Keywords:** catalase; cyclooxygenase 2; Mediterranean diet; oxidative stress; signal transduction; corneal epithelial cells; diabetes complications; inflammation; Cynara extracts; cytosolic phospholipases A<sub>2</sub>

## 1. Introduction

Diabetes mellitus (DM) is one of the major global health challenges today, due to the rapid increase in its incidence. This disease affects individuals of all ages, social conditions and demographic groups, and is spreading virtually everywhere in the world. DM is currently considered a disease with a large number of hidden cases, as those diagnosed represent only a small part of the real prevalence [1]. This chronic metabolic disorder develops when the pancreas fails to produce adequate amounts of insulin or when

body tissues cannot effectively respond to the hormone's action, leading to disturbances in glucose regulation. When diabetes is not kept under control, excessive glucose in the blood (hyperglycemia) occurs, which, over time, can seriously affect several body systems, especially blood vessels and the nervous system [2].

Approximately 50% of people with diabetes develop a form of corneal degeneration known as diabetic keratopathy [3]. The epithelial surface of the cornea is organized into 5–7 strata of non-keratinized squamous cells arranged in a stratified pattern which are essential for maintaining the



transparency and structural integrity of the cornea. Due to the avascular nature of the cornea, the supply of nutrients and glucose to the corneal epithelial cells occurs primarily by diffusion from the aqueous humor rather than from the tear film [4]. Because the aqueous humor contains high glucose (HG) concentrations under hyperglycemic conditions, corneal epithelial cells in patients with diabetes mellitus are subject to prolonged contact with glucose, which contributes to a spectrum of pathological epithelial changes [5]. Disturbance of the corneal homeostatic balance has been widely observed in patients with diabetes. This condition is associated with a malfunction of the epithelial barrier, changes in the structure of epithelial cells and reduced corneal sensitivity [6]. Even small changes in the diabetic cornea can impair healing processes, leading to prolonged recovery times or non-healing epithelial lesions. The delayed healing process exposes patients to serious visual complications, including gradual epithelial thinning, the onset of secondary damage, and the triggering of cytokines that facilitate fibrotic scar development [5].

Corneal epithelial cells form the outermost layer of the ocular surface, protecting the eye from microbes, allergens, and chemicals [7]. The prompt renewal of the epithelial layer, along with the restoration of its protective barrier, is crucial for maintaining corneal architecture and vision-related function [8]. It has been demonstrated that exposure to HG levels leads to dysfunction of the corneal epithelial barrier, leading to a notable reduction in transepithelial electrical resistance (TEER). Therefore, gaining insight into the pathophysiological processes affecting the cornea in patients with diabetes is crucial for maintaining its structural and functional integrity [9,10].

Moreover, increased oxidative stress resulting from elevated reactive oxygen species (ROS) production has been identified as a key factor in the development of diabetic complications [10]. The cornea, due to its location on the external part of the eye, is constantly exposed to high concentrations of oxygen and solar radiation, both sources of oxidative stress. To counteract this problem, the cornea contains various enzymatic antioxidant defenses, including superoxide dismutase (SOD) and catalase (CAT), and non-enzymatic ones, including glutathione (GSH). SOD catalyzes the conversion (dismutation) of the superoxide anion ( $O_2^{\cdot-}$ ) into the less harmful free radical hydrogen peroxide ( $H_2O_2$ ). As the only antioxidant capable of directly reacting with  $O_2^{\cdot-}$ , SOD is often considered the principal element of the cornea's antioxidant defense, this primary antioxidant plays a decisive role in neutralizing reactive oxygen species at the earliest stage of oxidative challenge. CAT is a heme-bound enzyme that catalyzes the breakdown of  $H_2O_2$  into water ( $H_2O$ ) and oxygen ( $O_2$ ), using Mn as a cofactor. In addition to detoxifying  $H_2O_2$ , CAT protects SOD from inactivation and may play a direct role in filtering UVB radiation. GSH is essential for the catalytic activity of the enzyme glutathione peroxidase (GPX), which performs a

similar function to CAT by reducing  $H_2O_2$  to  $H_2O$ , but it uses reduced glutathione (GSH) as an electron donor [11].

Studies have linked changes in ROS production to diabetic corneal problems, suggesting that HG and ROS production leads to systemic corneal disease, characterized by peripheral neuropathy, delayed corneal wound healing, and impaired cell migration [12,13]. Although oxidative stress has been widely studied, the specific mechanisms by which hyperglycemia modulates reactive oxygen species generation and interferes with the repair of corneal epithelial barrier function are not yet fully elucidated.

In previous studies we demonstrated, in HG-treated human retinal microvascular endothelial cells, an activation of cytosolic phospholipases A<sub>2</sub> (cPLA<sub>2</sub>) and of mitogen-activated protein kinase (MAPK) ERK1/2 [14] and an increase of cyclooxygenase-2 (COX-2) protein levels and release of inflammatory cytokines [15], highlighting that the activation of the ERK/cPLA<sub>2</sub>/COX-2 axis represents an early event after exposure to HG, mediating retinal damage. Cytosolic PLA<sub>2</sub> catalyzes the hydrolysis of membrane glycerophospholipids, primarily phosphatidylcholine, at the *sn*-2 position, releasing arachidonic acid (AA), which is a precursor for eicosanoids such as prostaglandins E<sub>2</sub> (PGE<sub>2</sub>) and leukotrienes (LTs). Cytosolic PLA<sub>2</sub> activation is triggered by various pro-inflammatory signals, initiating a  $Ca^{2+}$ -dependent translocation from the cytosolic space toward the perinuclear membranes. This process involves phosphorylation of Ser-505 in the catalytic domain by MAPK ERK1/2 and phosphorylation of Ser-727 by other MAPKs [16]. To date, few studies have elucidated the role of the signaling pathway in diabetic corneal diseases.

*Cynara cardunculus* L. var. *atilis* (DC.) is a perennial plant native to the Mediterranean region, popularly known as cultivated cardoon and closely related to the more widespread globe artichoke [*C. cardunculus* L. var. *scolymus* (L.) Fiori] and the wild cardoon [*C. cardunculus* L. var. *sylvestris* (Lamk) Fiori], the progenitor of both cultivated species. Traditionally grown for its edible, fleshy leaf midribs and for the use of its flowers as a source of vegetable rennet [17], the cultivated cardoon has recently attracted interest for its potential in the development of natural extracts for pharmacological applications [18]. The primary reason for this interest is its considerable abundance of bioactive molecules, including phenolic acids, flavonoids, and sesquiterpene lactones [19,20], which have been shown to possess antioxidant, anti-inflammatory, and hepatoprotective properties [21]. Among the three *C. cardunculus* taxa, cultivated cardoon is considered the most promising for the extraction of natural bioactive compounds, as it may produce more biomass than wild cardoon and globe artichoke [22].

It has been demonstrated that globe artichoke leaf extract can lower sorbitol levels in rat eye lenses exposed to high glucose conditions. Sorbitol buildup is a hallmark of diabetic retinopathy, and reducing its accumulation could

offer protective benefits against this eye condition. Experimental evidence from both *ex vivo* and *in vivo* models demonstrates that artichoke leaf extract plays a protective role by decreasing oxidative stress and suppressing inflammatory processes, while simultaneously enhancing the efficiency of the endogenous antioxidant defense system [23–25]. The cultivated cardoon, a versatile crop in the Mediterranean diet, is particularly rich in bioactive phenolic compounds, recognized for their antioxidant effects, as well as dietary fiber and essential minerals [26]. Polyphenols possess strong antioxidant properties that enable them to neutralize reactive oxygen species, thereby limiting oxidative stress. This protective effect can reduce the risk of oxidative damage to ocular tissues, which is a key factor in the development of age-related eye diseases such as cataracts and macular degeneration [23]. Clearly, these multifaceted actions cannot be performed by a single compound, but rather by a complex mixture of active ingredients, mainly mono- and dicaffeoylquinic acids and flavonoids such as luteolin, which exert additive or synergistic pharmacological effects.

In the present study, we investigated the early cellular and molecular responses of HG-treated human corneal epithelial cells to cultivated cardoon (CC) leaf extract. Our findings indicate that CC extract improved viability, reduced ROS production, increased antioxidant enzyme activities and migration and reduced the inflammatory process after glucose treatment, suggesting its potential as a preventive agent against the development of chronic diabetic complications. The results, obtained after 24- and 48-hour treatments, are preliminary to understanding CC's potential in diabetic keratopathy.

## 2. Materials and Methods

### 2.1 Reagents

All reagents were from Sigma (St. Louis, MO, USA) or Merck (Darmstadt, Germany) or VWR (Leighton Buzzard, UK). Standards were purchased from Extrasynthese (Lyon, France) or Roth (Karlsruhe, Germany).

### 2.2 Plant Sample Collection

The cultivated cardoon 'Altilis 41', developed by researchers at Catania University as part of a breeding program on *C. cardunculus* L., was selected as the target genotype due to its high production of biomass and bioactive compounds [26,27]. Seeds were placed in plastic pots (diameter: 10 cm; height: 8 cm) filled with a 1:1 (v/v) mixture of peat-based substrate (Profi-Substrate, Gramoflor, Germany) and sand. The pots were placed in an unheated greenhouse and irrigated, when necessary, with deionized water ( $EC \leq 0.01 \text{ dS m}^{-1}$ ). Upon reaching phenological stage 15 [28], per each replicate, seedlings from the five pots were harvested and pooled, after which the leaves were freeze-dried and mechanically ground to a fine powder at ambient temperature. Per each replicate, the ground leaf powders were sieved through a 500  $\mu\text{m}$  mesh, and the re-

sulting underflow fraction was stored at  $-20^\circ\text{C}$  until high-performance liquid chromatography (HPLC) analysis and biochemical tests.

### 2.3 Determination of Bioactive Polyphenolic Compounds

Per each replicate, a lyophilized sample ( $100 \pm 0.5 \text{ mg}$ ) of CC leaves was extracted using 1 mL methanol/water (70:30 v/v) containing 1 mM butylated hydroxytoluene and hesperetin as an internal standard. After shaking for 1 hour at  $25^\circ\text{C}$ , the mixture was centrifuged, and the supernatant was stored. This procedure was performed twice to improve compound recovery, and the collected supernatants were stored at  $-20^\circ\text{C}$  for analysis. Three independent replicates were prepared and analyzed.

Caffeoylquinic acids and flavone profiles were analyzed as already described [29] with some modifications, using a Zorbax Eclipse XDB-C18 column ( $4.6 \times 150 \text{ mm}$ ;  $5.0 \mu\text{m}$  particle size) and diode array detection. The chromatographic separation was performed using a mobile phase composed of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B), with a constant flow rate of  $0.4 \text{ mL min}^{-1}$ . The elution gradient was programmed to increase from 5% to 10% solvent B over the first 10 minutes, then to 25% at 42 minutes, and subsequently to 50% at 45 minutes, which was maintained until 50 minutes. Chromatograms were recorded at 280, 320 (caffeoylquinic acids), 330 (apigenins) and 350 (luteolines) nm. Quantification was based on calibration curves using available commercial standards. Mono- and di-caffeoylquinic acids were quantified using 5-*O*-caffeoylquinic acid (CGA) and 1,3-*O*-dicaffeoylquinic acid (1,3-DCQA) as references, respectively; apigenin and luteolin derivatives were quantified as their respective glucosides (i.e., apigenin-7-*O*-glucoside, A7GLC, and luteolin-7-*O*-glucoside, L7GLC). Compounds were identified by retention time, ultraviolet-visible spectroscopy/mass spectrometry (UV/MS) spectra, and comparison with commercial standards or internal reference (hesperetin) when standards were unavailable. MS/MS analyses were performed using a 6420 Triple Quadrupole Mass Spectrometer equipped with Mass Hunter software (Agilent Technologies, Inc, Santa Clara, CA, USA). The effluent was diverted to waste for the first 2 min of each run, after which negative electrospray ESI (–) was used to ionise compounds eluting from the column. Nitrogen was used both as drying gas at a flow rate of 6 L/min and as nebulising gas at a pressure of 55 psi. The drying gas temperature was  $300^\circ\text{C}$ , and a potential of 4000 V was applied across the capillary. The fragmentor voltage was 120 V, and the collision energies used were in the range 20 V. Quadrupole 1 filtered the known ionised masses of the compounds of interest, whilst quadrupole 2 scanned ions produced by collision in the range  $m/z$  100–1000 at a scan time of 500 ms cycle $^{-1}$ . The data on mono- and di-caffeoylquinic acids were expressed as grams of CGA equivalent and 1,3-DCQA

equivalent per kilogram of dry matter (DM), respectively. Apigenin and luteolin derivatives were expressed as grams of A7GLC and L7GLC per kilogram of DM, respectively.

#### 2.4 Human Corneal Epithelial Cell Culture and Experimental Protocol

Human corneal epithelial cells (HCEpiCs, Innoprot, P10871, Derio, Spain) were grown in Corneal Epithelial Cell Complete Medium (CECM, Innoprot, P60131). Cells were mycoplasma-free (Innoprot, REF: P10871-IM). To validate the authenticity of the cell lines, Innoprot employs Genetic Identity Verification through Short Tandem Repeat (STR) profiling, which is a standard method for human cells.

For high glucose treatments, cells were cultured for 24 h in their respective growth media supplemented with glucose to a concentration of 25 mM. Mannitol treatment was included to distinguish whether the observed effects were due to glucose metabolism or solely osmotic stress. Cells were incubated, for the same time period of 24 h, to the respective media supplemented with 25 mM mannitol.

CC leaf powders, obtained by freeze-drying the plant material, were dissolved in dimethyl sulfoxide (DMSO). The stock solution 0.1 mM, contained a mixture of chemicals extracted from the CC leaves. To evaluate the effect of CC leaf extracts, corneal cells were pre-incubated with the CC extract for 24 h before HG treatment. During the incubation of these cell cultures for the following 24 h, in the presence or absence of 25 mM glucose, the cultures were replaced with artichoke extract in fresh medium. Schematically, experiments were performed on five groups of corneal cultures, incubated with CECM containing 5.5 mM glucose (NG), as follows:

- (1) normal glucose group (NG): 24 h incubation with CECM + 24 h incubation with fresh CECM;
- (2) high glucose group (HG): 24 h incubation with CECM + 24 h incubation with fresh CECM containing glucose to a concentration of 25 mM;
- (3) mannitol group (M): 24 h incubation with CECM + 24 h incubation with fresh CECM containing mannitol to a concentration of 25 mM;
- (4) cultivated cardoon group (CC): 24 h incubation with CECM containing CC leaf extract + 24 h incubation with fresh CECM containing CC extract;
- (5) cultivated cardoon + high glucose group (CC + HG): 24 h incubation with CECM containing CC extract + 24 h incubation with fresh CECM containing CC leaf extract and HG.

#### 2.5 Viability Assay

Cell viability was determined by MTT assay (Chemicon, Temecula, CA, USA) as previously described [30].

#### 2.6 Transepithelial Electrical Resistance

HCEpiCs ( $7 \times 10^4$  cells/cm<sup>2</sup>) were seeded in transwells (0.4  $\mu$ m pore size, Costar, 3412, Washington, DC, USA). After a confluent monolayer has formed, cells were incubated with 10  $\mu$ g/mL or 20  $\mu$ g/mL of CC extract for 24 h. Transepithelial electrical resistance (TEER) was measured using the Millicell-ERS system (MERS 000 01; Millipore, AG, Volketswil, Switzerland), as previously described [31].

#### 2.7 Wound Healing Assay

The effect of CC leaf extract on cell migration after treatment with HG was evaluated by wound healing assay, as previously described [13].

#### 2.8 ROS Measurements

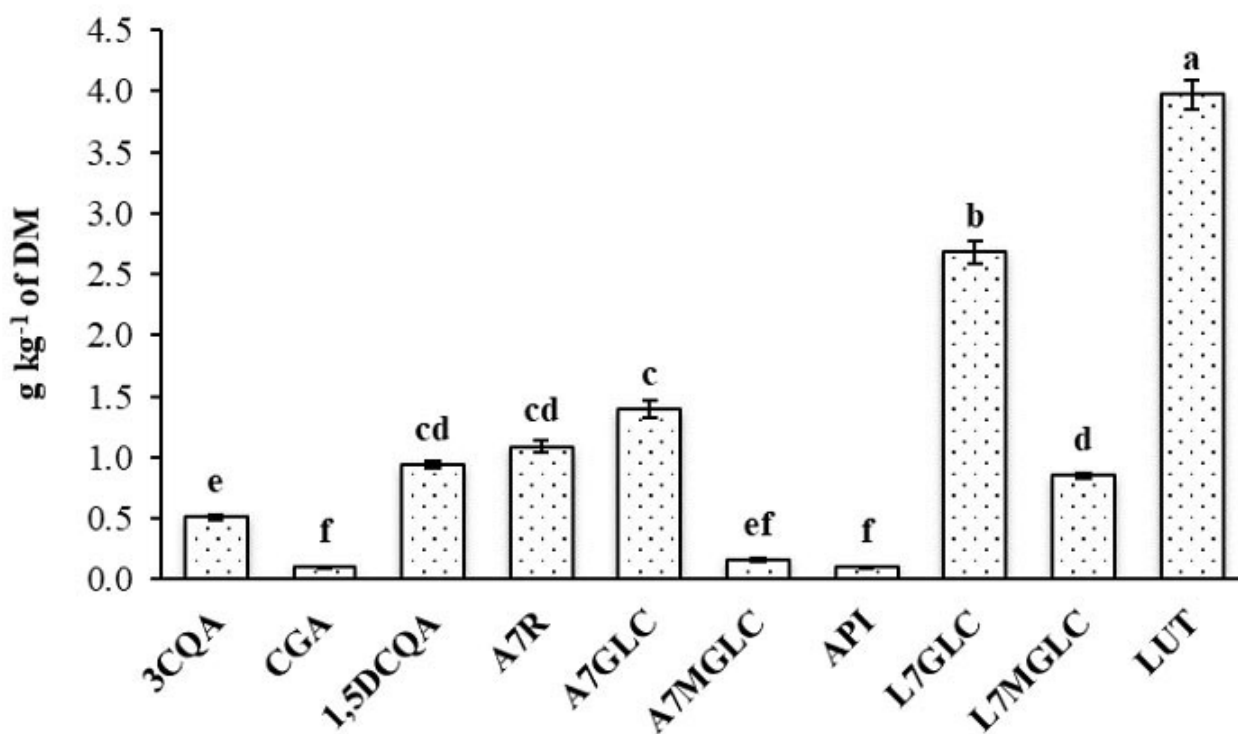
Reactive oxygen species (ROS) were detected using Cellular Reactive Oxygen Species Detection Assay Kit (ab113851, Abcam, Cambridge, UK) as previously described [32].

#### 2.9 Antioxidant Enzyme Assay

After different treatments, the activities of antioxidant enzymes Superoxide dismutase (SOD), catalase (CAT) and the levels of glutathione (GSH) were assayed using commercial kits (SOD Activity CS0009, CAT Activity MAK531 and GSH Assay MAK517, Sigma-Aldrich, Burlington, MA, USA), according to the manufacturer's instructions.

#### 2.10 Immunoblotting

Following treatments, HCEpiCs were lysed and Bradford assay was employed to quantify total protein content. A total of 40  $\mu$ g of protein from each sample was separated by SDS-PAGE and transferred to membranes according to established protocols [15]. Primary antibodies against cPLA<sub>2</sub> (1:1000, sc-376636, Santa Cruz, CA, USA) and phospho-cPLA<sub>2</sub> (1:1000, 2831, Cell Signaling Technology, Danvers, MA, USA), as well as ERK1/2 (1:1000, 4696, Cell Signaling Technology, Danvers, MA, USA) and phospho-ERK1/2 (1:1000, 4370, Cell Signaling Technology, Danvers, MA, USA),  $\beta$  Actin (1:1000, 4967, Cell Signaling Technology, Danvers, MA, USA) and COX-2 (1:1000, 4842, Cell Signaling Technology, Danvers, MA, USA) were used. Membranes were then treated with fluorescent secondary antibodies (1:20,000) for 1 h at room temperature. Membranes initially probed for total cPLA<sub>2</sub> and total ERK1/2 were subsequently stripped and re-probed for phospho-cPLA<sub>2</sub> (1:1000) and phospho-ERK1/2 (1:1000). BIORAD ChemiDoc™ XRS + software (BioRad, Laboratories, Hercules, CA, USA) and Image Quant TL software, v2003 (Image Lab™ Software, Version 6.0, Bio-Rad, Laboratories, Hercules, CA, USA) were used to detect and quantify the signals.



**Fig. 1. Content ( $\pm$  standard deviation) of bioactive phenolic compounds in cultivated cardoon leaves.** Lower case letters (a–f) above the bars indicate statistically significant difference at  $p < 0.05$ . “a” indicates the highest value and “f” indicates the lowest value. (LSD test,  $p < 0.05$ ). 3CQA, 3-*O*-caffeoylquinic acid; CGA, chlorogenic acid; 1,5DCQA, 1,5-*O*-dicafeoylquinic acid; A7R, apigenin-7-*O*-rutinoside; A7GLC, apigenin-7-*O*-glucoside; A7MGLC, apigenin-7-*O*-malonylglucoside; API, apigenin; L7GLC, luteolin-7-*O*-glucoside; L7MGLC, luteolin-7-*O*-malonylglucoside; LUT, luteolin. The data on mono- and di-caffeoylquinic acids were expressed as grams of CGA equivalent and 1,3-DCQA equivalent per kilogram of dry matter (DM), respectively. Apigenin and luteolin derivatives were expressed as grams of A7GLC and L7GLC per kilogram of DM, respectively.

### 2.11 Prostaglandin E2 and Interleukin-6 Release in Cell Media

Following treatments, supernatants of cell cultures were used to determine PGE2 (Cayman Chemical, Ann Arbor, MI, USA) and interleukin-6 (IL-6) levels (R&D Systems Inc., Minneapolis, MN, USA), according to the manufacturer’s instructions. Detection range was 7.8–1000 pg/mL for PGE2 and 3.1–300 pg/mL for IL-6.

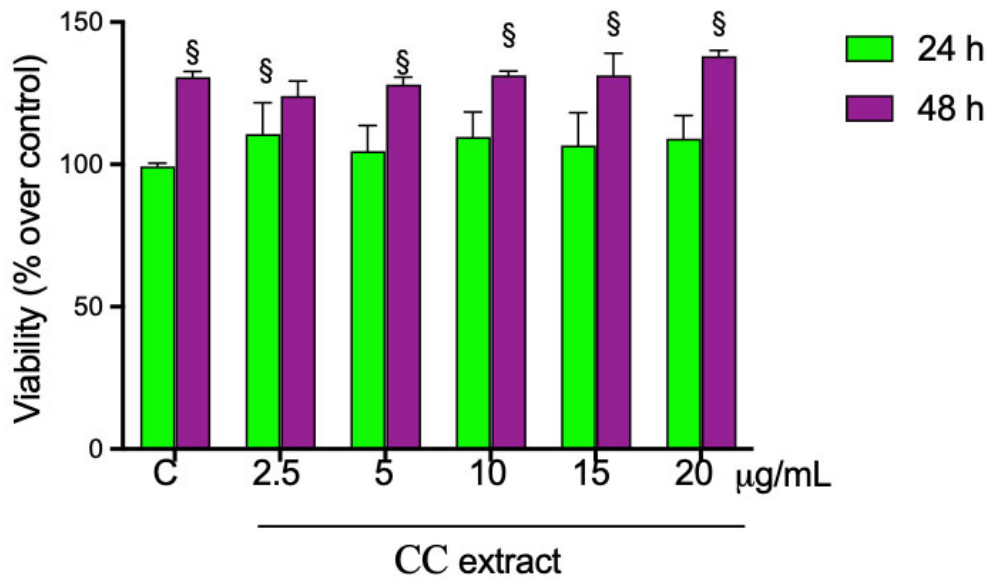
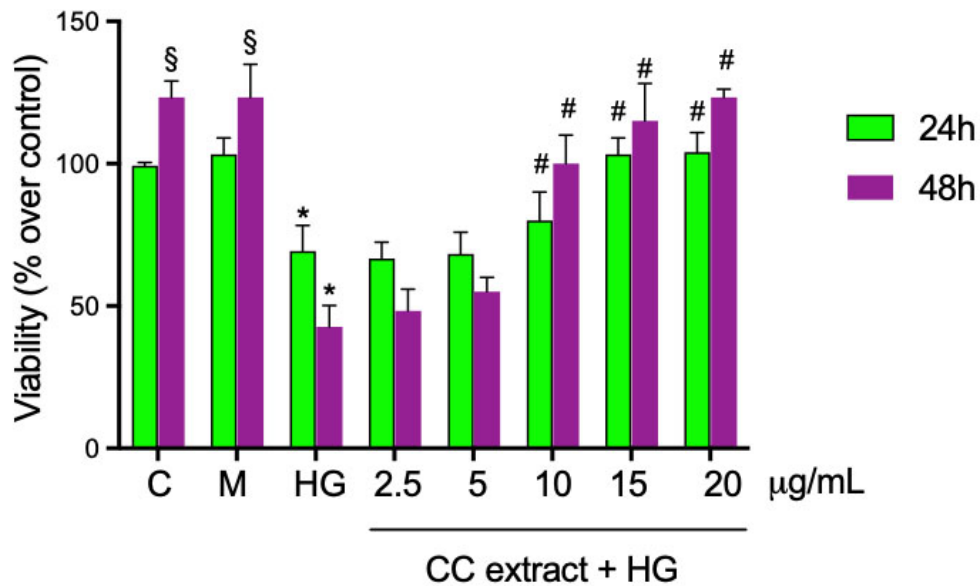
### 2.12 Statistical Analysis

Experiments were conducted in triplicate, each comprising four samples per group ( $n = 4$ ). Values are from mean  $\pm$  standard deviation (SD). Comparisons between experimental and control groups were performed using One-way ANOVA with Tukey–Kramer post hoc test ( $p$ -value  $< 0.05$ ). GraphPad Prism version 7.00 (GraphPad Inc., San Diego, CA, USA) was employed for both statistical analysis and graph construction.

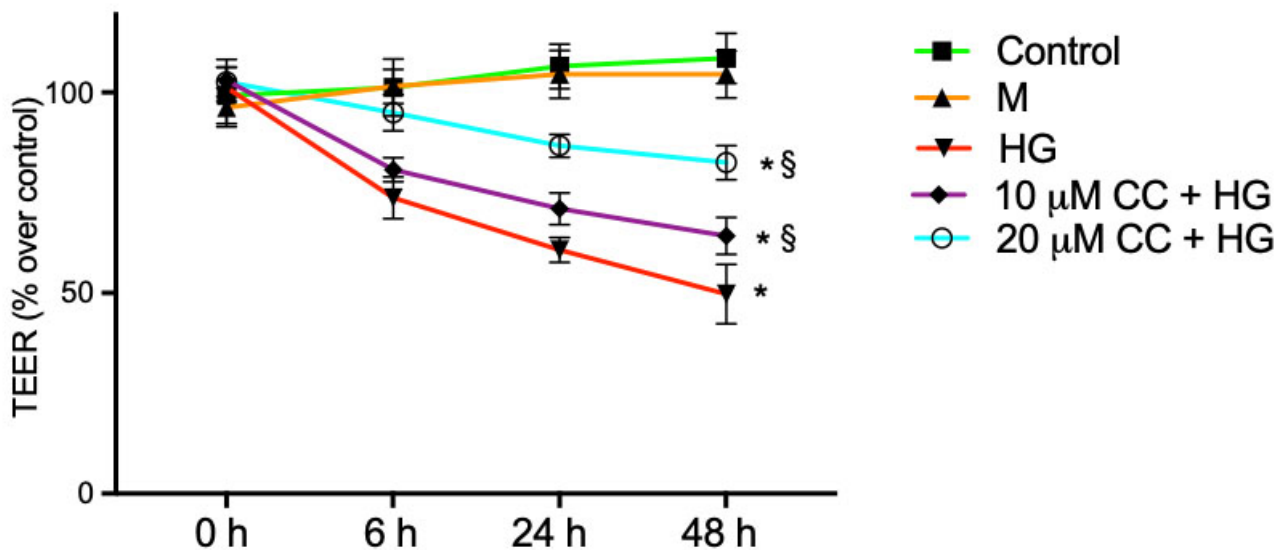
## 3. Results

### 3.1 Bioactive Polyphenolic Compounds in *Cynara cardunculus* var. *altilis*

HPLC-MS was employed to both identify and quantify ten distinct bioactive compounds present in the CC leaf extract, providing a detailed phytochemical profile, belonging to the groups of caffeoylquinic acids and flavones. The latter represented approximately 87% of the total quantified polyphenols, consistent with previous findings in similar species [33]. Among the flavones, luteolin and its derivatives were the most abundant, with luteolin and luteolin-7-*O*-glucoside reaching values of 3.97 and 2.68 g kg<sup>-1</sup> of dry matter, respectively (Fig. 1), in agreement with the findings of Sałata *et al.* [19]. Apigenin-7-*O*-glucoside and 1,5-*O*-dicafeoylquinic acid were the predominant compounds within the apigenin and caffeoylquinic acid groups, with amounts of 1.39 and 0.94 g kg<sup>-1</sup> of dry matter, respectively, as also observed in other phenolic-rich leaf extracts [26,34].

**A****B**

**Fig. 2.** Cell viability was evaluated by MTT assays in human corneal epithelial cells (HCEpiCs). Cells were treated for 24 and 48 h with cultivated cardoon (CC) leaf extract 2.5–5–10–15 and 20 µg/mL in the absence (A) and in the presence (B) of 25 mM glucose (HG). Mannitol 25 mM (M) was used as osmotic control. Results are presented as the mean ± standard deviation (SD) from triplicate measurements obtained from three separate experimental runs. \*  $p < 0.05$  vs. corresponding untreated cells grown for 24 h or 48 h in a medium containing 5.5 mM glucose (C), HG for 24 h (green bar) vs. respective control (C) 24 h (green bar) and HG for 48 h (purple bar) vs. respective control (C) 48 h (purple bar); §  $p < 0.05$  vs. cells with the same treatment at 24 h; #  $p < 0.05$  vs. HG-treated cells without CC leaf extract. One-way ANOVA with Tukey–Kramer post hoc test was used.



**Fig. 3. Transepithelial electrical resistance (TEER).** Barrier integrity was evaluated in HCEpiCs by TEER measurements after incubation for 24 h with 5.5 mM glucose (NG, Control) or 25 mM high glucose (HG) in absence or in presence of 10 µg/mL (10 µg/mL + HG) or 20 µg/mL (20 µg/mL + HG) of cultivated cardoon (CC) leaf extract. Mannitol 25 mM (M) was used as osmotic control. Results are presented as the mean ± standard deviation (SD) from three separate experiments, each performed in triplicate. \*  $p < 0.05$  vs. untreated cells grown in a medium containing 5.5 mM glucose (Control); §  $p < 0.05$  vs. HG-treated cells without extract. One-way ANOVA with Tukey–Kramer post hoc test was used.

### 3.2 *Cynara cardunculus* var. *altilis* Extract Improved the Viability of Human Corneal Epithelial Cells Under High Glucose

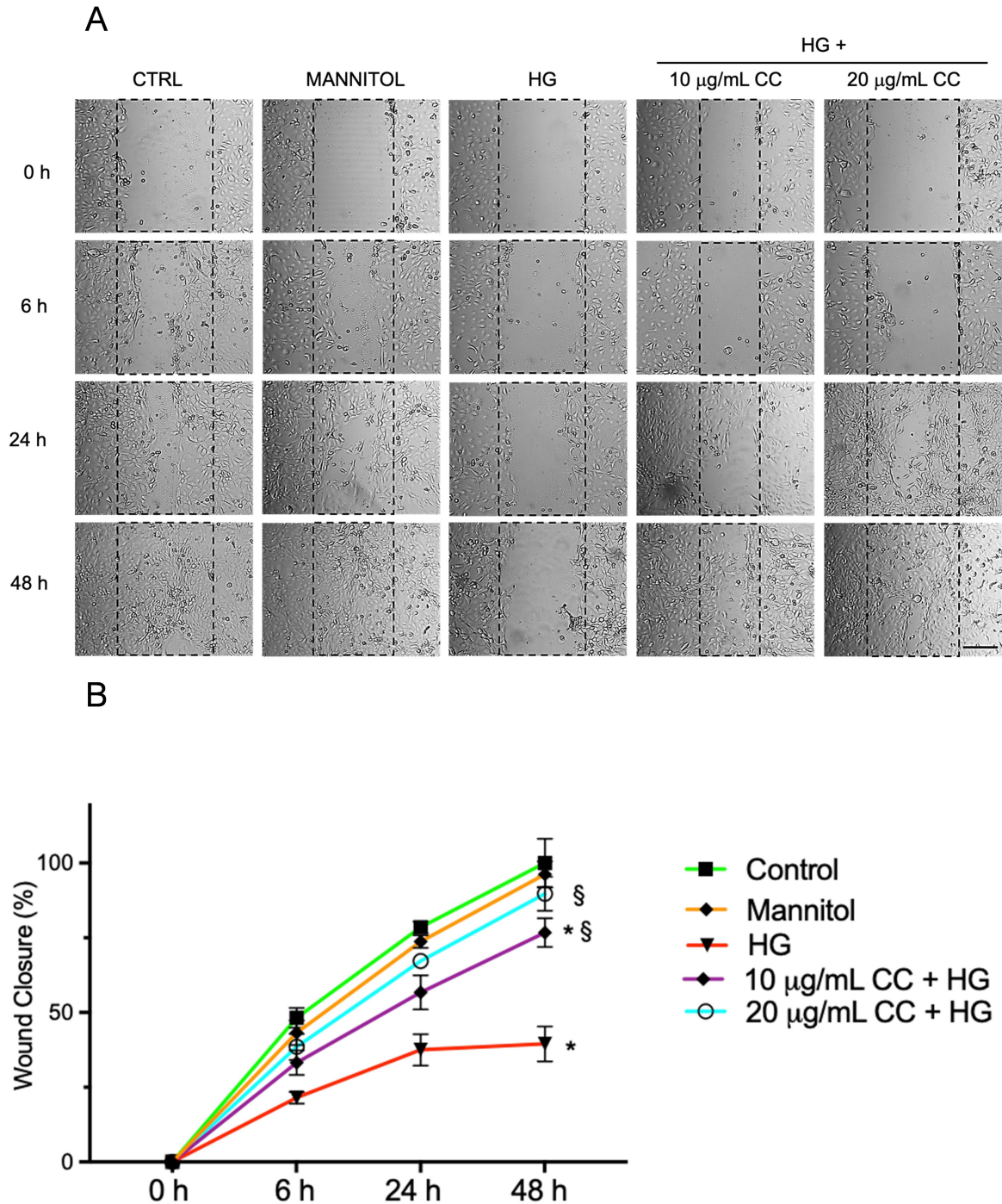
MTT-based cytotoxicity tests were performed to determine the effects of CC leaf extract on cell viability following treatment of HCEpiCs with increasing CC leaf extract concentrations (2.5–5.0–10–15–20 µg/mL). As shown in Fig. 2A, all concentrations caused no effect on cell viability after incubation for 24 and 48 h. In another set of experiments, HCEpiCs were cultured in a medium supplemented with 25 mM glucose (HG) for 24 h and 48 h in the absence and the presence of increasing concentrations of CC leaf extract (2.5–5.0–10–15–20 µg/mL), as schematically described in the Materials and Methods section. Mannitol treatment was included to distinguish whether the observed effects were due to glucose metabolism or solely osmotic stress. The treatment with HG for 24 and 48 h induced a decrease in cell viability by about 31% and 81%, respectively, in comparison with cells incubated in NG medium. Preincubation of the cells for 24 h with increasing concentrations of CC leaf extract 10–15–20 µg/mL and incubation for another 24 h with fresh medium containing CC leaf extracts in the presence of HG significantly increased cell viability by about 16%, 49% and 50%, respectively, when evaluated against the corresponding values observed in HG-treated cells (Fig. 2B). After 48 h in the presence of HG, 10–15–20 µg/mL of CC leaf extract induced an increase of cell viability by about 2.4, 2.7 and 2.9 fold, respectively, in comparison with cells treated with HG. The presence of

lower concentrations of CC leaf extract (2.5 and 5 µg/mL) did not significantly alter the viability compared with cells treated with HG. Treatment with mannitol 25 mM did not alter cell viability.

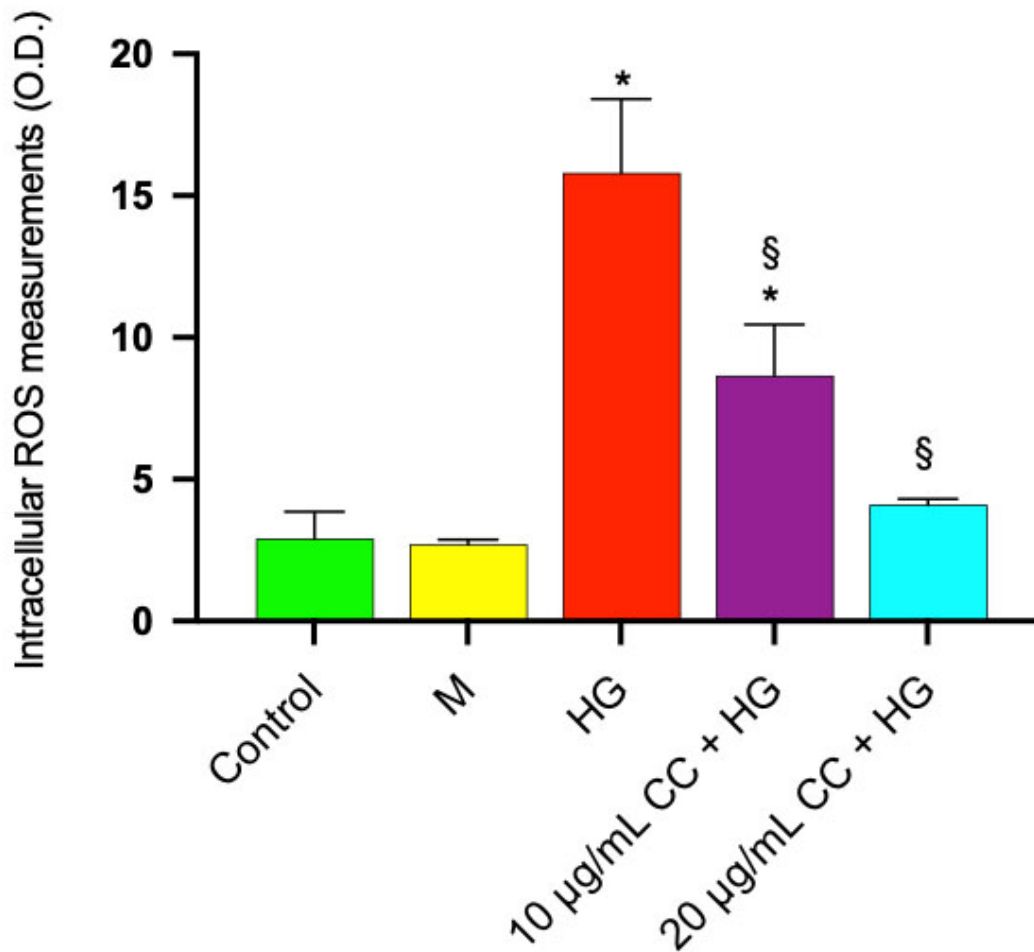
Following these findings, further treatments were performed using HG for 24 h, excluding the incubation for 48 h that was excessively harmful for HCEpiCs. Moreover, the CC leaf extract was used at concentrations of 10 and 20 µg/mL, which gave the best protection results after treatment with HG.

### 3.3 *Cynara cardunculus* var. *altilis* Extract Prevented HG-Induced Reduction of Transepithelial Electrical Resistance (TEER)

The effect of CC leaf extract on epithelial barrier integrity was evaluated by TEER assays. HCEpiCs were plated on Transwell membranes and incubated for 6 h, 24 h and 48 h with 25 mM glucose (HG) in the absence or in the presence of 10 µg/mL and 20 µg/mL of CC leaf extract. As shown in Fig. 3, the treatment with HG significantly reduced the values by approximately 28%, 42% and 54% after 6 h, 24 h and 48 h, respectively, compared with untreated cells. Preincubation of the cells for 24 h with 10 µg/mL of CC leaf extract and incubation with fresh medium containing 10 µg/mL of the CC leaf extract in presence of HG for 6 h, 24 h and 48 h, significantly increased TEER by about 10%, 16% and 30%, respectively, when evaluated in comparison with HG-treated cells. Even more, preincubation of the cells for 24 h with 20 µg/mL of CC leaf extract and in-



**Fig. 4. Migration of HCEpiCs after wound healing.** (A) selected images illustrating wound healing assays carried out in HCEpiCs incubated with normal glucose (NG, 5.5 mM glucose, Control), Mannitol 25 mM, high glucose (HG, 25 mM glucose), or with HG in presence of 10  $\mu\text{g/mL}$  (10  $\mu\text{g/mL}$  + HG) or 20  $\mu\text{g/mL}$  (20  $\mu\text{g/mL}$  + HG) of cultivated cardoon (CC) leaf extract, immediately after the wound at 0 h and after 6, 24 and 48 h. Images were captured using a Leica microscope at 20 $\times$  magnification, with a scale bar representing 200  $\mu\text{m}$ . (B) relative quantification of wound closure percentage after 6, 24 and 48 h of incubation of the cells with different treatments above described. Results are presented as the mean  $\pm$  standard deviation (SD) from three separate experiments, each performed in triplicate. \*  $p < 0.05$  vs. untreated cells grown in a medium containing 5.5 mM glucose (Control); §  $p < 0.05$  vs. HG-treated cells without extract. One-way ANOVA with Tukey–Kramer post hoc test was used.



**Fig. 5. ROS production was evaluated in HCEpiCs after incubation with DCFDA as described in Materials and Methods.** HCEpiCs were incubated for 24 h in the absence (Control) or in the presence of 25 mM glucose (HG) or in the presence of 10 µg/mL (10 µg/mL CC + HG) or in the presence of 20 µg/mL (20 µg/mL CC + HG) of cultivated cardoon (CC) leaf extract. Mannitol 25 mM (M) was used as osmotic control. Results are presented as the mean ± standard deviation (SD) from three separate experiments, each performed in triplicate. \*  $p < 0.05$  vs. untreated cells grown in a medium containing 5.5 mM glucose (Control); §  $p < 0.05$  vs. HG-treated cells without CC extract. One-way ANOVA with Tukey–Kramer post hoc test was used.

cubation with fresh medium containing 20 µg/mL of the CC leaf extract in presence of HG for 6 h, 24 h and 48 h, significantly increased TEER by about 30%, 42% and 67%, respectively, in comparison with HG-treated cells. Treatment with mannitol (M) 25 mM did not alter cell viability. The presence of 10 µg/mL or 20 µg/mL of CC leaf extract on control cells incubated for the same time in absence of HG did not modify TEER values compared with cells incubated without CC leaf extract (data not shown).

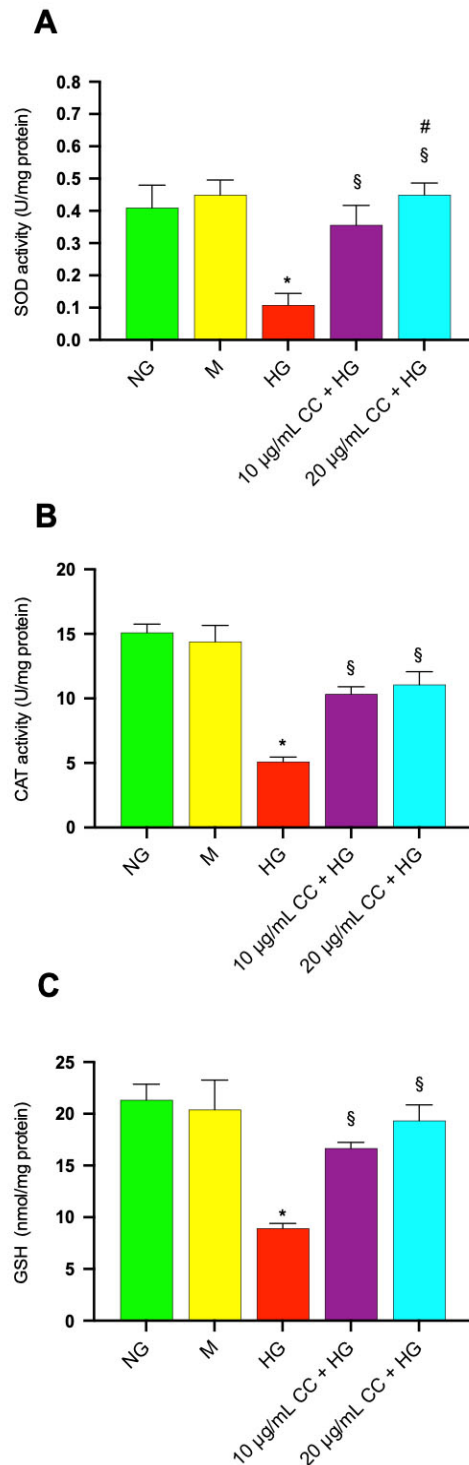
#### 3.4 *Cynara cardunculus* var. *atilis* Extract Improved the Migration of Human Corneal Epithelial Cells Under High Glucose

The wound healing assay was employed to evaluate the effect of CC leaf extract on HCEpiCs under high glucose environment. As shown in Fig. 4A,B, the treatment with HG reduced the wound closure by about 50% after 6 and 24 h and by 63% after 48 h, in comparison to untreated

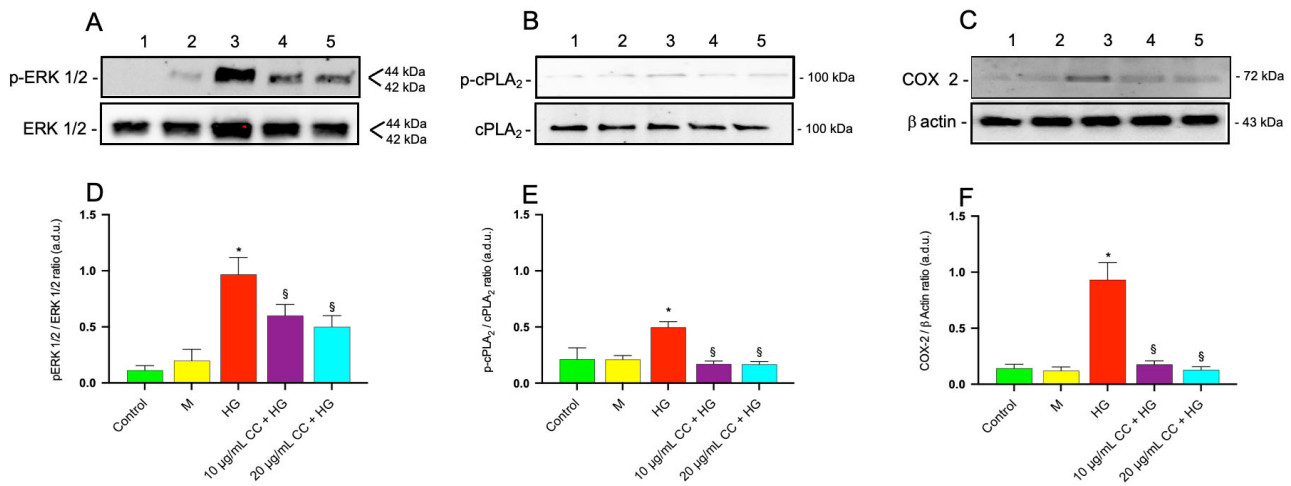
cells. Preincubation of the cells for 24 h with 10 µg/mL or 20 µg/mL of CC extract and incubation with fresh medium containing 10 µg/mL or 20 µg/mL of the CC leaf extract in presence of HG for 24 h induced an increase of 1.5 and 1.8 fold, respectively, and after 48 h, an increase of 2.0 and 2.4 fold, respectively, in comparison to HG-treated cells.

#### 3.5 *Cynara cardunculus* var. *atilis* Extract Significantly Decreased the Production of Reactive Oxygen Species (ROS)

High concentrations of glucose have been shown to cause the production of intracellular ROS, which leads to oxidative stress in rabbit corneal epithelial cells [35]. Our results also demonstrated a significant increase in ROS production after incubation of HCEpiCs with HG for 24 h compared with control cells incubated in a medium containing 5.5 mM glucose (NG). In Fig. 5, ROS production was significantly increased by 5.4 fold in comparison with con-



**Fig. 6. Effect of CC leaf extract on antioxidant defenses in HCEpiCs exposed to high glucose: modulation of SOD and CAT activities (A, B) and intracellular GSH levels (C).** (A) Superoxide dismutase (SOD), (B) catalase (CAT) antioxidant activity and (C) glutathione (GSH) content were evaluated in HCEpiCs incubated for 24 h in the absence (Control, NG) or in the presence of 25 mM glucose (HG) or in the presence of 10 µg/mL (10 µg/mL CC + HG) or in the presence of 20 µg/mL (20 µg/mL CC + HG) of cultivated cardoon (CC) leaf extract. Mannitol 25 mM (M) was used as osmotic control. Results are presented as the mean ± standard deviation (SD) from three separate experiments, each performed in triplicate. \*  $p < 0.05$  vs. untreated cells grown in a medium containing 5.5 mM glucose (Control); §  $p < 0.05$  vs. HG-treated cells without CC leaf extract. #  $p < 0.05$  vs. HG-treated cell in the presence of 10 µg/mL of leaf extract (10 µg/mL CC + HG). One-way ANOVA with Tukey–Kramer post hoc test was used.



**Fig. 7. Effect of CC leaf extract on ERK 1/2, cPLA<sub>2</sub> phosphorylation and COX-2 expression in HCEpiCs exposed to high glucose.** Immunoblot analysis was conducted using specific antibodies targeting p-ERK 1/2 and total ERK 1/2 (A), activated (phosphorylated, p-cPLA<sub>2</sub>) and total cPLA<sub>2</sub> (B), as well as COX-2 (C) proteins. The blots were also probed with an anti  $\beta$  Actin antibody to ensure equal loading of protein per lane (C). Densitometric analysis of the immunoblots was performed using Image J software (Version 1.52a, NIH, Bethesda, MD, USA) to quantify protein levels for each band (in arbitrary densitometry units, a.d.u.). Densitometric analysis of western blots assessing ERK 1/2 phosphorylation (ratio of p-ERK 1/2/ERK 1/2 in (D)) and cPLA<sub>2</sub> (ratio of p-cPLA<sub>2</sub>/cPLA<sub>2</sub> in (E)) are reported. For COX-2 protein, the data were normalized to  $\beta$  Actin (F). Panels represent different treatments, indicated by numbers (A–C) and by distinct colors (D–F): control cells in 5.5 mM glucose (1, green), 25 mM mannitol-treated cells (2, yellow), 25 mM glucose-treated cells, HG (3, red), 25 mM glucose (HG) in presence of 10  $\mu$ g/mL of cultivated cardoon (CC) leaf extract (4, purple) and 25 mM glucose (HG) in the presence of 20  $\mu$ g/mL of CC leaf extract (5, cyan). Results are presented as the mean  $\pm$  standard deviation (SD) from three separate experiments, each performed in triplicate. \*  $p < 0.05$  vs. untreated cells grown in a medium containing 5.5 mM glucose (Control); §  $p < 0.05$  vs. HG-treated cells without CC extract. One-way ANOVA with Tukey–Kramer post hoc test was used.

control cells incubated in NG medium. Preincubation with 10  $\mu$ g/mL and 20  $\mu$ g/mL of CC leaf extract reduced ROS production by 45% and 74%, respectively, in comparison with HG-treated cells. Treatment with mannitol (M) 25 mM did not induce ROS production.

### 3.6 *Cynara cardunculus* var. *atilis* Extract Affected Antioxidant Enzymatic Activities in HCEpiCs in a High Glucose Environment

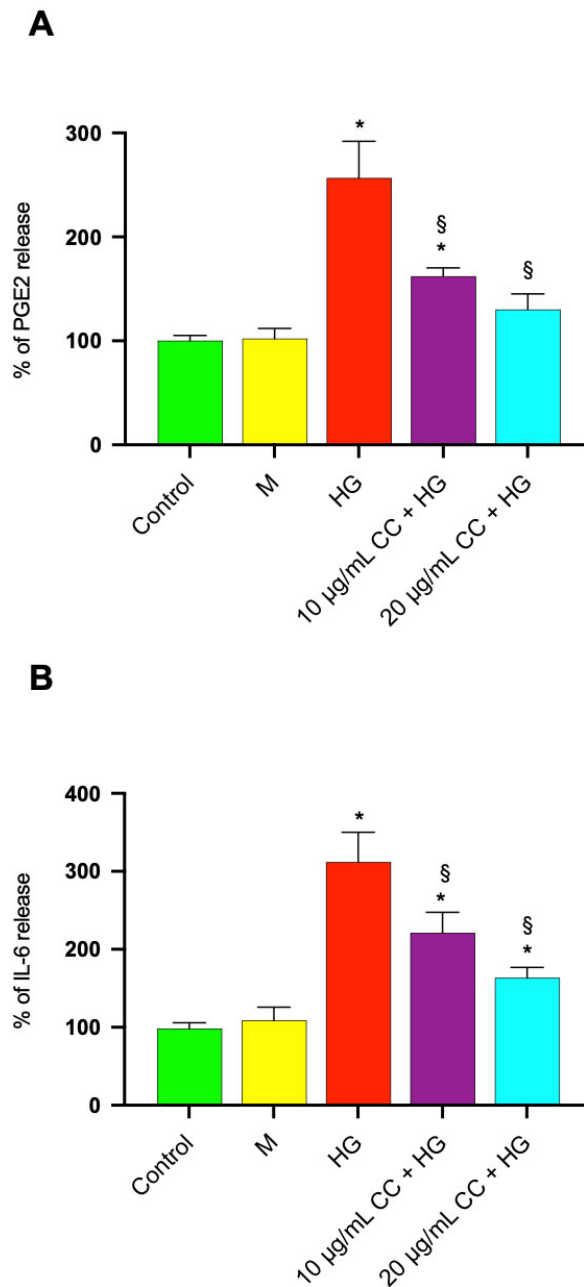
To determine whether CC leaf extract modulates the antioxidant defenses of HCEpiCs, we quantified the activities of superoxide dismutase (SOD) and catalase (CAT), as well as the intracellular concentration of glutathione (GSH). These biomarkers represent the primary enzymatic and non-enzymatic mechanisms responsible for scavenging reactive oxygen species within cells. The treatment with HG induced a significant decrease of SOD and CAT activities and GSH level. In Fig. 6A,B, the levels of SOD and CAT activity were reduced by 4.4 and 2.3 fold, respectively, after incubation with HG in comparison with control cells in normal glucose. Incubation of the cells with HG in association with 10  $\mu$ g/mL or 20  $\mu$ g/mL of CC leaf extract, induced an increase of SOD activity by 3.3 and 4.5 fold, respectively, and CAT activity by 1.8 and 2.2 fold, respectively, in comparison to HG-treated cells without CC leaf extract.

GSH content was also significantly reduced by 2.3 fold after HG treatment compared with untreated cells (Fig. 6C). When the HG-treatment was conducted in association with 10  $\mu$ g/mL or 20  $\mu$ g/mL of CC leaf extract, the GSH amount increased by 1.8 and 2.2 fold, respectively, in comparison to HG-treated cells without CC leaf extract, highlighting a protective effect.

### 3.7 *Cynara cardunculus* var. *atilis* Extract Down-Regulated the ERK1/2/cPLA<sub>2</sub>/COX-2 Axis in HCEpiCs Treated With HG

Since our *in vitro* study demonstrated that the activation of the ERK/cPLA<sub>2</sub>/COX-2 axis represents an early event after exposure of microvascular endothelial cells to HG [15], we investigated the potential involvement of ERK 1/2 and cPLA<sub>2</sub> in our experimental model. It is well established that the phosphorylation of cPLA<sub>2</sub> is essential for its activation, enabling it to release arachidonic acid from membrane phospholipids, a process that is regulated by ERK 1/2.

Immunoblots for p-ERK 1/2 and total ERK 1/2 are shown in Fig. 7A. Treatment of HCEpiCs with HG led to a significant 2.0 fold increase in phospho-ERK 1/2 levels compared with control cells, as shown in Fig. 7D (p-ERK 1/2/ERK 1/2 ratio). Incubation of the cells with HG in as-



**Fig. 8. Effect of CC leaf extract on PGE2 and IL-6 release in HCEpiCs exposed to high glucose.** Prostaglandins E2 (PGE2) and interleukin-6 (IL-6) release were evaluated in medium of HCEpiCs incubated for 24 h in the absence (Control) or in the presence of 25 mM glucose (HG) or in the presence of 10 µg/mL (10 µg/mL CC + HG) or in the presence of 20 µg/mL (20 µg/mL CC + HG) of cultivated cardoon (CC) leaf extract. Mannitol 25 mM (M) was used as osmotic control. After incubation, aliquots of supernatants were used for PGE2 (A) and IL-6 (B) determination by ELISA Assay Kits. Results are presented as the mean  $\pm$  standard deviation (SD) from three separate experiments, each performed in triplicate. \*  $p < 0.05$  vs. untreated cells grown in a medium containing 5.5 mM glucose (Control); §  $p < 0.05$  vs. HG-treated cells without CC extract. One-way ANOVA with Tukey–Kramer post hoc test was used.

sociation with 10 µg/mL or 20 µg/mL of CC leaf extract reduced the phosphorylation of ERK 1/2 by about 2.2 fold and 2.6 fold, respectively, compared with HG-treated cells.

Immunoblots for p-cPLA<sub>2</sub> and total cPLA<sub>2</sub> are shown in Fig. 7B and quantitative analysis of western blots (ratio of p-cPLA<sub>2</sub>/cPLA<sub>2</sub>) reported in Fig. 7E demonstrated a 3.2

fold increase in the phosphorylated (active) form of cPLA<sub>2</sub> in HCEpiCs treated with HG compared with control cells. Incubation of the cells with HG in the presence of 10 µg/mL or 20 µg/mL of CC leaf extract reduced the phosphorylation of cPLA<sub>2</sub> by 2.8 fold and 3.0 fold, respectively, compared with HG-treated cells. This HG-induced increase in cPLA<sub>2</sub>

phosphorylation supports enhanced enzyme activity, leading to more arachidonic acid being available for cyclooxygenase activity. Based on this, we assessed COX-2 protein levels after treating HCEpiCs with HG, both in the presence and the absence of CC leaf extract.

Immunoblots for COX-2 are shown in Fig. 7C. The quantitative analysis of immunoblots for COX-2, represented by the COX-2/ $\beta$ -Actin ratio (Fig. 7D), revealed that HG treatment led to a 3.3 fold increase in COX-2 levels compared with untreated control cells, confirming its inflammatory effect. The treatment with HG in the presence of 10  $\mu$ g/mL or 20  $\mu$ g/mL of CC leaf extract significantly reduced COX-2 levels by 3.2 and 4.1 fold, respectively, compared with HG-treated cells in the absence of CC leaf extract (Fig. 7F).

No significant change in p-ERK, p-cPLA<sub>2</sub> and COX-2 expression was observed in Mannitol treated cells compared with controls.

### 3.8 *Cynara cardunculus* var. *altilis* Extract Reduced PGE2 and IL-6 Release in HCEpiCs Treated With HG

Since COX-2 levels were significantly increased after HG treatment, PGE2 release was quantified in HCEpiC treated with HG in the absence/presence of 10  $\mu$ g/mL or 20  $\mu$ g/mL of CC leaf extract. Treatment of HCEpiCs with HG increased PGE2 release by 2.5 fold in comparison to untreated cells (Fig. 8A). The treatment with HG in association with 10  $\mu$ g/mL or 20  $\mu$ g/mL of CC leaf extract reduced the release by 1.6 and 1.9 fold, respectively. PGE2 levels in the medium of untreated HCEpiC were  $380 \pm 29$  pg/mL.

To confirm that treatment with CC leaf extract was able to reduce the inflammatory process, IL-6 levels were measured in the medium of HCEpiC treated with HG in the absence/presence of 10  $\mu$ g/mL or 20  $\mu$ g/mL of CC leaf extract. As shown in Fig. 8B, HG treatment increased IL-6 release by about 3.0 fold compared to untreated control cells. The treatment with HG in association with 10  $\mu$ g/mL or 20  $\mu$ g/mL of CC leaf extract reduced the release by 1.4 and 1.8 fold, respectively. IL-6 levels in the medium of untreated HCEpiC were  $127 \pm 10$  pg/mL.

No significant change in PGE2 and IL-6 release was observed in Mannitol treated cells compared with controls.

## 4. Discussion

The global rise in diabetes mellitus has led to a worrying increase in complications associated with the disease. Chronic hyperglycemia causes significant biochemical and metabolic changes that result in both macrovascular and microvascular damage. Macrovascular complications primarily affect the heart and brain, while microvascular damage contributes to neuropathy, nephropathy, and ocular tissue injuries [2]. Patients with diabetes often experience a range of ocular surface issues, including epithelial defects, recurrent corneal erosion, ulcers, corneal edema, superficial punctate keratitis, and delayed corneal epithelial

wound healing. In particular, diabetic keratopathy is an ocular complication of diabetic peripheral neuropathy, characterized by impaired corneal epithelial wound healing and reduced subbasal corneal nerve plexus density and hypoesthesia [6]. Clinically, this manifests as ocular surface discomfort, often described as burning or dryness, due to impaired corneal innervation and epithelial integrity [36]. Multiple studies have demonstrated that the diabetic corneal epithelium is characterized by increased cellular fragility, decreased epithelial cell density, reduced epithelial thickness, and compromised barrier integrity. These structural and functional abnormalities are indicative of disrupted cellular homeostasis, involving dysregulation of critical processes such as proliferation, differentiation and migration [37].

It has been shown that both oxidative stress and the inflammatory process are responsible for the damage to the ocular tissues of diabetic patients. Recognizing the significant role of oxidative stress in the progression of diabetic complications, extensive research has investigated the protective potential of various antioxidants, particularly those derived from plants. Natural compounds such as  $\alpha$ - and  $\gamma$ -tocopherol, retinol, carotenoids and phytochemicals including anthocyanins and polyphenols, have demonstrated beneficial effects. These substances help mitigate complications by inhibiting the synthesis of prostaglandins and pro-inflammatory cytokines and by reducing oxidative stress markers [38].

Polyphenols, a diverse class of secondary metabolites found in plant-based foods such as fruit, vegetables, nuts and tea, contribute to the maintenance of ocular physiological homeostasis and promote tissue repair under pathological conditions [39,40].

*Cynara cardunculus* L. botanical complex is recognized as a rich source of health-promoting compounds, including polyphenols, inulin, vitamins, and minerals [26, 27]. Leaf extracts of both globe artichoke and cardoons have traditionally been used extensively in folk medicine, particularly for the treatment of liver disorders. The therapeutic effects of these extracts have often been attributed to key phenolic constituents particularly present in the heads and leaves, such as 1caffeoylquinic acids and flavones [41].

In our study, CC leaf extract alone did not alter cell viability, as reported in Fig. 2A, nor did it affect any of the measured parameters (data not shown). The treatment of HCEpiCs with 25 mM glucose significantly reduced corneal cell viability, confirming results by others that demonstrated a decreased epithelial cell density in the cornea in the presence of elevated glucose levels, both in animal models and in human research studies [42,43]. To select the CC leaf extract concentration with the best protective effect on HG-treated HCEpiCs, cell viability was determined using MTT assay, which provides information on cellular metabolic activity, thereby discriminating between living and dead cells. The results demonstrated that the pre-

treatment of corneal cells with cultivated cardoon extract preserved their viability by protecting them from HG damage.

Physiologically, to fulfill its protective barrier function, the cornea maintains a high TEER, guaranteed by the presence of tight junctions between epithelial cells, as well as a marked regenerative capacity which, in response to an insult, activates mechanisms of cell proliferation and migration [4]. Ultrastructural analysis via scanning electron microscopy revealed disrupted intercellular architecture in diabetic rats, associated with compromised integrity among corneal cells, responsible of the epithelial barrier [44] and *in vitro* studies also demonstrated significantly lower TEER values in corneal epithelial cell monolayers under diabetic conditions [12]. Our findings confirmed an HG-induced significant reduction in TEER, indicating a compromise in the integrity of the corneal epithelial barrier, and demonstrated that CC leaf extract maintained high TEER values in the presence of 25 mM glucose. Furthermore, we confirmed that HG treatment slows down the wound healing of HCEpiC [13]. In the present study, the treatment with HG in the presence of CC leaf extract effectively accelerated migration and wound closure to normal levels. Rapid re-epithelialization is essential to ensure the physiological function of the cornea; CC leaf extract, by ensuring a re-epithelialization process, potentially prevents corneal damage. In diabetic patients, delayed epithelial healing increases the risk of ocular complication and microbial infections, particularly in the context of an already compromised immune system [45,46]. Consequently, considerable efforts have been directed toward identifying agents capable of enhancing epithelial repair in the diabetic cornea such as cytokines [47], silk-derived proteins [48] and natural substances [49]. Our findings showed the effect of CC extract in promoting and accelerating cell migration, counteracting the damage of HG, and represent a promising outcome in the context of therapies based on the use of natural substances.

A close correlation has been also shown between diabetic corneal dysfunction and increased ROS production. Prolonged hyperglycemia can trigger glucose-induced protein auto-oxidation and promote ROS generation [35].

Oxidative stress is the consequence of a disrupted balance in which ROS are generated at a rate that surpasses the cell's antioxidant capacity to neutralize or repair the resulting oxidative damage, particularly present in the conjunctiva and cornea, highly exposed to UV radiation and particularly vulnerable to oxidative stress [50]. In diabetic mice, corneal injury leads to heightened oxidative stress, which significantly hinder corneal epithelial healing and elevate the risk of persistent epithelial defects [51].

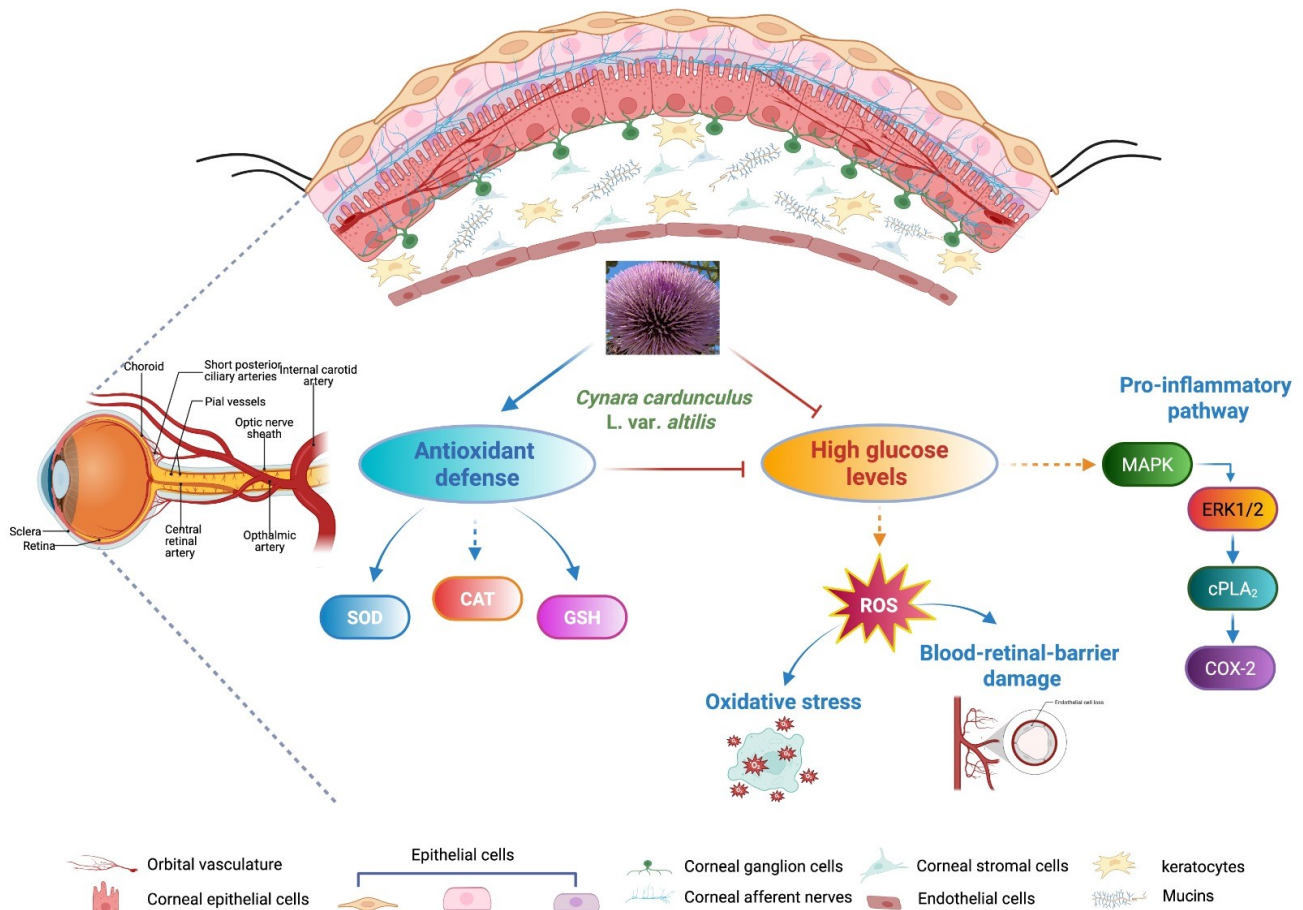
The endogenous antioxidant defense system includes both enzymatic and nonenzymatic antioxidants. Enzymatic antioxidants consist of SOD, CAT, glutathione peroxidase and glutathione oxidase, and, among the non-enzymatic an-

tioxidants, a significant role is played by GSH, synthesized by metabolic processes [52]. In patients with type 2 diabetes mellitus, insulin treatment has been shown to only partially reduce oxidative stress, as evidenced by persistently low GSH levels [10].

We therefore examined the effect of CC leaf extract on ROS concentrations and on several antioxidant enzyme activities and we found that CC leaf extract significantly decreased the production of ROS, increased SOD and CAT antioxidant activities as well as GSH content in HCEpiCs in a high glucose environment.

In eukaryotic cells, the bulk of oxidative metabolism is carried out in the mitochondria and endoplasmic reticulum. Within these organelles, standard redox reactions inevitably lead to the generation of free radicals, which, if not efficiently neutralized, can contribute to oxidative stress. The mitochondrial electron transport chain is responsible for producing approximately 90% of cellular ROS [53]. Moreover, metabolic pathways involving enzymes such as COX-2 and cPLA<sub>2</sub> play a key role in ROS generation. A study on an *in vivo/ex vivo* hypoxia model showed that the activation of cPLA<sub>2</sub> is involved in ventilator-induced diaphragm dysfunction by promoting mitochondrial ROS production, highlighting a positive interaction between cPLA<sub>2</sub> activity and oxidative stress at the mitochondrial level [54]. It also demonstrated in microglial cells, stimulated with lipopolysaccharide (LPS), an increase in the cPLA<sub>2</sub> activation alongside ERK1/2 activation and the ROS production induced by LPS was significantly reduced by cPLA<sub>2</sub> inhibitors such as arachidonyl trifluoromethyl ketone (AA-COCF<sub>3</sub>) but not by the iPLA<sub>2</sub> inhibitor BEL, indicating that cPLA<sub>2</sub> is specifically involved in mediating LPS-induced oxidative stress in microglial cells [55]. Moreover, in lens epithelial cells, cPLA<sub>2</sub> plays a crucial role in PDGF-stimulated cell growth, with its activation tightly regulated by calcium-dependent membrane translocation. This membrane targeting is essential for cPLA<sub>2</sub> to activate NADPH oxidase, leading to increased ROS production. These findings highlight that cPLA<sub>2</sub> plays a key role in linking calcium signaling to oxidative stress in corneal epithelial cells [56].

Therefore, elevated intracellular ROS not only damage macromolecules but also function as signaling molecules, triggering a cascade of biochemical and molecular responses that disrupt normal cellular signaling pathways [57,58]. Our results showed the significant inhibitory effect of CC extract on HG-induced cPLA<sub>2</sub> and ERK1/2 phosphorylated forms and COX-2 and on PGE<sub>2</sub> and IL-6 production. These findings suggest that CC extract may exert wide protective effects on glucose stressed HCEpiCs. One could hypothesize that, during ocular pathologies due to diabetes complications, free radicals could activate the ERK1/2/cPLA<sub>2</sub>/COX-2 signaling, triggering the inflammatory process and, at the same time, the activation of this pathway could lead to the production of other ROS, creat-



**Fig. 9. Potential signaling pathways modulated by *Cynara cardunculus* L. cultivated cardoon (CC) leaf extract in high glucose exposed human corneal cells.** *Cynara cardunculus* L. extract enhances the antioxidant defense enzymes including SOD, CAT activities and GSH content and inhibits ROS production and pro-inflammatory pathways, especially ERK 1/2/cPLA<sub>2</sub>/COX-2 signaling in high glucose exposed human corneal cells, highlighting the possibility that it could perform a protective function in the management of diabetic keratopathy. BioRender. Scuto, M. (2025) <https://BioRender.com/an2rc7d>.

ing a positive feedback loop (Fig. 9). We have not directly tested whether ROS act exclusively as upstream activators or are generated as a downstream product of this cascade. Further investigation will be necessary to validate the regulatory role of ROS in this context.

In this scenario, identifying therapeutics that could alleviate these conditions is crucial for advancing new treatment strategies for diabetic keratopathy. Our results indicate that CC leaf extract could have strong potential as a therapeutic agent, controlling both the inflammatory process and the production of ROS, thus maintaining intact the protective and regenerative functions of the cornea.

This study reports preliminary results on the effect of CC extract after *in vitro* treatment of corneal cells with HG. It is hoped that the results obtained will lay the foundation for future *in vivo* and clinical studies. Clearly, CC extract alone will not be able to replace the traditional therapy that diabetic patients undergo, but it could be used as a dietary supplement to reduce the damage in diabetic keratopathy.

Despite decades of research into oxidative stress as a

key pathogenic factor in the development of diabetes mellitus and its complications, this area remains highly relevant. Contemporary approaches to diabetes management are increasingly centered on personalized antioxidant therapies, incorporating targeted delivery systems and strategies that focus on specific sources of ROS.

Further investigation should be carried out to identify reliable biochemical markers of free radical activity and to explore the interplay between oxidative stress and inflammation, aiming to better understand their combined roles in ocular disease progression.

## 5. Conclusions

We investigated the effects of CC extract on cell migration and viability using an *in vitro* model of hyperglycemia-induced corneal cells. Our results demonstrated that cultivated cardoon leaf extract (i) reduced oxidative stress by lowering ROS production and enhancing SOD, CAT activities and GSH content in high glucose exposed human corneal cells; (ii) maintained a high TEER and

promoted corneal epithelial wound healing; (iii) induced down-regulation of the ERK 1/2/cPLA<sub>2</sub>/COX-2 signaling pathway involved in the inflammatory process and ROS production.

Collectively, these findings indicate that cultivated cardoon extract has the potential to enhance human corneal epithelial cell function. This provides a scientific basis for considering its development as a therapeutic agent in the prevention or treatment of diabetic keratopathy.

### Availability of Data and Materials

All data reported in this paper will also be shared by the lead contact upon request.

### Author Contributions

GL, ATS, and CDA designed the research study. AC, AA, CP, SL, GP and MS performed the research. GL, ATS, CDA and CL analyzed the data. GL, ATS, and CDA wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

### Ethics Approval and Consent to Participate

Not applicable.

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### Conflict of Interest

The authors declare no conflict of interest. Given her role as the Editorial Board member, Gabriella Lupo had no involvement in the peer-review of this article and has no access to information regarding its peer review. Given her role as the Guest Editor, Maria Scuto had no involvement in the peer-review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Adrian Gericke.

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