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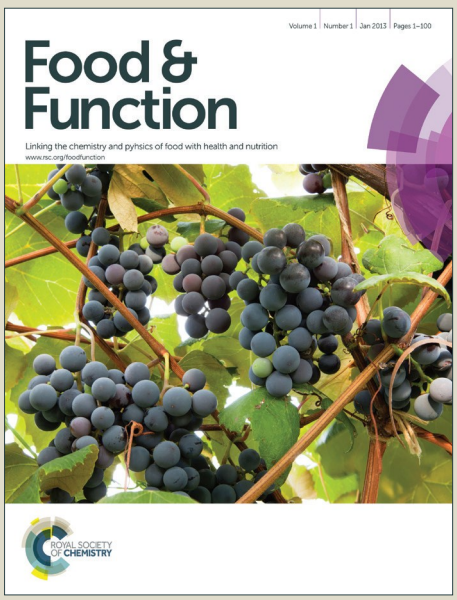

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Protective effect of anthocyanins and xanthophylls on UVB-induced damage in retinal pigment epithelial cells

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Abstract

Increased exposure to solar ultraviolet B (UVB) radiation produce oxidative damage that may promote age related macular degeneration (AMD) and other ocular pathologies. This study aimed to demonstrate protective effects of some anthocyanins and xanthophylls against UVB-induced oxidative damage to retinal epithelial cells (RPE). ARPE-19 cells were treated with 5 μM cyanidin-3-O-glucoside, delphinidin-3-O-glucoside, lutein, zeaxanthin or a mix of cyanidin3-O-glucoside:zeaxanthin prior to UVB exposure (500 J/m^2). Cell viability and mitogen-activated protein kinases (MAPKs) phosphorylation were determined by MTT and western blot analysis, respectively. Oxidative damage was evaluated by measuring intracellular reactive oxygen species (ROS). The data showed that UVB irradiation reduce the cell viability to 46% with increasing of intracellular ROS levels and phosphorylation of MAPKs. However, pre-treatment (60 min) with 5 μM cyanidin3-O-glucoside, lutein or zeaxanthin significantly reduced cellular ROS levels and phosphorylation of MAPKs (JNK1/2 and p38) mediated by UVB irradiation and subsequently increased cell viability. Thus, results show that UVB irradiation is able to induce apoptosis in ARPE19 cells through oxidative stress; however anthocyanins and xanthophylls pre-treatment can attenuate this damage. This suggests that cyanidin3-O-glucoside, lutein and zeaxanthin are effective in preventing UVB-induced damage in RPE cells and may be suitable as chemoprotective factors for prevention of ocular damage. The use of natural dietary antioxidants might reduce ocular oxidative damage mediated by UVB radiation.

Introduction

Oxidative damage and inflammation processes are related to the pathogenesis of different visual dysfunction, such as, cataracts or age-related macular degeneration (AMD). AMD is a complex multifactorial disease and one of the most common causes of severe visual loss in the elderly population[1-3]. Retinal pigment epithelial (RPE) cells, which are located in the outermost layer of the retina, are critical for the normal functioning and health of retinal photoreceptors and play an essential role in visual process; moreover their oxidative damage contributes to macular degeneration[4]. Exposure to solar ultraviolet (UV) radiation can induce DNA breakdown and oxidative stress with production of reactive oxygen species (ROS) whom may cause oxidative damage to RPE cells[5,6] inducing several retinal pathologies[7]. UV radiation is part of the sunlight spectrum and is divided into three different bands: UVA (320-400 nm, 90% of UVA radiation reaches the Earth's surface), UVB (280-320 nm, 10% of UVB radiation reaches the Earth's surface) and UVC (100-280 nm, UVC radiation is absorbed by the ozone layer). The damaging role of UVB has been extensively studied in skin pathologies due to the high risk of exposure of the skin to UVB, but eye is another organ strongly exposed to UVB radiation and little is known about the role of UVB radiation to the retina.

Recent attention has focused on the link between ocular diseases and intracellular oxidative stress[8]. Accumulation of high amounts of ROS may act as a secondary messenger to activate various signalling pathways by inducing stress-response genes or proteins inducing various biological processes, including apoptosis or programmed cell death[9,10]. The involvement of singlet oxygen in apoptosis has also been reported in UVB-irradiated RPE cells[11]. The major signalling pathways known to mediate UVB induced biological responses involve mitogen-activated protein kinases

(MAPKs)[12]. MAPKs are activated when apoptosis is UVB-induced in ARPE19 cells[5]. Although the mechanism by which oxidants mediate apoptosis is not well defined, the MAPKs pathways have been implicated in apoptosis induced by a wide range of apoptotic stimuli[13]. The MAPKs are a part of a phosphorylation cascade and include extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal protein kinase (JNK), and p38 kinase. ERK is generally considered to be a survival mediator involved in cell growth and differentiation[14], whereas p38 and JNK are usually referred to as stress-stimulated MAPKs, which are required for the induction of apoptosis by diverse stimuli such as UV irradiation, osmotic shock, and oxidants[13]. The down-regulation of MAPKs activity by specific inhibitors of these kinase families could be used in the treatment of MAPK-mediated diseases.

The regulation of ROS production might inhibit the tissue damage. Studies over the last few decades suggest that diet-derived antioxidants may help to prevent and/or delay progression of the pathogenic pathways, modifying the risk of suffer different diseases caused by oxidative damage. In support of this idea, several reports suggest that the administration of natural antioxidants compounds could reduce ROS and to be effective for preventing retinal injury induced by the oxidative damage[15-18].

Flavonoids and carotenoids are a class of natural biological compounds that have evolved to protect plants form oxidative damage induced by chronic exposure to UV light[19]. These phytochemicals have many human health benefits, and most of these beneficial effects were due to their potent antioxidant and free radical-scavenging properties. Several studies have shown the antioxidant effects of specific flavonoids and carotenoids on various types of cells inhibiting the cellular signalling pathways leading to different diseases[20,21]. Flavonoids cyanidin-3-O-glucoside (Cy) delphinidin-3-O-glucoside (Dp) and are members of a family of bioactive compounds known as

anthocyanins widely distributed in fruits and vegetables and are known to ameliorate oxidative stress. Previous studies have showed that delphinidin and cyanidin decreased oxidative stress in different cell lines scavenging free radicals[22,23], suppressing inflammation[24], and protecting against endothelial dysfunction[25]. On the other hand, lutein (Lu) and zeaxanthin (Ze) are xanthophyll carotenoids with antioxidant activity. In humans, lutein and zeaxanthin are distributed in the lens and macula and protects the ocular tissue by filtering blue light[26] and are believed to function as an antioxidant that quenches and scavenges photo induced ROS[27]. These carotenoids cannot be synthesized within human body and only source is diet including green vegetables, some fruits and egg yolks[28]. An inverse relationship between the serum concentration of lutein and zeaxanthin and ocular diseases, such as AMD and cataract, has been reported[29]. Recent studies have showed that lutein and zeaxanthin decreased oxidative stress in different cell lines (endothelial and retinal cells)[30-32] and is reported to have an anti-inflammatory effect in ocular tissue[16]. Thus, anthocyanins and carotenoids could suppress pathogenic signals through reducing ROS. Although studies have shown that delphinidin, cyanidin, lutein and zeaxanthin protects several cell types from oxidative stress, there are few studies on the protective effects of these compounds against UVB-induced damage in RPE cells.

In the present study, we evaluated the *in vitro* protective effects of delphinidin, cyanidin, lutein and zeaxanthin against UVB-induced damage responses in ARPE-19 cells, such as UVB-induced apoptosis, by attenuating MAPK signalling pathways developing a new *in vitro* model of retinal UVB protection.

Materials and methods

Materials

Cyanidin-3-O-glucoside, delphinidin-3-O-glucoside, lutein and zeaxanthin were purchased from Extrasynthese (Lyon, France). 3,4,5-dimethylthiazol-2,5-diphenyl-tetrazolium bromide (MTT), Bradford reagent, dichlorofluorescin (DCFH), dimethylsulfoxide (DMSO), phosphatase and protease inhibitor cocktails, sodium dodecyl sulfate (SDS), and ultra TMB-Blotting-solution were acquired from Sigma (Madrid, Spain). Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12), penicillin/streptomycin (5,000 U/mL) and trypsin/EDTA solution (170,000 U/L) were purchased from Lonza (Cultek S.L.U, Madrid, Spain). Fetal bovine serum (FBS) South American origin (Hyclone) was obtained from Thermo Scientific (Cultek). Materials and chemicals for protein electrophoresis and western blot were acquired from Invitrogen (Life technologies, Madrid, Spain). Cell culture dishes were obtained from BD Falcon (Madrid, Spain). Primary antibodies anti-JNK1/2, anti-phospho-JNK1/2, anti-p38, anti-phospho-p38, and secondary antibody goat anti-rabbit-HRP were purchased from Thermo Scientific (Cultek); anti-β-actin was purchased from Santa Cruz Biotechnology (Quimigen S. L, Madrid, Spain).

ARPE-19 cell cultures

Adult human retinal epithelial cells (ARPE-19) were used as the model to study the effect of UVB light on the retinal pigment epithelium and to see if anthocyanins and xanthophylls were able to protect the retina against this damage. ARPE-19 cells (a gift from Dr. Valverde, Instituto de Investigaciones Biomédicas, CSIC) were cultured in DMEM/F12 supplemented with 10% FBS and 1% penicillin/streptomycin (5,000 U/mL). Cells were plated at densities 1 x 10^6 cells in 75 cm^2 tissue culture flasks and maintained at 37°C under 5% CO_2 in a humidifier incubator until 90% of confluence. The culture medium was changed every 2 days. After a confluent monolayer appeared,

subculturing was carried out using a dissociating agent Trypsin/EDTA solution. For the experiments, cells were starved in serum-free DMEM for 24 h before they were subjected to further assays. All experiments were carried out between passage 10 to passage 30 to ensure cell uniformity and reproducibility.

Cell viability assays

The viability of the cells was determined by the MTT reduction assay. Cells were seeded into 96-well plates (5 x 10^3 cells/well) and allowed to adhere 24 h.

Cell viability after pre-treatment. Briefly, cell medium was replaced with serum-free medium containing 5 μM Dp, Cy, Lu, Ze or mix 2.5 μM Cy + 2.5 μM Ze (dissolved in DMSO) and the cells were incubated at 37°C under 5% CO_2 for 24 h. Control cells were incubated in serum-free medium with 0.1% DMSO.

Cell viability after UVB exposure. The culture medium was removed to avoid the formation of medium-derived toxic photoproducts induced by UVB light. ARPE19 cells were exposed to UVB (500 J/m^2) in PBS and incubated at 37°C under 5% CO_2 for another additional 24 h with fresh culture medium (control cells were not irradiated).

Cell viability after pre-treatment and UVB exposure. The cell medium was replaced with serum-free medium containing 5 μM Dp, Cy, Lu, Ze or mix 2.5 μM Cy + 2.5 μM Ze (dissolved in DMSO) and the cells were incubated at 37°C under 5% CO_2 for 60 min. After that, the medium was removed and cells were washed with PBS. Cells were exposed to a total UVB irradiation of 500 J/m^2. Immediately after UVB exposure, PBS was removed and serum-free medium was added to the cells and the cells incubated as for culturing at 37°C under 5% CO_2 for another 60 min (the control cells were not irradiated).

Thereafter 20 µL of a MTT solution in PBS (5 mg/mL) was added to each well for the quantification of the living metabolically active cells after four hours incubation Briefly, MTT is reduced to purple formazan in the mitochondria of living cells. Formazan crystals in the wells were solubilized in 200 µL DMSO. Absorbance was measured at 570 nm wavelength employing a microplate reader PowerWave™ XS (BioTek Instruments, Inc., Winooski, VT, USA). The viability was calculated considering controls containing a solvent control (0.1% DMSO) as 100% viable. Data represent the mean and standard deviation of three independent experiments (n=3).

Evaluation of reactive oxygen species (ROS) generation

Intracellular ROS levels were quantified following the method described by Martin and co-workers, using DCFH as fluorescent probe[33]. After being oxidized by intracellular oxidants, DCFH will become dichlorofluorescein (DCF) and emit fluorescence. To test for the protective effect, cells were seeded (5 x 10^4 cells per well) in 24-multiwell plates and grown until they reached 70% of confluence. Cells were treated with 5 µM Dp, Cy, Lu, Ze or mix 2.5 µM Cy + 2.5 µM Ze for 60 min. After that, cells were washed with PBS and incubated with culture medium and DCFH probe for 30 min. The unabsorbed probe was removed by washing with PBS and cells were then irradiated with UVB (500 J/m^2). After 60 min of incubation at 37°C under 5% CO_2, fluorescence at excitation wavelength of 485 nm and emission wavelength of 530 nm was measured in a plate reader (BioTek). The control cells were not irradiated. Data were presented as the mean and standard deviation of three independent experiments (n=3).

UVB irradiation

Cells were seeded in a culture dishes (19.5 cm^2) at a density of (5 x 10^5 cells) in 2 mL DMEM-F12 containing 10% FBS and 1% penicillin/streptomycin. This density provided 80–90% cell confluences 72 h after seeding. Before every experiment, the cells were starved in serum-free DMEM/F12 for 24 h. The medium was replaced with serum-free medium containing 5 μM Dp, Cy, Lu, Ze or mix 2.5 μM Cy + 2.5 μM Ze (dissolved in DMSO) and the cells were incubated at 37°C under 5% CO_2 for 60 min. Control cells were incubated in serum-free medium containing 0.1% DMSO. For UVB exposure, the medium was removed and cells were washed and gently overlaid with PBS. A specific UVB lamp emitting a peak wavelength of 312 nm with intensity of 5.8 W/m^2 (Vilber Lourmat, Moune La Vallee, France) was used. Cells were exposed to a total UVB irradiation of 500 J/m^2 (approximately 90 s exposure). Immediately after UVB irradiation, PBS was removed and serum-free medium was added to the cells and incubated as for culturing at 37°C under 5% CO_2 for 60 min (post-irradiation time). Control cells were treated identically except that they were exposed to ambient light rather than UVB. All the experiments were performed three times in triplicate.

Cell lysate preparation and western blot analysis

Cells were recovered with cold PBS and lysed at 4°C in a lysis buffer containing 25 mM Hepes (pH 7.5), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.1% Triton X-100, 200 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM PMSF, phosphatase and protease inhibitors. After incubation on ice for 15 min, the lysate was centrifuged at 10,000 rpm for 20 min at 4ºC. The supernatants were collected, assayed for protein concentration by using the Bradford reagent, aliquoted and stored at -20°C until used for Western blot analyses. Sample aliquots of 30 μg of protein were loaded on to 10% polyacrylamide gels and separated

213 at 125 V for 110 min. Gels were then overlaid with PVDF membranes (iBlot® Dry
214 Blotting System, Invitrogen, USA) and blotted. Gels were stained with Coomassie Blue
215 dye to confirm efficient transfer. Membranes were blocked in TBS-T (20 mm Tris pH
216 7.5, 150 mm NaCl, 0.05% Tween 20) 5% skim milk for 1 h at room temperature,
217 washed three times with TBS-T for 10 min. Membranes were probed with the
218 corresponding primary antibody (1:1000 dilution in TBS-T) by incubation over-night at
219 4ºC. After that, the membranes were washed three times with TBS-T for 10 min
220 followed by incubation with anti-rabbit-HRP secondary antibody (1:2500 dilution in
221 TBS-T) at room temperature for 1 hour and then washed with TBS-T three times for 10
222 min. Protein bands were visualized by using TMB as a substrate. Normalization of
223 western blot was ensured by β-actin protein (load control) and bands were quantified
224 using a scanner and densitometry software GelQuant.NET v1.8.2 provided by
225 biochemlabsolutions.com.
226
227 **Statistical analysis**
228 The results were reported as means ± standard deviation (SD) performed in
229 triplicate. The data was analysed by analysis of variance (ANOVA) with a post hoc
230 Duncan test. Differences were considered significant at $p<0.05$. All statistical tests were
231 performed with IBM SPSS Statistics for Windows, Version 19.0 (IBM Corp., Armonk,
232 NY, USA).
233
234 **Results**
235 *UVB radiation induces cell death on ARPE19*
236 To confirm the cytotoxic effect of UVB irradiation on ARPE19 cells, cultured
237 cells were exposed to increasing doses of UVB radiation (100, 200, 500 and 1000 J/m^2).

Cell viability was determined at 24 h after UVB irradiation by MTT assay. As we expected in our *in vitro* model, different percentages of cultured ARPE19 cells died after UVB exposure. The decrease of cell viability was dose-dependent, resulting in 73.9, 58.8, 46.1 and 25.4% of remaining surviving cells at 100, 200, 500 and 1000 J/m^2, respectively (Figure 1). Since 500 J/m^2 of UVB irradiation caused a decrease of about a half in cell viability (46%), this dosage was used in the following studies aimed to study the protective effect of the different bioactive compounds on UVB-induced cell damage.

Cytotoxic effect of anthocyanins and xanthophylls

To examine the non-cytotoxic effect of Dp, Cy, Lu, Ze (5 μM) and mix Cy (2.5 μM) + Ze (2.5 μM) on ARPE19 cells, a cell viability assay by MTT was performed. The effects of anthocyanins and xanthophylls on the ARPE19 viability are shown in Figure 2. Results showed that the different compounds and the tested mix did not significantly affect cell viability at 24 h after treatment. The data indicated that these compounds have no cytotoxic effects on ARPE19 cells. Therefore, 5 μM as total concentration was used in the further protective experiments. The concentration of anthocyanins and xanthophylls used in all experiments were selected based on previous reports, taking into account that the concentration of anthocyanins and xanthophylls observed in human plasma is in the nM to low μM range[34].

Anthocyanins and xanthophylls protect ARPE cell from UVB-induced death

To determine the protective effects of Dp, Cy, Lu, Ze (5 μM) and a mixture of Cy (2.5 μM) + Ze (2.5 μM) on ARPE19 cells after UVB exposure, a cell viability assay was performed (Figure 3). The MTT assay showed the cell viability of untreated ARPE19 cells was decreased after UVB exposure (41.0 % of viability respect to the

control cells). However, the viability decrease was significantly reversed (p<0.05) by pre-treatment with Cy, Lu, Ze and Cy+Ze mix (Figure 3), approximately 56, 69, 77, and 74% of cells were viable upon UVB exposure, respectively. On the other hand, cells pre-treated with 5 μM Dp did not show any significantly increase in ARPE19 viability (44.2% of cell viability). These results indicate that Cy, Lu, Ze and the mixture Cy+Ze are effective in the prevention of UVB-induced ARPE19 cell damage and these prevention is most effective when the cells are pre-treated with xanthophyll compounds in comparison with anthocyanins.

Effect of anthocyanins and xanthophylls on ROS production

In ARPE19 cells, UVB radiation is known for being a potent inducer of ROS formation[35]. Accordingly, ARPE19 cells exposed to UVB irradiation (500 J/m^2) for 60 min significantly enhanced ROS generation (Figure 4) and cell damage resulting in a remarkably decrease of ARPE19 cell viability (Figure 1). Pre-treatment of ARPE19 cells for 60 min with Cy, Lu, Ze and Cy+Ze mix evoked a significant (p<0.05) reduction in the cellular ROS generation (Figure 4). The significant decrease in ROS levels and, consequently, in the oxidative stress condition leads to a decline of ARPE19 cell death, as shown in Figure 3. However, pre-treatment of ARPE19 cells with 5 μM Dp did not provoke a significant (p>0.05) reduction in the intracellular ROS formation (Figure 4) neither in ARPE19 cell death (Figure 3). Once again, the higher decreases in the ROS formation were obtained when the cells were pre-treated with xanthophyll compounds. These results suggest that UVB irradiation activates ROS formation, and thus oxidative stress pathways may play role in UVB-induced cell death on ARPE19 dells.

Inhibition of UVB-induced MAPK activation

UV irradiation causes the activation of MAPKs, which may lead to ARPE cell damage and cell death[5]. To investigate the inhibitory effect of anthocyanins and xanthophylls on MAPKs phosphorylation, we pre-treated the cells with the different compounds and mix prior to UVB exposure. In our experimental model, the JNK1/2 and p38 phosphorylation were increased after UVB irradiation (Figure 5). However, JNK1/2 phosphorylation in ARPE19 cells was significantly (p<0.05) reduced by pre-treatment with Cy (Figure 5A) and Lu and Ze (Figure 5B). UVB-induced p38 phosphorylation was only significantly (p<0.05) reduced by the pre-treatment with Lu and Ze (Figure 5B). These results demonstrate that Cy, Lu and Ze at 5 μM inhibit MAPK activation (Figure 5A and 5B), reduce ROS generation (Figure 4) and increase cell viability (Figure 3) after UVB irradiation. The inhibitory effect on MAPK activation again was more notorious when the cells were treated with xanthophyll compounds. The pre-treatment with Cy+Ze mix was able to reduce the phosphorylation levels of JNK1/2 and p38 but the results were not significant respect to the UVB irradiated control (p>0.05)

Discussion

The health benefits of anthocyanins and xanthophylls have been associated with their potent antioxidant and anti-inflammatory properties[36], which is related to their hydrogen donating ability and the capacity to stabilize the radicals formed. *In vitro* studies have demonstrated these antioxidant effects in different human model cells[37,38]. The role of anthocyanins and xanthophylls at present seem promising for reversing oxidative stress and possibly improving diseases in which oxidative processes are involved, such as those affecting ocular degeneration. However, the evidence is lacking

and one should be aware that their dosage and efficacy in ocular diseases are still limited. Our study evaluated the ability of specific anthocyanins (delphinidin and cyanidin-3-glucosides) and xanthophylls (lutein and zeaxanthin) in protecting the UVB-induced RPE cell damage. We observed that cyanidin, and specially, lutein and zeaxanthin reduced the UVB-induced decrease in cell viability (Figure 3); lowered intracellular ROS generation and MAPKs activation (Figures 4 and 5).

It is well known that sunlight causes cell damage, which accumulates during lifetime, inducing cell death through phototoxic events. Intense light exposure has been used as an environmental stressor in testing human model cells of photoreceptor cell degeneration[39], particularly in the retinal pigment epithelium cells. The outcomes of these works have improved our understanding of both visual transduction and retinal degenerations. Good progress has been made in preventing retinal light damage with antioxidants and neurotrophic factors. Thus, although it has been gained insights into the process, the mechanism of light damage still eludes a thorough understanding. Three retinal light damage hypotheses, originally present by Noell[40], have guided the work in this area. These include: i) a toxic photoproduct formation during exposure to intense light; ii) a metabolic abnormality resulting from light exposure; iii) and light-induced oxidative reactions. Taken together with a new amount of additional work, the retinal light damage is a multi-factorial process involving both environmental and genetic factors.

The human ARPE19 cell line serves to study *in vitro* different strategies to protect the retina. These cells are susceptible to oxidative damage because of its high metabolic rate and oxygen consumption, phagocytosis of rod outer segments, and the presence of photosensitizing compounds[40]. Therefore, ARPE19 cells are potentially sensitive to UVB irradiation and have been used for many *in vitro* studies of UV-

induced damage to the retina and oxidative stress[5,17,18]. The role of oxidative stress has been implicated in many age-related diseases and a large number of studies support the key role of ROS in visual degeneration[42,43]. Our experimental model indicates that UVB irradiation (500 J/m^2) significantly increases intracellular ROS levels in ARPE19 cells (Figure 4) with a lower viability (Figures 1 and 3), which is consistent with that oxidative stress is involved in UVB-induced damage in RPE cells. In order to investigate the relationship between an increase in ROS generation, UVB-induced MAPK phosphorylations and cell death, anthocyanin and xanthophyll compounds as natural antioxidants were used.

It has been reported that the activation of MAPK is essential for UVB-induced apoptosis in RPE cells, which includes phosphorylation of ERK, JNK and p38 kinases[12]. It is generally accepted that ERK activation is essential for cell survival, whereas the activation of JNK and p38 is thought to play a role in apoptotic or cell death signalling. Our study confirm that UVB irradiation caused a large increased in intracellular ROS generation (Figure 4), as well as JNK1/2 and p38 phosphorylation in ARPE19 cells (Figure 5) 1 h after UVB irradiation and subsequently elevated cell death (Figure 1 and 3). However, the protective experiments showed that cyanidin, and specially, lutein and zeaxanthin were able to reduce intracellular ROS generation (Figure 4) and partially decrease cell death induced by UVB irradiation (Figure 3). Simultaneously, these compounds were able also to block, in part, the phosphorylation of JNK1/2 and p38 caused by the UVB irradiation (Figure 5). All these results suggest that oxidative stress is involved in UVB-induced cell death and that it occurs partially through influencing the signal transduction pathways of MAPKs such as, JNK1/2 and p38, but this process could be partially inhibited by natural antioxidants such as anthocyanins and xanthophylls.

Regarding the possible mechanisms by which the tested compounds inhibit UVB-induced RPE cell death, our results showed that it may act through its antioxidative effect (Figures 4 and 5). The biologic harmful effects of UVB are mediated by the generation of intracellular ROS. These active oxygen species mediate many damaging effects, such as DNA damage and cell death. In the present study, we confirm that UVB-induced intracellular ROS production was decreased by pre-treatment with Cy, Lu, Ze and mix Cy+Ze. This inhibition, in turn, contributes to the protective effect on UVB-induced ARPE19 cell death (Figure 3). However, we also found that UVB-induced intracellular ROS production cannot be completely inhibited by anthocyanin and xanthophylls pre-treatment. This may be the reason why these compounds could not completely reverse UVB-induced cell death (Figures 3 and 4).

In vivo and *in vitro* studies have confirmed that xanthophylls photo-protective role is related to its antioxidant activity or with the modulation of other endogenous antioxidant pathways in the cell. Moreover, it has been established that xanthophylls structure has a great influence in its antioxidant activity, being influenced by the presence of functional groups with increasing polarities, such as carbonyl and hydroxyl groups, in the terminal rings, as well as by the number of conjugated double bonds[44,45]. This way, xanthophylls antioxidant effectiveness increase as the number of conjugated double bonds increase. On the other hand, the antioxidat activities exhibited by anthocyanins are also influenced by their own structures, essentially by the number of hydroxyl constituents of the B-ring. Delphinidin usually exhibit higher antioxidant capacities than cyanindin, and this remarkable effect is associated with the fact that delphinidin has three hydroxyl groups atached to its B ring. However, our results using anthocyanins suggest that the inhibition of intracellular ROS production is not related with the number of hydroxyl groups on the B-ring. These findings are in accordance

388 with previous results described when hydroxyl radical scavenging activity[46] and oxygen
389 radical absorbance capacity (ORAC)[47] were evaluated using anthocyanin pigments.

390 Taken all together, the most promising results during the present study were
391 obtained when the cells were treated with the xanthophylls, lutein and zeaxanthin. These
392 results are in accordance with the previous obtained by Bian[16] using 10 μM of these
393 same compounds. The best results obtained with the xanthophylls may be due to a
394 higher accumulation in RPE cells[48]. Moreover, xanthophylls are dipolar carotenoids
395 which insert themselves in a mainly perpendicular orientation into the retinal cell
396 membrane and this orientation is thought to offer optimal protection against oxidation[49].
397 Lutein and zeaxanthin are also excellent quenchers of singlet oxygen of photoactive
398 molecules. ARPE-19 cells actively take up lutein and zeaxanthin from the medium[16].
399 Epidemiologic studies suggest that dietary lutein and zeaxanthin play significant
400 protective roles against visual loss[50] and lower serum levels of these carotenoids are
401 associated with increased risk for AMD[51,52]. In our study, the greater protective effect of
402 lutein and zeaxanthin may be related to their higher cellular accumulation. The
403 differential uptake and retentions of lutein and zeaxanthin in the retina may be related to
404 the expression of their binding proteins in the retinal cells[53,54]. An increase in dietary
405 intake of lutein and zeaxanthin would increase the macular pigment optical density and
406 provide better protection against oxidation. It has long been suspected that accumulation
407 of lutein and zeaxanthin in the retina may protect against eye damage by two different
408 mechanisms: blocking harmful UVB radiation and quenching ROS. Bian[16] indicate that
409 supplementation with lutein or zeaxanthin can partially protect against oxidative stress
410 and inflammatory response in RPE cells by modulating inflammation-related genes.

411 Because dietary anthocyanins have anti-inflammatory and antioxidant properties
412 they could have also potential benefits in inhibiting retinal diseases associated with

oxidative stress and inflammation. Anthocyanins have shown limited absorption *in vivo* in comparison to xanthophylls; however, some studies in animals have showed accumulation of anthocyanins in the brain, liver, and ocular tissues[55,56]. These results confirm the potential anthocyanins bioavailability in the eye tissues. Of the two anthocyanins tested in the present study, cyanidin was able to reduce the intracellular ROS generation and contributes to inhibit partially the activation of JNK1/2 inducing higher cell viability after UVB irradiation. These results are according with previous studies[23,57] using higher concentrations (up to 10 μM), where cyanidin inhibited the ROS generation and cellular necrosis without a significant reduction in the cell viability. Thus, our data suggests that cyanidin may have benefits to prevent proliferative retinopathies. p38 MAPK also participates in cellular responses to mitogenic stimuli, including oxidative stress or UV exposure, during cell differentiation and apoptosis. A previous report suggested that delphinidin and cyanidin at 10 μM can regulated the activation of p38 MAPK[23]. However in our experimental model the anthocyanins used at 5 μM were not able to inhibit p38 phosphorylation (Figure 5A). Because low concentrations of anthocyanins and xanthophylls (5 μM) did not decrease the viability of the cells (Figure 2), we assume that normal and moderate intake of the compounds as natural food ingredients will have no deleterious consequences in the RPE cells, and may have even beneficial effects, in the cases of cyanidin, lutein and zeaxanthin.

In summary, our study provides the evidence that the anthocyanin cyanidin-3-glucoside, and specially, xanthophylls lutein and zeaxanthin are potential protective agents for RPE cells against UVB irradiation. The data demonstrate that these natural antioxidant compounds can inhibit UVB-induced intracellular ROS generation and MAPK activation. Moreover, these compounds increase ARPE19 cell survival after exposure to UVB radiation, which suggests a preventing effect from UVB-induced

438 damage and may be suitable to be further developed as chemoprotective agents and can
439 play an important role in the prevention of oxidation or UVB-induced ocular disorders.
440 Thus, modulating RPE cell damage through ingestion of selective natural antioxidants
441 may be a useful strategy for reducing the oxidative damage that occurs in ocular
442 pathologies. These results should be supported by *in vivo* experiments in order to
443 confirm these findings. Future experiments using a chronic photo-oxidation model and
444 physiologically relevant concentrations of anthocyanins and xanthophylls are needed.
445

446 **Conclusion**

447 The present study demonstrates that UVB irradiation induce cellular oxidative
448 stress in ARPE19 cells by increasing intracellular ROS production, altering activities of
449 MAPKs and by subsequently inducing cell death. Although, some anthocyanins and
450 xanthophylls have shown to protect against oxidative damage in RPE cells, the present
451 report shows that the anthocyanin cyanidin and especially the xanthophylls lutein and
452 zeaxanthin are effective against UVB-mediated RPE oxidative damage.
453

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458

459 **References**

460 1. N. M. Bressler, Age-related macular degeneration is the leading cause of blindness,
461 *JAMA*, 2004, **291**, 1900-1901.
462 2. N. G. Congdon, D. S. Friedman, T. Lietman, Important causes of visual impairment
463 in the world today, *JAMA*, 2003; **290**, 2057-2060.
464 3. J. Z. Nowak, Age-related macular degeneration (AMD): pathogenesis and therapy,
465 *Pharmacol. Rep.* 2006, **58**, 353-363.
466 4. F. Q. Liang, B. F. Godley, Oxidative stress-induced mitochondrial DNA damage in
467 human retinal pigment epithelial cells: a possible mechanism for RPE aging and
468 age-related macular degeneration, *Exp. Eye Res.,* 2003, **76**, 397–403.
469 5. R. Roduit, D. F. Schorderet, MAP kinase pathways in UV-induced apoptosis of
470 retinal pigment epithelium ARPE19 cells, *Apoptosis,* 2008, **13**, 343-353.
471 6. K. S. Tratsk, S. Thanos, UV irradiation causes multiple cellular changes in cultured
472 human retinal pigment epithelium cells, *Graefes Arch. Clin. Exp. Ophthalmol.*,
473 2003, **241**, 852-859.
474 7. K. V. Chalam, V. Khetpal, R. Rusovici, S. Balaiya, A review: role of ultraviolet
475 radiation in age-related macular degeneration, *Eye Contact Lens,* 2011, **37**, 225–
476 232.
477 8. Y. Imamura, S. Noda, K. Hashizume, K. Shinoda, M. Yamaguchi, S. Uchiyama, T.
478 Shimizu, Y. Mizushima, T. Shirasawa, K. Tsubota, Drusen, Choroidal
479 neovascularization, and retinal pigment epithelium dysfunction in SOD1-deficient
480 mice: A model of age-related macular degeneration, *Proc. Natl. Acad. Sci.*, 2006,
481 **103**, 11282-11287.
482 9. S. Fulda, A. M. Gorman, O. Hori, A. Samali, Cellular stress responses: cell survival
483 and cell death, *Int. J. Cell Biol.,* 2010, Article ID 214074.

10. O. Trachootham, W. Lu, M. A. Ogasawara, N. Rivera-Del Valle, P. Huang, Redox regulation of cell survival, *Antioxid. Redox Signal*, 2008, **10**, 1343-1374.
11. H. G. Yu, G. S. Choe, J. W. Heo, Oxidative damage induced by UV in cultured retinal pigment epithelial cells, *J. Korean Ophthal. Society* 2003, **44**, 2890-2895.
12. A. M. Bode, Z. Dong, Mitogen-activated protein kinase activation in UV-induced signal transduction, *Sci. STKE,* 2003, **2003**, RE2.
13. T. Wada, J. M. Penninger, Mitogen-activated protein kinases in apoptosis regulation, *Oncogene*, 2004, **23**, 2838–2849.
14. P. Dent, A. Yacoub, P. B. Fisher, M. P. Hagan, S. Grant, MAPK pathways in radiation responses, *Oncogene*, 2003, **22**, 5885-5896.
15. S. Aimjongjun, M. Sutheerawattananonda, N. Limpeanchob, Silk lutein extract and its combination with vitamin E reduce UVB-mediated oxidative damage to retinal pigment epithelial cells, *J. Photochem. Photobiol., B*, 2013, **124**, 34–41.
16. Q. Bian, S. Gao, J. Zhou, J. Qin, A. Taylor, E. J. Johnson, G. Tang, J. R. Sparrow, D. Gierhart, F. Shang, Lutein and zeaxanthin supplementation reduces photooxidative damage and modulates the expression of inflammation-related genes in retinal pigment epithelial cells, *Free Radic. Biol. Med.,* 2012, **53**, 1298–1307.
17. G. Cao, M. Chen, Q. Song, Y. Liu, L. Xie, Y. Han, Z. Liu, Y. Ji, Q. Jiang, EGCG protects against UVB-induced apoptosis via oxidative stress and the JNK1/c-Jun pathway in ARPE19 cells, *Mol. Med. Rep.*, 2012, **5**, 54-59.
18. C. M. Chan, J. H. Huang, H. H. Lin, H. S. Chiang, B. H. Chen, J. Y. Hong, C. F. Hung, Protective effects of (-)-epigallocatechin gallate on UVA-induced damage in ARPE19 cells, *Mol. Vis*., 2008, **14**, 2528-2534.

19. F. Delgado-Vargas, A. R. Jiménez, O. Paredes-López, Natural pigments: carotenoids, anthocyanins, and betalains--characteristics, biosynthesis, processing, and stability, *Crit. Rev. Food Sci. Nutr.*, 2000, **40**, 173–289.

20. A. Hanneken, F. F. Lin, J. Johnson, P. Maher, Flavonoids protect human retinal pigment epithelial cells from oxidative-stress-induced death, *Invest. Ophthalmol. Vis. Sci.*, 2006, **47**, 3164-3177.

21. E. Middleton, C. Kandaswami, T. C. Theoharides, The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer, *Pharmacol. Rev.*, 2000, **52**, 673-751.

22. Y. Noda, T. Kaneyuki, A. Mori, L. Packer, Antioxidant activities of pomegranate fruit extract and its anthocyanidins: delphinidin, cyanidin, and pelargonidin, *J. Agric. Food Chem.*, 2002, **50**, 166–171.

23. K. Ogawa, Y. Kuse, K. Tsuruma, S. Kobayashi, M. Shimazawa, H. Hara, Protective effects of bilberry and lingonberry extracts against blue light-emitting diode light-induced retinal photoreceptor cell damage *in vitro*, *BMC Complement. Altern. Med.*, 2014, **14**,120.

24. S. G. Lee, B. Kim, Y. Yang, T. X. Pham, Y. K. Park, J. Manatou, S. I. Koo, O. K. Chun, J. Y. Lee, Berry anthocyanins suppress the expression and secretion of proinflammatory mediators in macrophages by inhibiting nuclear translocation of NF-κB independent of NRF2-mediated mechanism, *J. Nutr. Biochem.*, 2014, **25**, 404–411.

25. I. Serraino, L. Dugo, P. Dugo, L. Mondello, E. Mazzon, G. Dugo, A. P. Caputi, S. Cuzzocrea, Protective effects of cyanidin-3-O-glucoside from blackberry extract against peroxynitrite-induced endothelial dysfunction and vascular failure, *Life Sci.*, 2003, **73**, 1097–1114

26. J. van de Kraats, M. J. Kanis, S. W. Genders, D. van Norren, Lutein and zeaxanthin measured separately in the living human retina with fundus reflectometry, *Invest. Ophthalmol. Vis. Sci*., 2008, **49**, 5568–5573.
27. A. J. Chucair, N. P Rotstein, J. P. Sangiovanni, A. During, E. Y. Chew, L. E. Politi, Lutein and zeaxanthin protect photoreceptors from apoptosis induced by oxidative stress: relation with docosahexaenoic acid, *Invest. Ophthalmol. Vis. Sci.*, 2007; **48**, 5168-5177.
28. O. Sommerburg, J. E. Keunen, A. C. Bird, F. J. van Kuijk, Fruits and vegetables that are sources for lutein and zeaxanthin: the macular pigment in human eyes, *Br. J. Ophthalmol*., 1998, 82, 907-910.
29. A. Alves-Rodrigues, A. Shao, The science behind lutein, *Toxicol Lett*, 2004, **150**, 57–83.
30. D. K. Lee, R. N. Grantham, J. D. Mannion, A. L. Trachte, Carotenoids enhance phosphorylation of Akt and suppress tissue factor activity in human endothelial cells, *J. Nutr. Biochem.*, 2006, **17**, 780–786.
31. M. Sasaki, K. Yuki, T. Kurihara, S. Miyake, K. Noda, S. Kobayashi, S. Ishida, K. Tsubota, Y. Ozawa, Biological role of lutein in the light-induced retinal degeneration, *J. Nutr. Biochem.*, 2012, **23**, 423–429.
32. X. Xu, L. Hang, B. Huang, Y. Wei, S. Zheng, W. Li, Efficacy of ethanol extract of *Fructus lycii* and its constituents lutein/zeaxanthin in protecting retinal pigment epithelium cells against oxidative stress: *in vivo* and *in vitro* models of age-related macular degeneration, *J. Ophthalmol*., 2013, DOI: 10.1155/2013/862806
33. M. A. Martín, I. Cordero-Herrera, L. Bravo, S. Ramos, L. Goya, Cocoa flavanols show beneficial effects in cultured pancreatic beta cells and liver cells to prevent the onset of type 2 diabetes, *Food Res. Inter.*, 2014, 63, 400–408.

558 34. E. Pojer, F. Mattivi, D. Johnson, C. S. Stockley, The case for anthocyanin
559 consumption to promote human health: A review, *Compr. Rev. Food Sci. F.*, 2013,
560 **12**, 483–508.
561 35. D. E. Heck, A. M. Vetrano, T. M. Mariano, J. D. Laskin, UVB light stimulates
562 production of reactive oxygen species: unexpected role for catalase, *J. Biol. Chem.*,
563 2003, 278, 22432-22436.
564 36. J. He, M. Giusti, Anthocyanins: natural colorants with health-promoting properties.
565 *Annu. Rev. Food Sci. Technol*., 2010, **1**,163-187.
566 37. S. de Pascual-Teresa, D. A. Moreno, C. García-Viguera, Flavanols and
567 anthocyanins in cardiovascular health: a review of current evidence, *Int. J. Mol.*
568 *Sci*., 2010, **11**, 1679-703.
569 38. A. Kaulmann, T. Bohn, Carotenoids, inflammation, and oxidative stress-
570 implications of cellular signaling pathways and relation to chronic disease
571 prevention, *Nutr. Res.,* 2014, **34**, 907-929.
572 39. D. T. Organisciak, D. K. Vaughan, Retinal light damage: mechanisms and
573 protection, *Prog. Retin Eye Res*., 2010, **29**, 113–134.
574 40. W. K. Noell, V. S. Walker, B. S. Kang, S. Berman, Retinal damage by light in rats,
575 *Invest Ophthalmol*., 1966, **5**, 450-473.
576 41. G. Dou, R. Kannan, D. R. Hinton, Endoplasmic reticulum response to oxidative
577 stress in RPE, in *Studies on retinal and choroidal disorders,* eds. R. D. Stratton, W.
578 W. Hauswirth and T. W. Gardner, Human Press, Springer, New York, 2012, pp.
579 241-258.
580 42. S. Balaiya, R. K. Murthy, V. S. Brar, K. V. Chalam, Evaluation of ultraviolet light
581 toxicity on cultured retinal pigment epithelial and retinal ganglion cells, *Clin.*
582 *Ophthalmol.,* 2010, **4**, 33–39.

583 43. W. W. Chou, K. C. Chen, Y. S. Wanga, J. Y. Wang, C. L Liang, S. H. Juo, The role
584 of SIRT1/AKT/ERK pathway in ultraviolet B induced damage on human retinal
585 pigment epithelial cells, *Toxicol. in Vitro*, 2013, **27**, 1728–1736.
586 44. F. Delgado-Vargas, A. R. Jiménez, O. Paredes-López, Natural pigments:
587 Carotenoids, anthocyanins, and betalains - Characteristics, biosynthesis, processing,
588 and stability, *Crit. Rev. Food Sci. Nutr.*, 2000, **40**, 173–289.
589 45. S. H. Lee, D. B. Min, Effects, quenching mechanisms, and kinetics of carotenoids
590 in chlorophyll-sensitized photooxidation of soybean oil, *J. Agric. Food Chem.*,
591 1990, **38**, 1630-1634.
592 46. T. Tsuda, K. Shigu, K. Ohhim, S. Kuwakishi, T. Osawa, Inhibition of lipid
593 peroxidation and the active oxygen radical scavenging effect of anthocyanin
594 pigments isolated from *Phaseoluss vulgaris* L., *Biochem. Pharmacol.*, 1996, **52**,
595 1033-1039.
596 47. H. Wang, G. Cao, R. L. Prior, Oxygen radical absorbing capacity of anthocyanins,
597 *J. Agric. Food Chem.*, 1997, **45**, 304–309.
598 48. A. During, S. Doraiswamy, E. H. Harrison, Xanthophylls are preferentially taken
599 up compared with β-carotene by retinal cells via a SRBI-dependent mechanism, *J.*
600 *Lipid Res.*, 2008, **49**, 1715–1724.
601 49. W. K. Subczynski, A. Wisniewska, J. Widomska, Location of macular xanthophylls
602 in the most vulnerable regions of photoreceptor outer-segment membranes, *Arch.*
603 *Biochem. Biophys.*, 2010, **504**, 61-66.
604 50. J. M. Seddon, U. A. Ajani, R. D. Sperduto, R. Hiller, N. Blair, T. C. Burton, M. D.
605 Farber, E. S. Gragoudas, J. Haller, D. T. Miller, Dietary carotenoids, vitamins A, C,
606 and E, and advanced age-related macular degeneration. Eye disease case-control
607 study group, *JAMA*, 1994, **272**, 1413-1420.

608 51. J. A. Mares-Perlman, A. I. Fisher, R. Klein, M. Palta, G. Block, A. E. Millen, J. D.
609 Wright, Lutein and zeaxanthin in the diet and serum and their relation to age-related
610 maculopathy in the third national health and nutrition examination survey, *Am. J.*
611 *Epidemiol.*, 2001, **153**, 424–432.
612 52. L. Robman, H. Vu, A. Hodge, G. Tikellis, P. Dimitrov, C. McCarty, R. Guymer,
613 Dietary lutein, zeaxanthin, and fats and the progression of age-related macular
614 degeneration, *Can. J. Ophthalmol.*, 2007, **42**, 720–726.
615 53. P. Bhosale, A. J. Larson, J. M. Frederick, K. Southwick, C. D. Thulin, P. S.
616 Bernstein, Identification and characterization of a Pi isoform of glutathione S-
617 transferase (GSTP1) as a zeaxanthin-binding protein in the macula of the human
618 eye, *J. Biol. Chem.,* 2004, **279**, 49447–49454.
619 54. P. Bhosale, B. Li, M. Sharifzadeh, W. Gellermann, J. M. Frederick, K. Tsuchida, P.
620 S. Bernstein, Purification and partial characterization of a lutein-binding protein
621 from human retina, *Biochem.*, 2009, **48**, 4798–4807.
622 55. W. Kalt, J. Blumberg, J. McDonald, M. Vinqvist-Tymchuk, S. Fillmore, B. Graf, J.
623 O'Leary, P. Milbury, Identification of anthocyanins in the liver, eye, and brain of
624 blueberry-fed pigs, J. *Agric. Food Chem.*, 2008, **56**, 705–712.
625 56. H. Matsumoto, Y. Nakamura, H. Iida, K. Ito, H. Ohguro, Comparative assessment
626 of distribution of blackcurrant anthocyanins in rabbit and rat ocular tissues, *Exp.*
627 *Eye Res.*, 2006, **83**, 348–356.
628 57. R. Chen, M. Hollborn, A. Grosche, A. Reichenbach, P. Wiedemann, A. Bringmann,
629 L. Kohen, Effects of the vegetable polyphenols epigallocatechin-3-gallate, luteolin,
630 apigenin, myricetin, quercetin, and cyanidin in primary cultures of human retinal
631 pigment epithelial cells, *Mol. Vis.*, 2014, **20**, 242-258.
632

633 **Figures**

634 **Figure 1.** Effect of UVB irradiation on ARPE19 cells viability. Cells were exposed to
635 UVB radiation at indicated doses and incubated in serum-free medium for 24 h. The
636 results are expressed as percentage of control and are represented by mean ± SD (n=3).
637 Asterisk indicates $p<0.05$ versus control cells no irradiated.

638

639 **Figure 2.** Cytotoxic effects of delphinidin (Dp), cyanidin (Cy), lutein (Lu), zeaxanthin
640 (Ze) and cyanidin-zeaxanthin mix (Cy+Ze). ARPE19 cells were treated for 24 h, and
641 cell viability was assessed by MTT assay. The results are expressed as percentage of
642 control and are represented by mean ± SD (n=3).

643

644 **Figure 3.** Protective effect delphinidin (Dp), cyanidin (Cy), lutein (Lu), zeaxanthin
645 (Ze), and cyanidin-zeaxanthin mix (Cy+Ze) on ARPE19 cells after UVB irradiation.
646 ARPE19 cells were pre-treated with 5 μM Dp, Cy, Lu, Ze or 2.5 μM Cy + 2.5 μM Ze
647 for 60 min before being exposed to UVB radiation. Cell viability was determined by
648 MTT assay 24 h after UVB radiation (500 J/m^2). The control bar means the cells were
649 not neither treated with any compound nor exposed to UVB radiation. The results are
650 expressed as percentage of control and are represented by mean ± SD (n=3). Asterisk
651 indicates $p<0.05$ versus UVB-exposed cells without any compound pre-treatment.

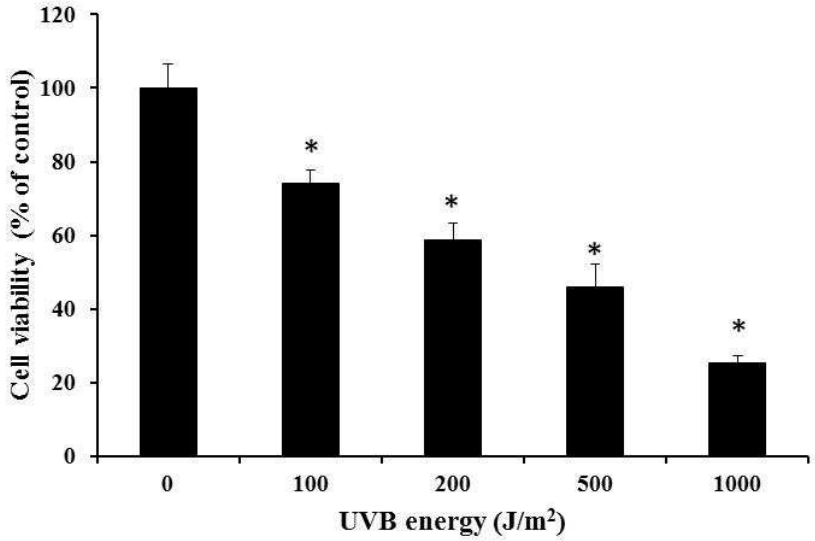
652

653 **Figure 4.** Protective effect of delphinidin (Dp), cyanidin (Cy), lutein (Lu), zeaxanthin
654 (Ze), and cyanidin-zeaxanthin mix (Cy+Ze) on intracellular ROS generation after UVB
655 exposure. ARPE19 cells were pre-treated with 5 μM of Dp, Cy, Lu, Ze or 2.5 μM Cy +
656 2.5 μM Ze for 60 min before being exposed to UVB radiation (500 J/m^2) and tested for
657 ROS production. Values are expressed as a percent relative to the control condition.

658 Asterisk indicates significant reduction of ROS production compared to UVB-exposed
659 cells without any compound pre-treatment ($p<0.05$).
660
661 **Figure 5.** Effect of delphinidin (Dp) and cyanidin (Cy) (**A**), lutein (Lu) and zeaxanthin
662 (Ze) (**B**) and cyanidin-zeaxanthin mix (Cy+Ze) (**C**) on UVB-induced JNK and p38
663 phosphorylations. ARPE19 cells were pre-treated with the compounds for 60 min
664 before being exposed to UVB radiation (500 J/m^2). After 60 min of incubation cells
665 were collected and lysates were analysed by western blot analysis. Each result is
666 representative of three experiments. Asterisk indicates significant reduction of MAPK
667 phosphorylation compared to UVB-exposed cells alone without any compound pre-
668 treatment ($p<0.05$).
669

670 **Figure 1.**

671

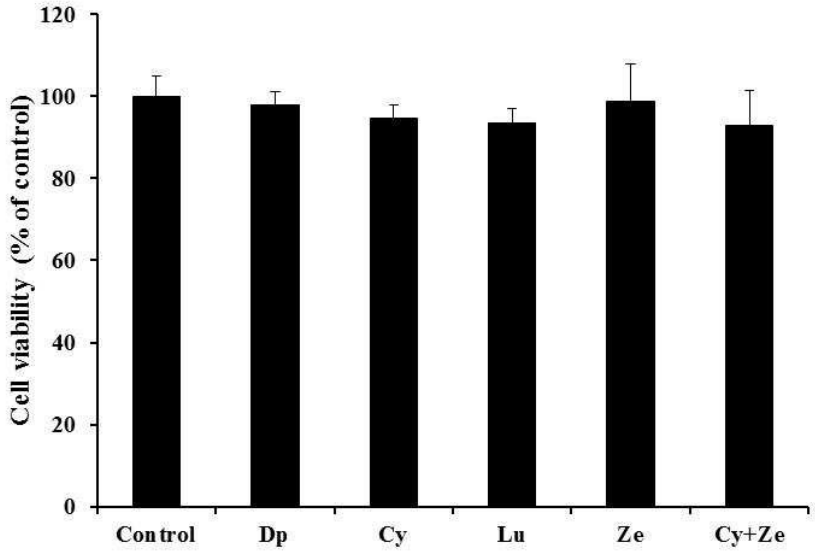


672

673

674 **Figure 2.**

675



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678 **Figure 3.**

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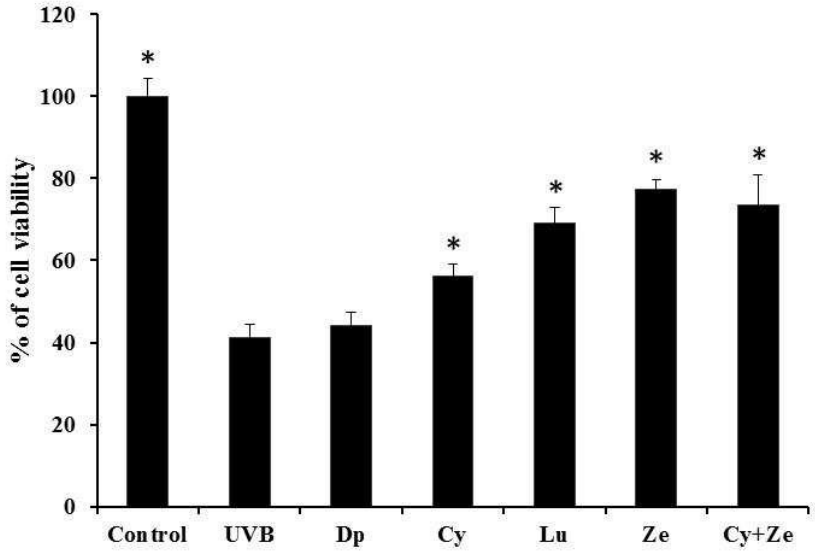

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682 **Figure 4.**

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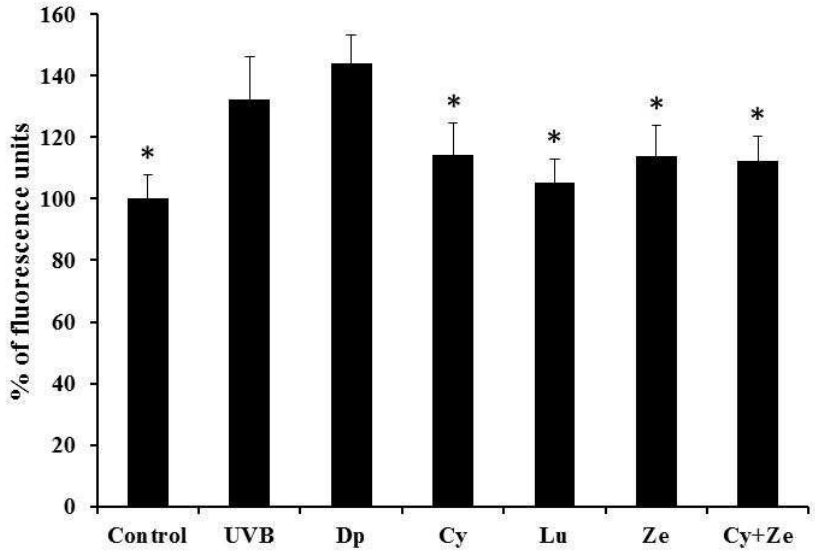

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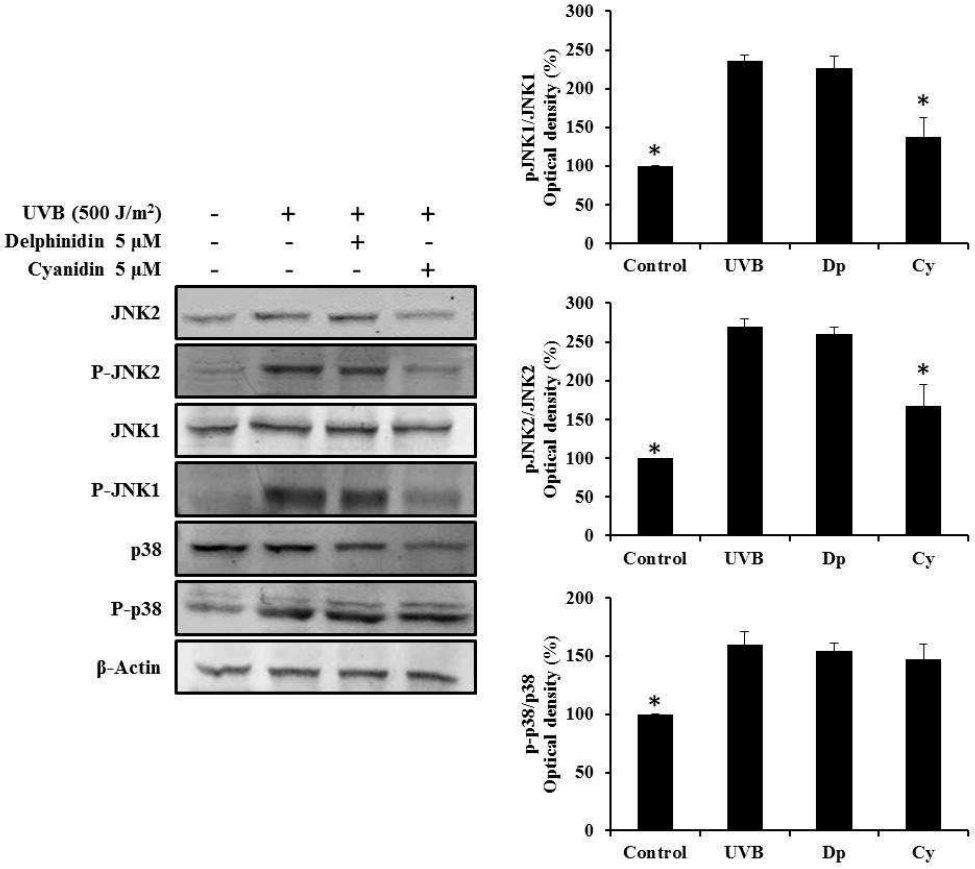
686 **Figure 5.**

687 **A)**



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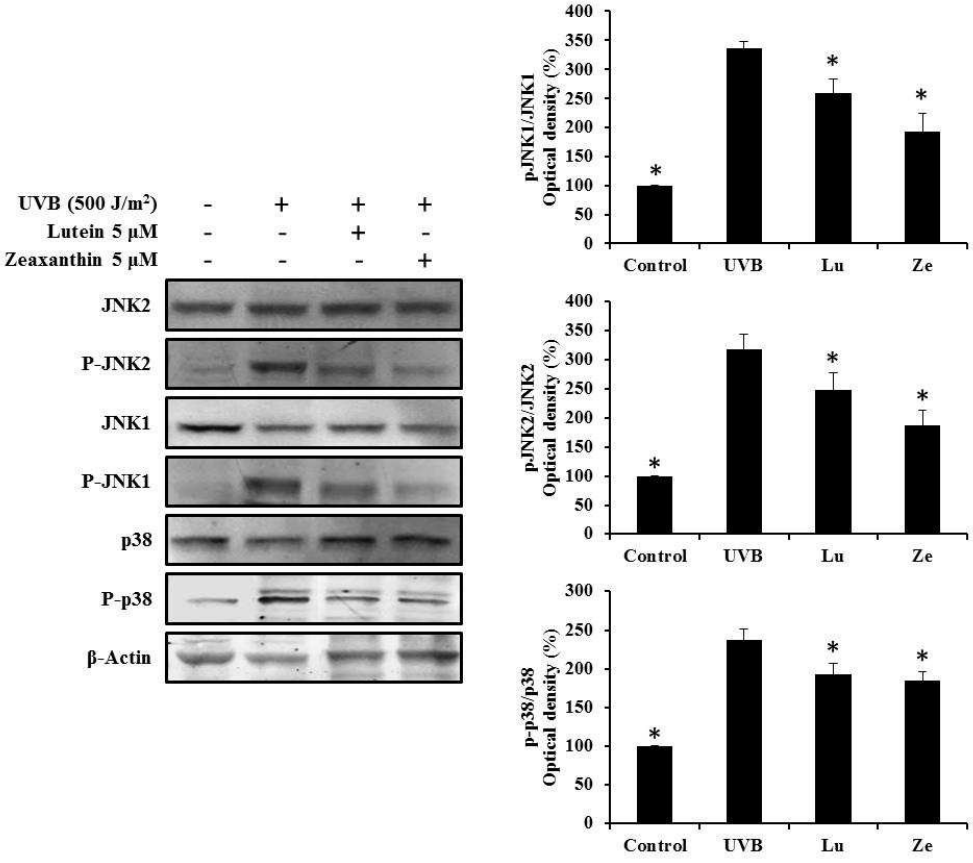
690 **Figure 5.**

691 **B)**



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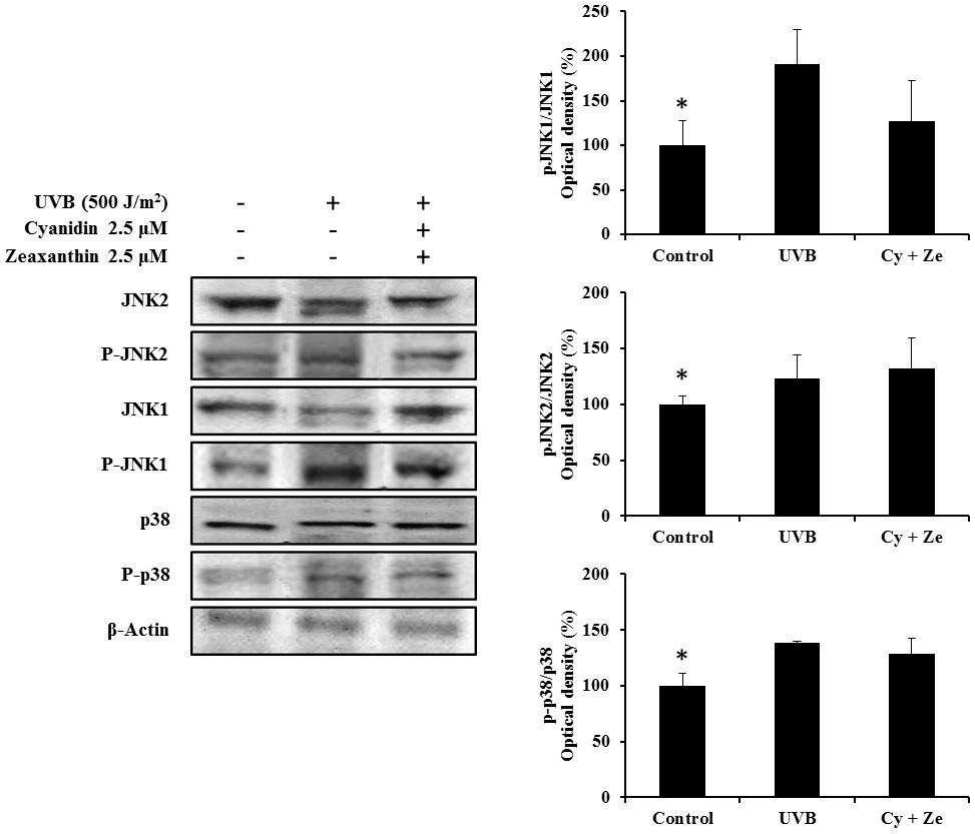
694 **Figure 5.**

695 **C)**



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