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Hydrocaffeic and *p*-coumaric acids, natural phenolic compounds, inhibit UV-B damage in WKD human conjunctival cells *in vitro* and rabbit eye *in vivo*

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Abstract

This paper studied the effect on UV-B ocular damage of 10 μM hydrocaffeic acid (HCAF) alone and as a mixture (MIX) (5 μM HCAF + 5 μM *p*-coumaric acid). Since ocular UV-B damage is mediated by reactive oxygen species, the aim was to test if HCAF and MIX could reduce oxidation damage in human conjunctival cells (WKD) *in vitro* and in cornea and sclera of rabbits *in vivo*. After UVB irradiation (44 J/m^2) of WKD cells, 8-oxodG levels in DNA were markedly increased and this effect was attenuated by HCAF and MIX. Rabbit eyes were treated by application of HCAF and MIX drops before UV-B exposure (79 J/m^2). Corneal and scleral DNA oxidation damage, xanthine-oxidase (XO) activity and malondialdehyde levels (MDA) in corneal tissue and prostaglandin E_2 (PGE_2) in the aqueous humour were reduced by HCAF alone and in combination with *p*-coumaric acid, showing their potential as a topical treatment against UV-B damage.

Keywords: *UV-B, 8-oxo-2′-deoxyguanosine, phenolic acids, reactive oxygen species, XO*

Abbreviations: *UV-B, Ultraviolet B radiation; ROS, Reactive oxygen species; 8-oxodG, 8-oxo-2'-deoxyguanosine; dG, 2'-deoxyguanosine; p-COUM, p-coumaric acid; HCAF, hydrocaffeic acid; DNPH, 2,4-dinitrophenylhydrazine; TEP, 1,1,3,3-tetraethoxypropane; WKD, Wong Kilbourne derived cells; MDA, Malonyldialdehyde; PBS, Phosphate buffer solution.*

Introduction

The depletion of the ozone layer has caused a considerable increase in UV-B radiation (280–315 nm) at the earth level, causing acute and chronic pathological effects on the skin, eye and immune system of humans and other living organisms [1]. Worldwide some 12–15 million people are blinded annually by cataracts, 20% of which may be caused or enhanced by sun exposure [2]. High exposure to UV-B is particularly hazardous for the eye, since it causes photokeratitis, cataract, pterygium, pinguecula and retinal damage [3,4]. UV-B also induces corneal inflammation and increases pro-inflammatory cytokines IL-1, IL-6, IL-8, TNF-*a* [5] and prostaglandins [6].

Classical studies suggest that dipyrimidine photoproducts are a major type of DNA lesion due to UV-B and are directly responsible for increased cytotoxicity and mutations [7]. Nowadays it is also known that UV-B induces ROS-mediated DNA damage and alteration of proteins and lipids [8,9]. Guanine is the most easily oxidized base in DNA, having the lowest oxidation potential of the four canonical bases [10]. An oxidized form of guanine is 8-oxo-2'-deoxyguanosine (8-oxodG), that is generated by

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ROS and is widely used as a marker of oxidative DNA damage [11,12]. DNA damage is elevated in eye pathologies such as macular degeneration [13,14] and increased levels of 8-oxodG have been found also in pterygium [15].

The corneal epithelium contains many antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase [16] and endogenous antioxidants like L-ascorbic acid, α-tocopherol and β-carotene, which decrease when the eye is exposed to UV-B [17–19]. On the other hand, mechanical damage is the major cause of opthalmological morbidity and unilateral blindness worldwide [20]. In the US alone, 2.4 million ocular injuries occur every year and 14.4% of the population suffers an ocular injury at least once in a lifetime [21].

Taking this into account, eye drops containing a molecule or a mixture of natural molecules having combined antioxidant and anti-inflammatory effects could be effective in preventing damage caused by UV-B, mechanical damage or both.

Polyphenols are secondary plant metabolites widely distributed in the vegetal kingdom. Some of them have antioxidant, free-radical scavenging, anti-inflammatory and anti-cancer effects [22–24]. The phenolic acid *p*-coumaric (p-COUM) prevents free radical damage in corneal cells *in vitro* [25] and exerts anti-oxidant and anti-inflammatory effects on the colon mucosal cells in rats [26,27]. Among phenolic acids, hydrocaffeic acid (HCAF), a caffeic acid metabolite, has been insufficiently studied and its antioxidant [28] and anti-proliferative effects have been described only on colon cancer cells and at high doses [29].

The purpose of the present study was to investigate the effect of HCAF or of a mixture (here defined as MIX) of HCAF and p-COUM on oxidation damage and inflammation caused by UV-B *in vitro* and *in vivo*. When we were in the process of writing this report, a paper was announced in the literature on the protective effects of HCAF on keratinocytes exposed to UV light *in vitro* [30].

Materials and methods

Reagents

HCAF and p-COUM, hydroferulic acid, nuclease P1, alkaline phosphatase, RNAse, proteinase K, 2′-deoxyguanosine (2dG), 8-oxo-2′-deoxyguanosine (8-oxodG), 2,4-dinitrophenylhydrazine (DNPH) and 1,1,3,3-tetraethoxypropane (TEP) were purchased from Sigma (Sigma Chemical Co., Milan, Italy). EIA prostaglandin-kit was obtained from Cayman Chem (San Diego, CA). Cell culture reagents were from Gibco (Milan, Italy).

Cell culture and exposure to UV-B

Human conjunctival epithelial cells (Wong Kilbourne, WKD, ECACC 93120839) were cultured in Eagle's MEM containing 2 mM glutamine, 100 units/mL-1 penicillin and 100 μg/mL streptomycin and supplemented with 10% foetal bovine serum. The cell line was maintained at 37°C in a 95% oxygen 5% CO_2 atmosphere. Cells were grown in 60 mm plates until confluence; before UV exposure, cells were rinsed twice with PBS and the medium was replaced by red phenol-free medium without serum containing 10–100 μM HCAF or a mixture of 5 μM HCAF + 5 μM p-COUM (MIX) and incubated for 30 min. A dose-effect experiment was performed to choose the appropriate UV-B dose and to check the effect on cell viability. Cells were then irradiated for 20, 30, 45 and 60 s with UV-B generated by a Vilber Lourmat Lamp (Cedex, France) with a wavelength set at 280–350 nm (emission peak at 312 nm and intensity of 2.2 W/m^2). The total UV-B irradiation doses were 44, 66, 99 and 132 J/m^2, respectively. After exposure, cells were incubated at 37°C with 5% CO_2 for 45 min and trypsinized. Viability was measured using the trypan blue exclusion method. Cells were finally centrifuged and pellets were lysed with 1 mL of 10 mM Tris-HCl buffer pH = 8, 10 mM EDTA, 10 mM NaCl and 0.5% SDS (DNA lysis buffer).

Cellular uptake

Cells were grown until confluence and incubated in serum-free media containing either 10 μM of HCAF or MIX (5 μM HCAF + 5 μM p-COUM). After 30 and 75 min media were recovered and prepared for LC-MS/MS analysis, according to Larrosa et al. [31]. Cells were washed twice with PBS, lysed, scraped with 2 ml methanol, sonicated on ice for 5 min and centrifuged at 3000 rpm. The supernatant was concentrated under reduced pressure and an aliquot (100 μL) was analysed by LC-MS/MS. The LC system was a Perkin Elmer Series 200 (Perkin Elmer, Italy), consisting of a binary-pump and an autosampler. Samples were separated onto a reverse phase C18 Phenomenex Luna column (150 mm × 4.60 mm, 5 μM particle size). The mobile phase was water with 0.1% formic acid (solvent A) and acetonitrile: water:formic acid 96.9:3:0.1 v/v (solvent B) at a flow rate of 1 mL min^{-1}. The gradient started with 10% B in A for 3 min to reach 80% B in A at 13 min, 80% B in A at 14 min and 90% of B in A at 15 min; chromatographic run time was 30 min per injection. API 365 triple quadrupole mass spectrometer was used for MS detection. The mass spectrometer was operated in negative-ion, multiple reaction monitoring (MRM) mode. The temperature and flow rate of the turbo gas was adjusted to 250°C and 8 L min^{-1}, respectively. The ionization was set to −4000 V. Nitrogen was used as curtain gas, nebulizing gas and

collision gas; their flows were at instrument settings of 6, 7 and 3, respectively. Collision energy was 26 V. Singly charged precursor-product ion (MS/MS) transitions were monitored at m/z 181→137, 181→59, for HCAF and 163→119 for *p*-COUM. Dwell time was 200 ms for all compounds.

Animals, treatment and UV exposure

All assays were carried out by fulfilling the Declaration of Helsinki related to animal welfare and experimental guidelines. Fourteen male New Zealand White albino rabbits (1.6–1.9 kg) were obtained from Charles River (Calco, Como, Italy). Three days prior to UV-B exposure we administered two drops (25 µl × 2) of saline as vehicle, HCAF (10–100 µM) or MIX (5 µM HCAF and 5 µM p-COUM) twice a day (b.i.d.) in the conjunctiva of each rabbit eye. Previously results in our laboratory have demonstrated an effect of p-COUM acid when administrated at 164 ng per day [31]. Taking this into account we decided to administer 182 ng or 1820 ng doses of HCAF or 91 ng of HCAF plus 82 ng of p-COUM for each eye.

To check if HCAF or p-COUM caused damage or irritation during the days preceding UV-B exposure, eyes were observed with a slit lamp microscope (Nikon, Japan) and in each eye the following parameters were measured: vessel reactivity (as hyperemia of the iris and sclera and limbal vessels); epithelial layer damage (as fluorescein staining); inflammatory reaction (as corneal opacity). At each time point, photographs were taken to document the responses. Before UV-B exposure animals were anaesthetized with sodium pentobarbital (30 mg/kg e.v.) and 50 µl of aqueous humour were obtained with a sterile needle (gauge: 0.45 × 12 mm) from each eye to determine the levels of PGE_2. Each eye (except control eyes) was exposed for 36 s to a UV-B emitted by the Vilber Lourmat Lamp described above according to the Lodovici et al. [32] method. Total irradiation dose for each eye was 79.2 J/m^2. The treatment with eye drops containing phenolic acids was continued for 5 days after UV-B exposure. A scheme of the experimental design is shown in Figure 1.

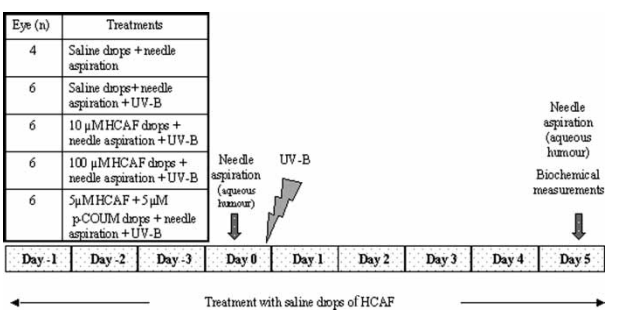

Figure 1. Scheme of *in vivo* experiments. Animals were New Zealand White albino rabbits.

Assay of 8-oxodG in WK cells, cornea and sclera

DNA was isolated with a previously published procedure [33] with some modifications. Cells, cornea or sclera tissues were homogenized in a Ultraturrax T-25 equipment at 24 000 rev/min for 1 min with 1 mL of DNA lysis buffer and then incubated at 37°C for 1 h with 10 µL of 20 µg/mL RNAse. We then added 50 µL of proteinase K (> 800 units mL^{-1}) and samples were incubated for an additional 3 h. Samples were then extracted twice with 1 mL chloroform/isoamyl alcohol (5:1, v/v) and DNA was precipitated by adding 1:10 volumes of 3 M ammonium acetate and 2 volumes of ethanol, kept at −20°C overnight and then centrifuged 1 h at 20 000 × g at 4°C. DNA was solubilized in 10 mM Tris-HCl pH 7.3 and aliquots of 100 µg of DNA were hydrolysed with 10 µL of nuclease P1 (1100 U/mL) for 2 h and 14 µL of alkaline phosphatase (750 U/mL) for 30 min. The hydrolysed mixture was filtered by Micropure-EZ enzyme remover (Amicon, Millipore) and 50 µg were injected into the HPLC. The nucleosides were separated by C18 reverse-phase column (Supelco, 5 µM, ID 0.46 × 25 cm). The levels of 8-oxodG and 2dG were detected using an ESA Coulochem II electrochemical detector in line with a UV detector as previously reported [34]. The 8-oxodG levels were expressed as the ratio of 8-oxodG/dG × 10^{-6}.

Xanthine-oxidase (XO) activity

Corneal tissue was homogenized in 1 mL of 50 mM PBS containing 10 µL of 0.1 mM dithiothreitol in Ultraturrax T-25 equipment and centrifuged for 15 min at 20 000 × g. A fraction of supernatant was used for XO activity determination according to Corte and Stirpe [34]. XO activity was assayed measuring the increase of absorbance at 286 nm deriving from uric acid production. Enzymatic activity was expressed as mU/mg protein (one unit of XO activity corresponds to 1 µmol of uric acid per min). Protein was determined by the Bio-Rad DC Protein colourimetric assay according to the manufacturer's specifications.

Malonyldialdehyde (MDA) levels in corneal tissue

MDA levels were determined according to the method of Mateos et al. [35]. Corneal tissue was homogenized as described above and 250 µL of supernatant were submitted to alkaline hydrolysis with 50 µL of 6 M NaOH for 30 min a 60°C. Protein was then precipitated with 30% trichloroacetic acid followed by centrifugation at 2800 × *g* for 10 min. A volume of 250 µL supernatant was mixed with 25 µL of 5 mM DNPH and incubated at 30 min at room temperature, protected from light. The reaction mixture (200 µL) was injected into an HPLC equipped with an LC-10 AD Shimadzu pump, a

UV/vis Perkin-Elmer LC 290 detector and with a Water Spherisorb ODS-2 column (4.6 mm × 150 mm I.D., 5 μM particle size).

PGE_2 assay

PGE_2 levels in aqueous humour were measured using an inmunoenzymatic method (EIA kit, Cayman Chem, San Diego, CA) according to the manufacturer's specifications. Aqueous humour was extracted prior to UV-B irradiation and before sacrifice. No dilution was used.

Statistical analysis

All analyses were carried out using SPSS® 14.0 software. All data are expressed as the mean ± SD of independent measurements. Statistical significance was determined by Student's *t*-test, using $p < 0.05$ as the level of significance.

Results

HCAF and p-COUM effects on DNA oxidation damage induced by UV-B in WKD cells

Increasing doses of UV-B irradiation (44, 66, 99 and 132 J/m^2) induced 8-oxodG higher levels in DNA of conjunctival cells and decreased cell viability (Figure 2). The 44 J/m^2 dose was chosen to be used in subsequent experiments since it induced DNA damage without causing a considerable decrease in viability, as measured by trypan blue exclusion test (Figure 2). As shown in Figure 3 the content of 8-oxodG in untreated cells was $1.25 \pm 0.12 \times 10^{-6}$ dG (mean ± SD) and increased to $6.06 \pm 0.41 \times 10^{-6}$ dG (4.8-fold) after UV-B irradiation. The addition to the incubation medium of HCAF (10 μM and 100 μM) significantly decreased (68% and 73%, respectively) 8-oxodG levels, restoring them to levels close to control. No significant differences were found between 10 μM and 100 μM HCAF, indicating that DNA protective effect may have been maximal at 10 μM. The MIX (5 μM HCAF + 5 μM p-COUM) induced a 77% decrease in 8-oxodG content, indicating a probable synergistic effect of the two compounds.

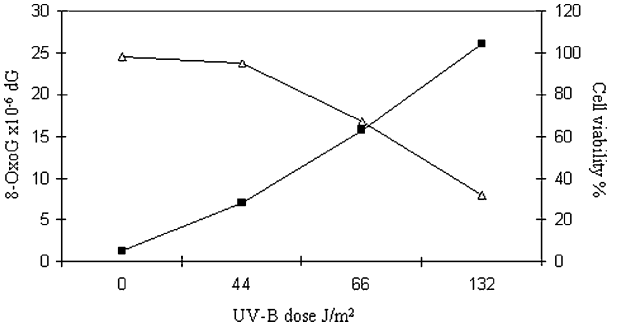

Figure 2. Effect of increasing doses of UV-B exposure (44, 66, 99 and 132 J/m^2) on cell viability △ (% respect to controls) and 8-oxodG × 10^{-6} dG levels ■. Cells were exposed to UV-B light and 45 min later were tripsinized and cell viability and 8-oxodG × 10^{-6} dG levels were determined. The data are average of three determinations.

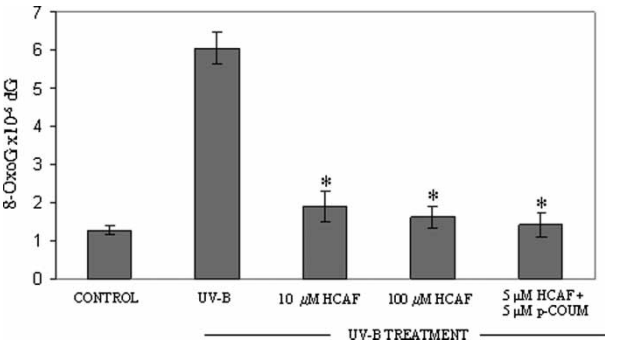

Figure 3. DNA oxidative damage in WKD human conjunctival cells after UV-B (44 J/m^2) exposure (*$p < 0.05$; $n = 3$).

Cell metabolism of HCAF and p-COUM acid

To study the uptake and metabolism of HCAF and p-COUM in WKD cells we analysed culture media and cells incubated for 30 and 75 min with these compounds. The concentration of HCAF decreased rapidly in cell media (from 10 μM to 4.6 μM in 75 min); a similar trend was observed for the 5 μM initial concentration (Figure 4). At 75 min we detected a $[M\text{-}H]^-$ ion at m/z 195 in cell media incubated with 10 μM HCAF. The MS^2 analysis produced the main daughter ions m/z 152 and m/z 136, suggesting an intracellular methylation of HCAF. The identity of this peak was confirmed injecting standards of hydroferulic acid (the methylated form of hydrocaffeic acid). Nevertheless, hydroferulic acid was not quantified in the incubation media, since its concentration was too low. The concentration of p-COUM did not vary in the cell media during 75 min of incubation, suggesting that this compound is not metabolized by WKD cells, at least in this period of time. Coherently with these results, in cells extracts no trace of p-COUM acid was detected. HCAF

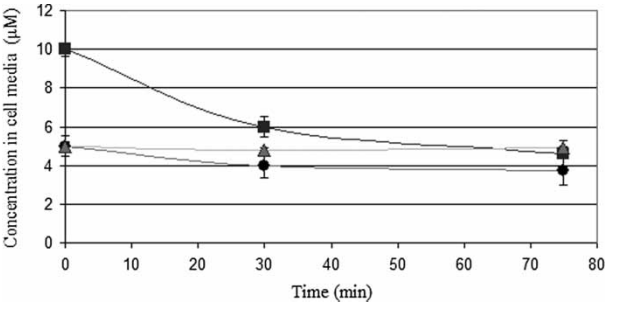

Figure 4. Cell uptake of HCAF ■ (10 μM) and MIX (5 μM HCAF ● + 5 μM p-COUM ▲) from cell media. Cells were treated with HCAF or MIX for 30 and 75 min. After that media and cell extract were purified and analysed by LC-MS-MS ($n = 3$).

was found in cell lysates at 1.5 μM concentration incubating cells with 10 μM initial concentration. Hydroferulic acid was not detected in cell extracts, possibly because it was below the detection limit.

HCAF and p-COUM effects on UV-B induced oxidative DNA damage to cornea and sclera in vivo

Administration of eye drops containing HCAF or MIX for 3 days before UV-B exposure did not induce hyperemia, epithelial damage or corneal opacity in rabbit eyes, in comparison with eyes that only received eye drops of vehicle (data not shown). After 3 days of pre-treatment, rabbit eyes were irradiated with UV-B for 36 s (total dose of 79.2 J/m^2). With this single UV-B exposure the levels of 8-oxodG in the cornea, measured 5 days after exposure, were increased ~ 21-fold in the cornea and ~ 14-fold in the sclera (Figure 5). We found smaller damage to the sclera relative to the cornea, probably because of a larger exposure surface of the cornea to UV-B radiation. As observed with WKD cells *in vitro*, the inhibition of oxidative DNA damage observed after HCAF-containing eye drops *in vivo* was maximal at 10 μM concentration. When eyes were treated with the MIX we observed a higher protective effect than with HCAF alone ($p < 0.05$). This indicates a synergistic effect *in vivo*.

XO activity after UV-B exposure and HCAF and p-COUM treatment

The activity of XO was enhanced significantly 5 days after UV-B irradiation. Treatment with 100 μM HCAF alone or with MIX significantly reduced XO activity ($p < 0.05$), restoring it to the range of control levels (Figure 6).

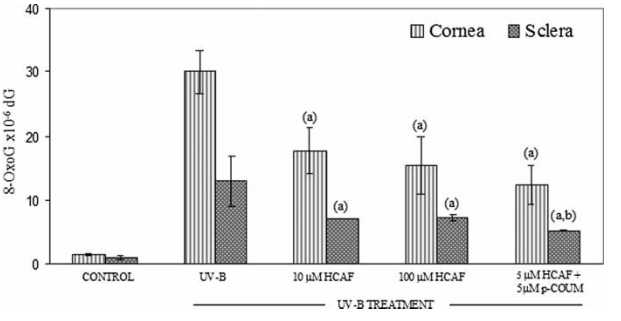

Figure 5. DNA oxidative damage in corneal and scleral tissue *in vivo*. Rabbit eyes were treated 3 days before irradiation and 5 days after UV-B (79.2 J/m^2) with eye drops twice daily (b.i.d., 50 μl); 182–1820 ng of HCAF (10–100 μM, respectively) were administered (ng/d/eye) or 91 ng HCAF+82 ng p–COUM, (MIX 5 μM of each compound). (a) significant vs eyes exposed to UV-B, (b) significant vs eyes treated with 10–100 μM HCAF ($p < 0.05$; $n = 6$).

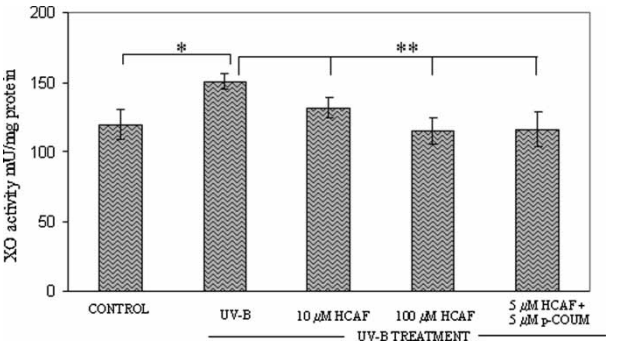

Figure 6. XO activity in cornea tissue 5 days after UV-B irradiation. Rabbit eyes were treated with the eye drops containing phenolics 3 days before and 5 days after UV-B. *significant vs control, **significant vs UV-B ($n = 6$).

HCAF and p-COUM effect on MDA levels in cornea tissue

MDA levels (Table I) were increased in cornea tissue. Lipid oxidation was reduced significantly by the treatment with 10–100 μM HCAF and with MIX (Table I).

HCAF and p-COUM effect on PGE_2 production in aqueous humour after UV-B rays

Five days after mechanical damage caused by needle aspiration of the aqueous humour, PGE_2 levels increased in the aqueous humour and UV-B irradiation caused a small, but significant, increase in PGE_2 (Table I). Treatment with 10 μM HCAF reduced PGE_2 production by ~ 60% and 100 μM was equally effective (Table I). Unlike what was observed with 8-oxodG levels, no synergistic effect on PGE_2 was obtained with the MIX treatment.

Discussion

The present study demonstrates that HCAF alone or in combination with p-COUM at relatively low concentration is effective in blocking UV-B induced damage. HCAF was able to decrease 8-oxodG formation in WKD cells *in vitro*. Similar results were previously reported for p-COUM on cornea-derived cells [25]. It has also been shown before that genistein, luteolin and quercetin reduce damage in calf thymus DNA irradiated with UV [36] and that *Calophyllum inophyllum* oil containing polyphenols also inhibits UV-induced DNA damage [37]. In a recent study, Park and Lee [38] demonstrate a protective effect of resveratrol in UV-B irradiated keratinocytes, associated with increased cell survival and reduced ROS production.

In the present study we showed that HCAF was taken up by WKD cells. These results are in agreement with data of Lekse et al. [39], who measured an intracellular concentration of 10 M of

Table I. MDA levels in the corneal tissues after needle aspiration of the aqueous humour, UV-B exposure and application of anti-inflammatory phenolic acids. Basal PGE_2 level in the aqueous humour and 5 days after needle aspiration and UV-B irradiation. The treatment with eye drops containing phenolics started 3 days before the needle aspiration.

Treatment	MDA(nmol/mg prot)	Basal level PGE_2(pg/mL)	Day 5 PGE_2(pg/mL)
Needle aspiration	0.94±0.13	11±5.5	236±86
Needle aspiration+UV-B	1.65±0.33 [a]	6±4.3	383±54[a]
Needle aspiration+UV-B+10 μM HCAF	1.07±0.19 [b]	12±7.0	148±32 [a,b]
Needle aspiration+UV-B+100 μM HCAF	0.92±0.16 [b]	20±13	147±50 [a,b]
Needle aspiration+UV-B+5 μM HCAF+5 μM p-COUM	1.04±0.21 [b]	10±3.7	200±24 [a,b]

[a] significant vs needle aspiration; [b] significant vs needle aspiration+UV-B exposure ($p<0.05$; $n=6$).

HCAF in erytrocytes incubated for 1 h in a buffer containing 20 M HCAF. Similarly, Huang et al. [28] showed the uptake of HCAF by human endothelial cells. We demonstrate here that HCAF was methylated by WKD cells, in agreement with observations by Poquet et al. [30] in human keratinocytes.

p-COUM acid was not taken up or metabolized by WKD cells, at least during the studied time. Similar results were obtained by Kern et al. [40] in the colon cell line Caco-2, where p-COUM concentrations in cell media showed a slight decrease in 24 h and only traces of methyl *p*-coumarate-sulphate and *p*-coumaric acid sulphate were detected.

We showed that the administration of eye drops with MIX (5 μM HCAF+5 μM p-COUM) *in vivo* suppressed 8-oxodG formation up to 77%. This effect of MIX was stronger than p-COUM acid administered alone at 10 μM dose [32] and than the effect of HCAF at 10 μM suggesting a synergistic effect between both compounds. This marked decrease in UV-B induced oxidative DNA damage could be relevant in eye pathology. In fact, ROS-induced damage is relevant in the eye and high levels of 8-oxodG occur in light-induced pathological processes such as pterygium [37]. In our study the prevention of DNA oxidation *in vivo* was accompanied by a reduction in lipid peroxidation (expressed as MDA levels). Similar effects were found by Tomaino et al. [41] in UV-B exposed epidermal cells treated with wine polyphenols.

The activity of XO, a pro-oxidant enzyme, is increased after UV-B irradiation [42]. Our results demonstrate that HCAF alone or MIX (HCAF+p-COUM) are able to reduce XO activity in the cornea after UV-B irradiation. These results are in agreement with the data of Chang et al. [43] who report that caffeic acid and p-COUM are competitive inhibitors of XO and also potent superoxide-supressive agents. These effects could explain in part the strong activity of these compounds on UV-B damage in our experiments.

In terms of inflammatory reactions, it is known that many stimuli, including UV and trauma, increase PGE_2 levels in the cornea [44,45]. In our experiments, mechanical damage due to the insertion of a needle for aspiration of the aqueous humour increased PGE_2 levels 22-fold; PGE_2 levels were elevated in the aqueous humour up to 32-fold in eyes irradiated with UV-B. Accordingly, Andley et al. [46] found a 121-fold increase in PGE_2 in the aqueous humour after irradiating rabbit eyes with 2800 J/m^2. In cell culture studies 5 mM tannic acids decrease PGE_2 concentration in keratinocytes exposed to 100 J/m^2 UV-B [47]. Eye drops containing HCAF, even at the lowest dose used, decreased PGE_2 production in the aqueous humour. This is in agreement with the data of Staniforth et al. [48] in mouse skin, who demonstrated that caffeic acid (precursor of HCAF) inhibits UV-B induced COX-2 protein, an enzyme evolved in PGE_2 synthesis. Moreover, Nagasaka et al. [49] report an anti-inflammatory effect of hydroxycinnamic acid derivatives in macrophages.

Taking into account all our results we can hypothesize that HCAF exerts its protective effect against UV-B radiation with three different mechanisms. It probably acts as a direct UV-B screen, since it shows an absorption spectrum from 250–300 nm with a maximum peak at 283 nm. HCAF also enters the cells, where it can interfere with radiation-induce oxidation damage (decreasing XO activity, DNA oxidative-damage and lipoperoxidation). Finally it can act as an anti-inflammatory compound, since it decreases PGE_2 levels in the aqueous humour. HCAF has shown an anti-inflammatory effect also in human fibroblasts stimulated with interleukin-1 *in vitro*.

p-COUM, which is not metabolized by WKD cells, seems to be active mainly as an UV screen, absorbing radiation and performing a scavenging action in cell media. In fact p-COUM absorb UV-B light from 250–350 nm, with a maximum peak at 312 nm.

In conclusion, we discovered that the phenolic compound HCAF protects against DNA oxidation due to UV-B *in vitro* and against ocular inflammation and cell oxidation damage induced by UV-B *in vivo*. It seems to exert this effect through manifold mechanisms, as discussed above.

HCAF is produced by the human colon microflora after consumption of food containing polyphenols (fruits and vegetables mainly), which are a part of the normal human diet. However, after dietary

consumption of fruits and vegetables the levels of HCAF would not reach a systemic concentration high enough to block UV-B damage to the eye. However, if HCAF is administered as a topical preparation (eye drops), it could be used to prevent UV-B damage and, possibly, to reduce inflammation induced by mechanical wear and tear of the eye. The present results show a synergistic effect of HCAF and p-COUM at low concentrations in preventing oxidative DNA damage. Both compounds are available at low cost, are non-toxic and not covered by registered patents for ocular use. Therefore, when subsequent experiments will be done to confirm these results in humans, they could represent a possible easy prevention measure if administered as eye-drops against UV-B ocular damage.

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***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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