Journal of Photochemistry and Photobiology B: Biology 136 (2014) 12–18

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

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

Protective effects of citrus and rosemary extracts on UV-induced damage in skin cell model and human volunteers

A. Pérez-Sánchez [a,1], E. Barrajón-Catalán [a,1], N. Caturla [b], J. Castillo [c,e], O. Benavente-García [c,e], M. Alcaraz [d,e], V. Micol [a,*]

[a] Skin Research Platform (SRP), Instituto de Biología Molecular y Celular (IBMC), Universidad Miguel Hernández, Avenida de la Universidad s/n, E-03202 Elche, Alicante, Spain
[b] Monteloeder S.L., Miguel Servet 16, nave 17, 03203 Elche, Alicante, Spain
[c] Nutrafur S.A., Camino Viejo de Pliego, km.2, 30820 Alcantarilla, Murcia, Spain
[d] Radiology and Physical Medicine Department, Faculty of Medicine, University of Murcia, 30100 Espinardo, Murcia, Spain
[e] Institute of Research Into Aging, University of Murcia, 30100 Espinardo, Murcia, Spain

ARTICLE INFO

Article history:
Received 24 February 2014
Received in revised form 5 April 2014
Accepted 7 April 2014
Available online 20 April 2014

Keywords:
UV radiation
Oral photoprotection
ROS
DNA damage
Rosemary
Citrus
Polyphenols

ABSTRACT

Ultraviolet radiation absorbed by the epidermis is the major cause of various cutaneous disorders, including photoaging and skin cancers. Although topical sunscreens may offer proper skin protection, dietary plant compounds may significantly contribute to lifelong protection of skin health, especially when unconsciously sun UV exposed. A combination of rosemary and citrus bioflavonoids extracts was used to inhibit UV harmful effects on human HaCaT keratinocytes and in human volunteers after oral intake. Survival of HaCaT cells after UVB radiation was higher in treatments using the combination of extracts than in those performed with individual extracts, indicating potential synergic effects. The combination of extracts also decreased UVB-induced intracellular radical oxygen species (ROS) and prevented DNA damage in HaCaT cells by comet assay and decreased chromosomal aberrations in X-irradiated human lymphocytes. The oral daily consumption of 250 mg of the combination by human volunteers revealed a significant minimal erythema dose (MED) increase after eight weeks (34%, $p < 0.05$). Stronger protection was achieved after 12 weeks (56%, $p < 0.01$). The combination of citrus flavonoids and rosemary polyphenols and diterpenes may be considered as an ingredient for oral photoprotection. Their mechanism of action may deserve further attention.

1. Introduction

UV radiation from the sun induces several harmful responses, including erythema, immunosuppression, edema, sunburn, hyperplasia, hyperpigmentation, premature aging and skin cancer. Only UVA (320–400 nm) and UVB (280–320 nm) are harmful to our skin. UVA accounts for more than 90% of the total UV radiation reaching us and is constant throughout the year, but UVB photons are one thousand times more capable of causing sunburn than UVA and increase considerably in the summer [1]. UVA is thought to play a crucial role in photoaging and causes epidermal hyperplasia, stratum corneum thickening and the synthesis of inflammatory cytokines and metalloproteinases (MMPs) [2,3]. Meanwhile, UVB causes sunburn, sun tanning, pigmented spots and wrinkles and accelerates skin aging.

Both UVA and UVB contribute significantly to photoaging. Photoaging is the superposition of chronic UV-induced damage on intrinsic skin aging and accounts for most age-related changes in skin appearance [4]. The molecular mechanism of photoaging is mostly triggered by ROS-mediated activation of cell surface receptors leading to stimulation of stress-associated kinase pathways, which are mediated by protein 1 (AP-1) nuclear transcription factor [5]. This factor prompts prostaglandin release, keratinocyte proliferation and a loss of collagen synthesis [6]. ROS also lead to the activation of NF-κB transcription factor, which induces the expression of pro-inflammatory cytokines and metaloproteinases (MMPs). Finally, ROS-induced mitochondrial DNA deletions compromise mitochondrial function [4]. All these factors together with direct protein oxidation contribute to accelerate extracellular matrix degradation. Although UVA is suspected to play a major role in photoaging, UVB activates AP-1 and causes direct DNA damage, inflammation, immunosuppression, angiogenesis and elastin degradation.

Major protective systems in human skin cells are the natural pigment melanin, which absorbs and scatters UV radiation, and

* Corresponding author. Tel.: +34 96 6658430; fax: +34 96 6658758.
E-mail address: vmicol@umh.es (V. Micol).
[1] These authors have contributed equally.

http://dx.doi.org/10.1016/j.jphotobiol.2014.04.007
1011-1344/

skin cells' antioxidant enzymes (catalase, superoxide dismutase and glutathione peroxidase) [4]. Moreover, p53 protein plays a crucial role in UVB protection, acting as a transcription factor that controls genes involved in the cell cycle, apoptosis and DNA repair [7].

Human skin is inadvertently exposed to approximately 2/3 of the cumulative erythemal ultraviolet (UV) dose/year when no topical protection is used [8]. Therefore, beyond topical sunscreen protection when consciously exposed [9], photoprotection by dietary compounds via endogenous delivery to the skin may significantly contribute to lifelong protection of skin health. Several plant phytochemicals have been found to be effective in preventing UV-induced DNA oxidative damage through a ROS scavenging mechanism *in vitro* and in animal models [1]. Rosmarinic acid [10–12], a phenolic acid derivative, the isoflavone genistein [13] and silibin from milk thistle [14] have exhibited skin protective effects against UVB-induced ROS on mice and cell models through p53 activation and decreased DNA damage. Chrysin (5,7-dihydroxyflavone) attenuates apoptosis, ROS generation and cyclooxygenase 2 (COX-2) expression and diminishes aquaporin-3 downregulation induced by UVB and UVA [15]. Rosemary diterpenes display strong *in vitro* antioxidant and anti-inflammatory effects, together with *in vivo* skin protective effects [16–18], and forskolin protects keratinocytes from UVB-induced apoptosis independently of melanin content [19].

In the present study, a specific combination of a rosemary extract, enriched in polyphenols and diterpenes, and a citrus bioflavonoid extract, containing flavonoids, was used for the first time to inhibit UVB's harmful effects on human keratinocytes. The capacity of this combination to decrease UVB-mediated cell death, ROS formation and DNA damage in human keratinocytes, and genotoxicity induced by X-rays in human lymphocytes, was assessed. A human intervention study using 250 mg of this combination by oral administration for three months was also established to determine the capacity of the ingredient to increase the minimal erythema dose (MED) of the volunteers upon UV exposure.

2. Materials and methods

2.1. Materials

The human keratinocytes HaCaT (a spontaneously immortalized cell line) were obtained from CLS Cell Lines Service GmbH (Eppelheim, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin–streptomycin were obtained from Gibco® (Life Technologies Co., Europe). Phytohemagglutinin, cytochalasin B (Cyt B) and the rest of the reagents were purchased from Sigma–Aldrich (Europe).

2.2. Extracts

Herbal extracts were kindly provided by Monteloeder, Inc. Citrus extract, obtained from immature grapefruit, was enriched in citrus bioflavonoids as declared by the manufacturer and showed a phenolic content of 22.57 ± 2.65 expressed as GAE (gallic acid equivalents)/100 g dry weight (dw). The rosemary extract contained phenolic compounds and diterpenes, as declared, and a phenolic content of 57.16 ± 1.25 GAE/100 g dw was determined. The combination of extracts (NutroxSun®) used in the cellular assays and the human intervention study contained the two extracts at a 1:1 ratio and had a phenolic content of 36.32 ± 3.91 GAE/100 g dw. Powered extracts were stored at RT under controlled humidity conditions in a desiccator. Rosemary extract was dissolved into sterile distilled water and citrus extract and the combination were dissolved into DMSO at desired concentrations. Solutions were sterile filtered and freshly prepared for every cellular assay.

2.3. Keratinocyte cell culture

HaCaT cells were grown in DMEM with 10% (v/v) FBS and 1% (v/v) penicillin–streptomycin (0.1 mg/ml penicillin and 100 U/ml streptomycin) in a humidified atmosphere with CO_2 (5% v/v) at 37 °C. The HaCaT cells were trypsinized every third day, following the manufacturer's instructions, and seeded in 96- or six-well plates, depending on the assay.

2.4. Treatment of cells, UVB irradiation and MTT survival assay

Cells were cultured in 96-well plates and maintained in medium for 24 h. For treatments, cells at 50–70% confluence were washed with phosphate-buffered saline (PBS) and treated with PBS containing the selected extract (12.5–100 μg/ml, no toxicity was observed at these concentrations), followed by treatment with UVB light emitted from a Bio-Link Crosslinker BLX-E312 (Vilber Lourmat, France) at 800 or 1200 J/m^2 [20]. Afterward, the PBS was replaced with fresh medium, and the cells were incubated for 72 h for viability assays or for 2 h for comet or ROS assays. The MTT assay was used for determining cellular viability [21]. The cell protection level was calculated as the percentage of cell viability recovered under a certain condition, where 100% was the difference between non-irradiated cells and irradiated cells in the absence of the extract.

2.5. Total phenolic content determination

Total polyphenolic content was determined using the Folin–Ciocalteu method, using gallic acid as the standard [22,23].

2.6. ROS generation evaluation

2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular probes®, Life Technologies Co., Europe) was used to monitor the intracellular ROS generation induced by UVB radiation [21]. Promptly, cells were cultured in a 96-well black plate until 90–100% confluence was reached. The cells were then treated with a thin layer of PBS containing the combined extract (75–100 μg/ml), followed by treatment with UVB light (800 or 1200 J/m^2) and labeled with H_2DCFDA.

2.7. Single-cell gel electrophoresis (comet assay)

Comet assay was essentially performed as previously reported [11]. A total of 50 nuclei by duplicate were utilized for determination of Comet score. The gels were analyzed by fluorescence microscopy (Nikon Eclipse TE2000U). DNA damage was quantified as described in Supplementary information section.

2.8. Cytokinesis-blocked micronucleus (CBMN) assay and genotoxicity

Human whole blood samples were obtained and treated in the presence and in the absence of the extracts and exposed to 2 Gy doses of X-rays (see Supplementary information for details). After X-irradiation, the micronucleus assay was performed on the irradiated lymphocytes using a reported CBMN method [24,25], adapted by the International Atomic Energy Agency (2011).

2.9. Human intervention study

The human intervention assay was developed on 10 healthy human volunteers by EVIC HISPANIA, Centro Experimental de Evaluación Cutánea, S.L. (Spain) to study the protective activity of the combination (NutroxSun®) after oral ingestion by the calculation of MED at different times (reference code 10-0266/0).

The compliance of the product with the European regulations in force was guaranteed after reviewing the procedure, undertaking letter, informed consent and previous documentation on the tested product (see Supplementary information for details).

2.10. Statistical analysis

The data were expressed as the mean ± SD of 4–8 determinations, depending on the assay. One-way analysis of variance (ANOVA) and statistical comparisons of the different treatments were performed using Tukey's test in GraphPad Prism version 5.0 (GraphPad Software). In the CBMN study, the degree of dependence and correlation between variables was assessed using ANOVA complemented by a contrast of means ($p < 0.05$). For human intervention study, a Student's t test for paired series was used.

3. Results and discussion

3.1. Effects of the combined extract and its individual components on the viability of HaCaT cells exposed to UVB

To study the protective effects of citrus and rosemary extracts and that of their combination, HaCaT cell viability after UVB irradiation (800 or 1200 J/m^2 dose) in the presence of the extracts, was determined using the MTT assay (Fig. 1). At 800 J/m^2, the presence of citrus extract steadily increased cell protection until saturation observed approximately at 50 μg/ml (Fig. 1A). A level of maximum protection of approximately 40% was reached compared with a control irradiated in the absence of the extract. When the UVB dose was increased to 1200 J/m^2, protection gradually increased with extract concentration until reaching a similar level of protection, i.e., 50%, but at higher concentrations (100 μg/ml). Rosemary extract exerted a lower level of protection than citrus extract, especially at high radiation dose (Fig. 1B). Levels of protection of 30% and 13% were observed at 800 and 1200 J/m^2, respectively, at the maximum extract concentration assayed (100 μg/ml).

The dissimilar polyphenolic compositions of citrus and rosemary extracts might provide with complementary protective mechanisms by reaching different cellular targets. To test this hypothesis, a mixture at a 1:1 ratio was evaluated in UV protection assays, as previously described, in a concentration range of 12.5–100 μg/ml (Fig. 1C). The combination showed a higher level of protection at all concentrations compared with individual citrus or rosemary extracts. When cells were exposed to 800 J/m^2, the combined extracts promoted a significant level of protection upon an increase in extract concentration, reaching a protection level of 70% at the highest concentration used. At 1200 J/m^2, the combination was also extremely potent and reached a nearly 60% protection level at 100 μg/ml compared with cells irradiated in the absence of it. When protection levels were compared at 800 J/m^2 (Supplementary Table 1), citrus and rosemary extracts at 50 μg/ml accomplished 40% and 13% protection, respectively (a theoretical additive effect of 53%). However, the combined extract achieved 70% protection at 100 μg/ml (which contained 50 μg/ml of each extract). These results indicate that the combination of citrus and rosemary extracts provided stronger protective effects in the cell model than the sum of the formulation's separate components. An even more striking result was observed when cells were irradiated with a 1200 J/m^2 dose. The combination achieved 60% protection at 100 μg/ml, whereas individual citrus and rosemary extracts at 50 μg/ml accomplished 13% and 5%, respectively. Detailed protection percentages for every concentration and extract are shown in Supplementary Table 1. These results strongly support the hypothesis that both extracts act synergistically in photoprotection since the combination exhibited better protection results than the sum of the effects of the individual components. Synergic effects have been previously described for plant polyphenols in order to explain abnormally enhanced antimicrobial, antioxidant and hypotriglyceridemic activities [21,26].

The rosemary polyphenols rosmarinic acid, carnosic acid and carnosol have been postulated to be effective agents for attenuating cell-damaging effects against UV radiation and also ionizing radiation [10–12,27,28]. Moreover, naringenin, a citrus bioflavonoid, was able to increase the survival of UVB-irradiated keratinocytes [29]. Nevertheless, the current study is the first one to report the synergic effect between these plant compounds. Rosemary and citrus polyphenols show a significant absorption within UVB range, so it seems reasonable to assume that part of their protective effect is based on a direct absorption and scattering of UVB radiation. Nevertheless, since some of these compounds may rapidly reach intracellular targets, we propose that they may be able to scavenge superoxide radical anions ($O_2^{\cdot-}$), hydroxyl radicals ($^{\cdot}OH$) and lipoperoxy radicals (R–OO$^{\cdot}$), which subsequently generate DNA damage and protein oxidation. Due to these preliminary results in a cell model, the combined extract was selected for further assays in which the formulation's capability to attenuate specific harmful effects related to photoaging, such as ROS generation and DNA damage, were analyzed.

3.2. Antioxidant activity and attenuation of ROS generation in UVB-irradiated HaCaT cells by rosemary and citrus combined extract

Intracellular ROS are one of the most damaging effects of both UVB and UVA radiations on skin, with subsequent damage to lipids, proteins and mitochondrial and nuclear DNA. Both citrus bioflavonoids and rosemary compounds have well-documented ROS-scavenging capacity in several models, including human skin cells.

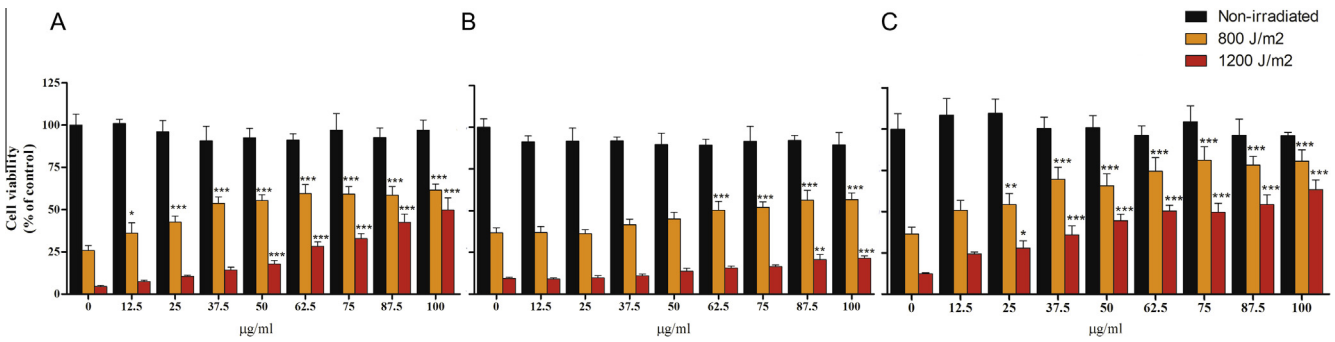

Fig. 1. Survival of human keratinocytes after irradiation with 800 or 1200 J/m^2 UVB in the presence of citrus extract (A), rosemary extract (B) or the combination of both (C). The data are expressed as the mean of 6–8 replicates ± SD. $^{*}(p < 0.05)$, $^{**}(p < 0.01)$ and $^{***}(p < 0.001)$ indicate statistically significant differences compared with an irradiated sample in the absence of the extracts.

Naringenin inhibits apoptosis and pyrimidine dimers upon UVB radiation [29]. Several rosemary compounds have shown a potent radical-scavenging capacity, primarily against lipid peroxidation [16], and the prevention of UV-induced ROS generation and MMP expression by carnosic acid has been reported [30].

To study the potential protective effect of the combination of extracts on the intracellular ROS generation induced by UVB radiation, cells were exposed to UVB at an 800 or 1200 J/m^2 dose in the presence of two different concentrations of the combined extract. ROS generation was then measured with H_2DCFDA, a fluorescent probe that becomes fluorescent when oxidized by free radicals and that is especially sensitive to H_2O_2, ·OH and peroxynitrite at the intracellular level [31]. Fig. 2 shows the ROS generation in HaCaT cells after UVB radiation in the absence and in the presence of various concentrations of the combined extract. As observed, a certain fluorescence signal was observed due to basal metabolism of HaCaT cells in the absence of irradiation and extract supplementation (black bar). When cells were irradiated either with 800 J/m^2 or 1200 J/m^2 in the absence of the extract, fluorescence signal showed a significant increase of 36% and 62%, respectively, compared with basal condition (0%) due to UVB-induced ROS generation ($p < 0.001$). Although UVA is more effective in inducing oxidative damage [4], these results strongly demonstrate that UVB is able to produce ROS species at intracellular level in cell model. In contrast, when cells were irradiated with 800 J/m^2 in the presence the combined extract, fluorescence intensity decreased significantly ($p < 0.001$) as concentration increased compared to irradiated and non-treated cells, i.e., 42% ROS decrease at 75 μg/ml and 50% ROS decrease at 100 μg/ml of the combined extract. The capacity of the combination to inhibit ROS generation was even stronger at 1200 J/m^2 UVB dose, showing 64% ROS decrease at 75 μg/ml and 71% ROS decrease at 100 μg/ml of the combination compared to irradiated and non-treated cells. Besides the evident UVB absorption capacity of the polyphenols present in the combination, these results clearly indicate that an additional mechanism of the combined extract photoprotective properties may be related to its capacity to decrease the generation of intracellular radical species such as H_2O_2, ·OH or peroxynitrite, which have been directly linked to DNA oxidative damage [32].

3.3. Effects of the combined extract on UVB-induced DNA damage

To confirm the hypothesis that inhibition of UV-induced intracellular ROS was concomitant with a certain level of DNA protection, we studied the level of nuclear DNA damage in individual cells exposed to UVB using the comet assay. Fig. 3 shows representative pictures of the effect of combined extract on comet electrophoresis gels for HaCaT cells that were either irradiated at 1200 J/m^2 or non-irradiated and the corresponding gels' quantification. The incubation of human keratinocytes with 100 μg/ml of combined extract did not significantly affect the comet score when cells were not irradiated (Fig. 3C) compared with the control (Fig. 3B), indicating the absence of genotoxicity for the extract. When control cells were irradiated, the comet score increased considerably (Fig. 3D) indicating an increased DNA damage. In contrast, the presence of 100 μg/ml of the combined extract (Fig. 3E) significantly reduced the DNA damage ($p < 0.05$), i.e., by 26% compared with irradiated and untreated cells. Therefore, our results reveal that the combination of rosemary and citrus extracts can decrease the number of UV-induced DNA breaks and hence either attenuates the genotoxic effects of UV radiation or protects the DNA repair machinery.

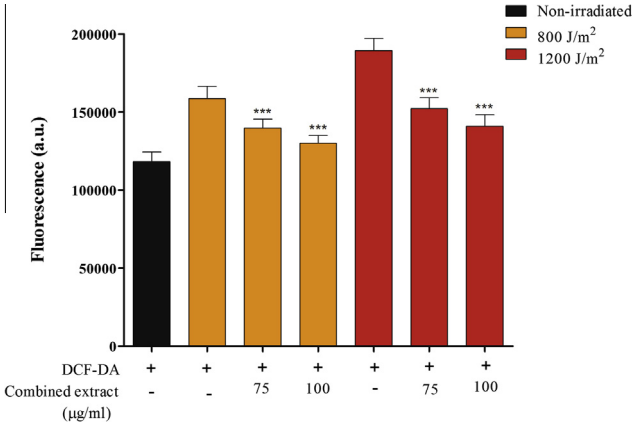

Fig. 2. Measurement of UV-induced ROS generation using H_2DCFDA fluorescent probe. Total fluorescence is expressed as arbitrary units. The data are expressed as the mean ± SD. The black bar indicates the fluorescence signal under basal conditions in the absence of irradiation (0%). ***($p < 0.001$) indicates significant differences compared with irradiated cells at the same UVB dose in the absence of the extract combination.

UVB acts on the epidermal basal cell layer of the skin; is absorbed by chromophores (such as DNA, RNA, proteins and melanin); and has been experimentally demonstrated to cause DNA damage, pyrimidine dimers, 8-OHdG formation, p53 induction, protein oxidation and the generation of ROS [33,34]. Incorrect repair of these lesions leads to mutations, which may cause the development of cancer cells [35]. The excited oxygen electrons of the ROS induced by UVB alter the red/ox state of the cell and also damage mitochondria, inducing apoptosis and tissue injury and contributing to altered cell growth and differentiation or to the development of skin cancer [33,36]. Considering all of the above mentioned *in vitro* evidence, i.e., the cell survival increase and the decrease in intracellular ROS generation and DNA damage by the combined extract, we suggest that the combination of rosemary and citrus extracts may be able to attenuate further major events leading to photoaging and skin cancer.

3.4. The genoprotective effects of combined extract against X-ray-induced ROS and DNA damage

Ionizing radiation generates reactive oxygen and nitrogen species within minutes of exposing cells to radiation, leading to compromised mitochondria viability, MAPK pathways activation and DNA damage [37]. Therefore, we have used X-ray as a model to test whether the combination of the extracts would be able to protect against a massive generation of radical species induced by X-ray exposure through micronuclei (MN) measurement.

Human whole-blood samples were X-ray-irradiated in the presence or absence of combined extract, and the number of lymphocytes bearing MN was determined after cell culture. When whole blood was exposed to 2 Gy in the absence of the combined extract, the number of lymphocytes bearing MN increased considerably compared with non-irradiated cells (Fig. 4). In contrast, combined extract caused a significant reduction of MN/500 CB frequency ($p < 0.001$) when administered either before or after irradiation (Fig. 4) compared with irradiated and non-treated cells. The protection factor exhibited a value of 39% protection when the combined extract was utilized before irradiation and 32% protection when the combined extract was used immediately after irradiation. These results agree with those obtained in the comet assay and corroborate the genoprotective and antimutagenic capacity of rosemary and citrus extracts combination against both UV-induced DNA damage and X-ray-induced ROS/RNS formation and subsequent chromosomal damage. The mechanism of this protection is most likely based on the capacity of the compounds present in the combination to scavenge the massive number of free radicals generated by X-ray, which leads to chromosomal aberrations. It has been postulated that both hydrophilic and lipophilic

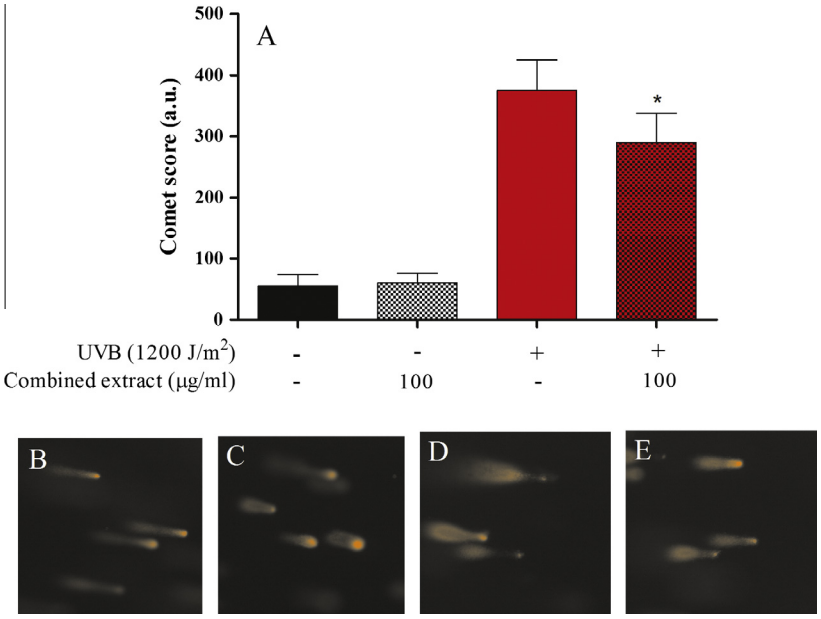

Fig. 3. Rosemary and citrus combination decreases UVB-induced DNA strand break formation in HaCaT cells (A). Keratinocytes were treated with the combination (100 μg/ml) and exposed to UVB (1200 J/m^2). To evaluate DNA damage, 50 cells (nuclei) per slide were analyzed. Total damage was expressed in arbitrary units and determined as described in the materials and methods section. Control consisting of non-irradiated HaCaT cells (B), non-irradiated cells in the presence of the combination (100 μg/ml) (C), irradiated cells at 1200 J/m^2 (D) and irradiated cells in the presence of the combination (100 μg/ml) at 1200 J/m^2 (E). The data are expressed as the mean ± SD. $^{*}(p < 0.05)$ indicates significant differences compared with irradiated cells in the absence of the combination.

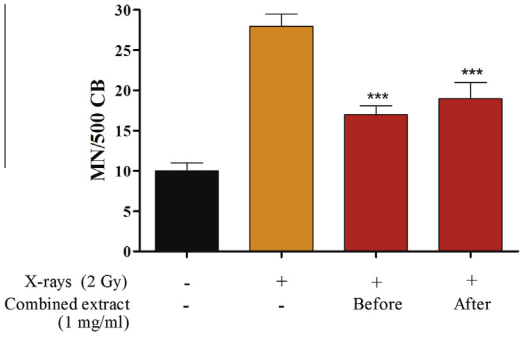

Fig. 4. Influence of rosemary and citrus combination administered before or after X-ray irradiation on the frequency of MN in irradiated and non-irradiated human lymphocytes. The number of MN was expressed in arbitrary units and determined as described in the materials and methods section. The data are expressed as the mean ± SD. $^{***}(p < 0.001)$ indicates significant differences compared with irradiated and non-treated cells.

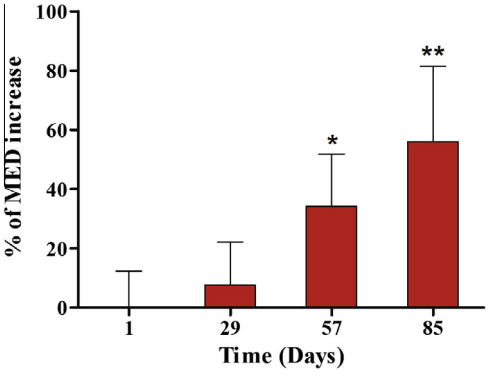

Fig. 5. Evaluation of the average MED of human volunteers measured at days 29, 57 and 85 of receiving the dietary supplement containing the combination compared with the starting value. The data are expressed as the mean ± SD. $^{*}(p < 0.05)$ and $^{**}(p < 0.01)$ indicate significant differences compared with the first day.

antioxidants from rosemary protect human lymphocytes from gamma-radiation when added before irradiation due to their capacity to eliminate the massive generation of superoxide anion and hydroxyl radicals upon irradiation. Interestingly, only oil-soluble antioxidants such as rosemary diterpenes seem to protect after irradiation probably due to their capacity to inhibit lipid peroxidative processes leading to chromosomal oxidative damage [27,28]. Therefore, we propose that the lipophilic antioxidants are the major contributors to DNA protective effect after irradiation, and that both, hydrophilic polyphenols and lipophilic antioxidants, contribute to the increased protection when added before irradiation.

3.5. Human intervention study results

To test the photoprotective efficacy of the combined extract, a human intervention study was performed by the oral administration to volunteers for whom the MED was determined after exposure to UV radiation. Details of the study protocol and MED variation results are described in the materials and Supplementary sections. None of the volunteers discontinued oral administration, so no exclusions were considered. The average results for MED variation during the trial are shown in Fig. 5. No significant differences in the MED value were by day 29th of consumption of combined extract compared with the initial values ($p = 0.429$). In contrast, at the day 57th of treatment, a significant increase in the MED was observed (34%, $p < 0.05$), indicating that after approx. eight weeks of oral ingestion of the combined extract, higher doses of UV radiation were needed to cause an erythematous skin reaction. The MED also increased after 85 days of treatment (56%, $p < 0.01$), indicating that longer oral treatments can improve UV protection effects. Long treatments with the supplement can most likely maintain steady-state systemic concentrations of active metabolites that protect skin cells from the inflammatory reaction appearing after UV radiation.

Several studies have reported the capacity of dietary supplements to prevent UV-induced skin damage in human volunteers. The oral consumption of β-carotene (90 mg/d) for 24 weeks resulted in a modest increase in the MED [38,39], as for high daily doses of α-tocopherol (1000–3000 IU) and ascorbate (2–3 g) [40,41]. An increase in the MED by 15% after the consumption of a combination of lycopene and a probiotic for six weeks has also been reported [42]. A high dose of squalene (13.5–27 g/day) for 90 days showed significant effects on photoprotection after measurements of skin biopsies [43]. In contrast, a combination of carotenoids, vitamins C and E and proanthocyanidins did not show significant differences in the MED compared with a placebo, but a significant decrease in MMP-1 and MMP-9 was observed in the group receiving the supplement [44]. Considering the reported Folin values, daily dose of total polyphenols in the combination used in the present human trial was approximately 100 mg, which supposes a very reasonable and safe antioxidants dose, and much lower compared to most of the abovementioned studies. In most studies on photoprotection based on nutritional ingredients, there is a time frame of approximately 6–10 weeks until protection against erythema becomes significant [8]. This data that reveals relatively long consumption periods is in agreement with our results, which show that the oral ingestion of a combination of rosemary and citrus extracts for eight weeks provides improved protection against UV-induced erythema. The most likely reason for this behavior seems to be that skin biological turnover requires several weeks to incorporate photoprotective nutrients.

In conclusion, although rosemary and citrus extracts have individually demonstrated cellular protective properties against the damaging radical species induced by UVB radiation, the extract combination used in this study showed a synergistic behavior in increasing the survival of human keratinocytes. The combination drastically decreased the generation of UVB-induced intracellular ROS and was also capable of preventing UV-induced DNA damage in the comet assay, which may contribute to decrease the risk for further skin disorders. Combination also showed genoprotective and antimutagenic properties in a model for massive generation of radical species using ionizing radiation. Finally, the photoprotective properties of this combination were corroborated in human volunteers through a 37% increase in the MED ($p < 0.05$) after eight weeks of oral ingestion, and even stronger MED increase after twelve weeks (56%, $p < 0.01$). This study is the first to show that the oral ingestion of a combination of rosemary and citrus extracts can reduce some molecular events related to skin photodamage, such as intracellular ROS generation and DNA damage. The results may contribute to understand how dietary polyphenols may provide a skin protective effect against UV, further than the daily use of topical sunscreens.

4. Disclosure statement

NC works for Monteloeder, SL. JC and OB-G work for Nutrafur, SA.

5. Abbreviations

UV	ultraviolet
ROS	reactive oxygen species
GAE	gallic acid equivalents
dw	dry weight
H_2DCFDA	2′,7′-dichlorofluorescein diacetate
PBS	phosphate-buffered saline
DMSO	dimethyl sulfoxide
MED	minimal erythema dose
MN	micronuclei

Acknowledgements

This work was supported by grants AGL2011-29857-C03-03 (Spanish Ministry of Science and Innovation), PROMETEO/2012/007 and ACOMP/2013/093 from Generalitat Valenciana (GV) and CIBER (CB12/03/30038, Fisiopatología de la Obesidad y la Nutrición, CIBERobn, Instituto de Salud Carlos III). The authors are grateful to GV for VALi+D fellowship ACIF/2013/064. We also thank Monteloeder and Nutrafur for their financial support of the human trial.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jphotobiol.2014.04.007.

References

[1] A. Svobodova, J. Psotova, D. Walterova, Natural phenolics in the prevention of uv-induced skin damage. A review, Biomed. Pap. Med. Fac. Univ. Palacky. Olomouc. Czech Repub. 147 (2003) 137–145.
[2] R.M. Lavker, G.F. Gerberick, D. Veres, C.J. Irwin, K.H. Kaidbey, Cumulative effects from repeated exposures to suberythemal doses of uvb and uva in human skin, J. Am. Acad. Dermatol. 32 (1995) 53–62.
[3] M. Wlaschek, G. Heinen, A. Poswig, A. Schwarz, T. Krieg, K. Scharffetter-Kochanek, Uva-induced autocrine stimulation of fibroblast-derived collagenase/mmp-1 by interrelated loops of interleukin-1 and interleukin-6, Photochem. Photobiol. 59 (1994) 550–556.
[4] M. Yaar, B.A. Gilchrest, Photoageing: mechanism, prevention and therapy, Br J. Dermatol. 157 (2007) 874–887.
[5] Y. Xu, Y. Shao, J.J. Voorhees, G.J. Fisher, Oxidative inhibition of receptor-type protein-tyrosine phosphatase kappa by ultraviolet irradiation activates epidermal growth factor receptor in human keratinocytes, J. Biol. Chem. 281 (2006) 27389–27397.
[6] G.J. Fisher, S.C. Datta, H.S. Talwar, Z.Q. Wang, J. Varani, S. Kang, J.J. Voorhees, Molecular basis of sun-induced premature skin ageing and retinoid antagonism, Nature 379 (1996) 335–339.
[7] D. Decraene, The Uvb-Response in Human Skin and its Modulation by the Proto-Oncogenic Akt Signaling Pathway, Leuven University Press, 2004.
[8] H. Sies, W. Stahl, Nutritional protection against skin damage from sunlight, Ann. Rev. Nutrit. 24 (2004) 173–200.
[9] M.S. Latha, J. Martis, V. Shobha, R. Sham Shinde, S. Bangera, B. Krishnankutty, S. Bellary, S. Varughese, P. Rao, B.R. Naveen Kumar, Sunscreening agents: a review, J. Clin. Aesthet. Dermatol. 6 (2013) 16–26.
[10] J. Vostálová, A. Zdarilová, A. Svobodová, Prunella vulgaris extract and rosmarinic acid prevent uvb-induced DNA damage and oxidative stress in hacat keratinocytes, Arch. Dermatol. Res. 302 (2010) 171–181.
[11] J. Psotova, A. Svobodova, H. Kolarova, D. Walterova, Photoprotective properties of *prunella vulgaris* and rosmarinic acid on human keratinocytes, J. Photochem. Photobiol. B 84 (2006) 167–174.
[12] M. Sanchez-Campillo, J.A. Gabaldon, J. Castillo, O. Benavente-Garcia, M.J. Del Bano, M. Alcaraz, V. Vicente, N. Alvarez, J.A. Lozano, Rosmarinic acid, a photoprotective agent against uv and other ionizing radiations, Food Chem. Toxicol. 47 (2009) 386–392.
[13] H. Wei, X. Zhang, Y. Wang, M. Lebwohl, Inhibition of ultraviolet light-induced oxidative events in the skin and internal organs of hairless mice by isoflavone genistein, Cancer Lett. 185 (2002) 21–29.
[14] R.P. Singh, R. Agarwal, Mechanisms and preclinical efficacy of silibinin in preventing skin cancer, Eur. J. Cancer 41 (2005) 1969–1979.
[15] N.L. Wu, J.Y. Fang, M. Chen, C.J. Wu, C.C. Huang, C.F. Hung, Chrysin protects epidermal keratinocytes from uva- and uvb-induced damage, J. Agric. Food Chem. 59 (2011) 8391–8400.
[16] P. Sapra, E.H. Moase, J. Ma, T.M. Allen, Improved therapeutic responses in a xenograft model of human b lymphoma (namalwa) for liposomal vincristine versus liposomal doxorubicin targeted via anti-cd19 igg2a or fab′ fragments, Clin. Cancer Res. 10 (2004) 1100–1111.
[17] E.A. Offord, J.C. Gautier, O. Avanti, C. Scaletta, F. Runge, K. Kramer, L.A. Applegate, Photoprotective potential of lycopene, beta-carotene, vitamin e, vitamin c and carnosic acid in uva-irradiated human skin fibroblasts, Free Radic. Biol. Med. 32 (2002) 1293–1303.
[18] S. Reuter, S. Eifes, M. Dicato, B.B. Aggarwal, M. Diederich, Modulation of anti-apoptotic and survival pathways by curcumin as a strategy to induce apoptosis in cancer cells, Biochem. Pharmacol. 76 (2008) 1340–1351.
[19] T. Passeron, T. Namiki, H.J. Passeron, E. Le Pape, V.J. Hearing, Forskolin protects keratinocytes from uvb-induced apoptosis and increases DNA repair independent of its effects on melanogenesis, J. Invest. Dermatol. 129 (2009) 162–166.

[20] A.O. Abu-Yousif, K.A. Smith, S. Getsios, K.J. Green, R.T. Van Dross, J.C. Pelling, Enhancement of uvb-induced apoptosis by apigenin in human keratinocytes and organotypic keratinocyte cultures, Cancer Res. 68 (2008) 3057–3065.
[21] M. Herranz-Lopez, S. Fernandez-Arroyo, A. Perez-Sanchez, E. Barrajon-Catalan, R. Beltran-Debon, J.A. Menendez, C. Alonso-Villaverde, A. Segura-Carretero, J. Joven, V. Micol, Synergism of plant-derived polyphenols in adipogenesis: Perspectives and implications, Phytomedicine 19 (2012) 253–261.
[22] D. Huang, O.U. Boxin, R.L. Prior, The chemistry behind antioxidant capacity assays, J. Agric. Food Chem. 53 (2005) 1841–1856.
[23] E. Barrajon-Catalan, S. Fernandez-Arroyo, D. Saura, E. Guillen, A. Fernandez-Gutierrez, A. Segura-Carretero, V. Micol, Cistaceae aqueous extracts containing ellagitannins show antioxidant and antimicrobial capacity, and cytotoxic activity against human cancer cells, Food Chem. Toxicol. 48 (2010) 2273–2282.
[24] M. Fenech, The cytokinesis-block micronucleus technique: a detailed description of the method and its application to genotoxicity studies in human populations, Mutat. Res. 285 (1993) 35–44.
[25] M. Fenech, A.A. Morley, Measurement of micronuclei in lymphocytes, Mutat. Res. 147 (1985) 29–36.
[26] L. Tomás-Menor, A. Morales-Soto, E. Barrajón-Catalán, C. Roldán-Segura, A. Segura-Carretero, V. Micol, Correlation between the antibacterial activity and the composition of extracts derived from various spanish cistus species, Food Chem. Toxicol. 55 (2013) 313–322.
[27] M.J. Del Bano, J. Castillo, O. Benavente-Garcia, J. Lorente, R. Martin-Gil, C. Acevedo, M. Alcaraz, Radioprotective-antimutagenic effects of rosemary phenolics against chromosomal damage induced in human lymphocytes by gamma-rays, J. Agric. Food Chem. 54 (2006) 2064–2068.
[28] M. Alcaraz, C. Acevedo, J. Castillo, O. Benavente-Garcia, D. Armero, V. Vicente, M. Canteras, Liposoluble antioxidants provide an effective radioprotective barrier, Br J. Radiol. 82 (2009) 605–609.
[29] M.A. El-Mahdy, Q. Zhu, Q.E. Wang, G. Wani, S. Patnaik, Q. Zhao, S. Arafa el, B. Barakat, S.N. Mir, A.A. Wani, Naringenin protects hacat human keratinocytes against uvb-induced apoptosis and enhances the removal of cyclobutane pyrimidine dimers from the genome, Photochem. Photobiol. 84 (2008) 307–316.
[30] M. Park, J. Han, C.S. Lee, B.H. Soo, K.M. Lim, H. Ha, Carnosic acid, a phenolic diterpene from rosemary, prevents uv-induced expression of matrix metalloproteinases in human skin fibroblasts and keratinocytes, Exp. Dermatol. 22 (2013) 336–341.
[31] O. Myhre, J.M. Andersen, H. Aarnes, F. Fonnum, Evaluation of the probes 2′,7′-dichlorofluorescin diacetate, luminol, and lucigenin as indicators of reactive species formation, Biochem. Pharmacol. 65 (2003) 1575–1582.
[32] M. Ichihashi, M. Ueda, A. Budiyanto, T. Bito, M. Oka, M. Fukunaga, K. Tsuru, T. Horikawa, Uv-induced skin damage, Toxicology 189 (2003) 21–39.
[33] J. Krutmann, P. Humbert, Nutrition for Healthy Skin: Strategies for Clinical and Cosmetic Practice, Springer, 2011.
[34] S. Liardet, C. Scaletta, R. Panizzon, P. Hohlfeld, L. Laurent-Applegate, Protection against pyrimidine dimers, p53, and 8-hydroxy-2′-deoxyguanosine expression in ultraviolet-irradiated human skin by sunscreens: Difference between uvb + uva and uvb alone sunscreens, J. Invest. Dermatol. 117 (2001) 1437–1441.
[35] L. Marrot, J.R. Meunier, Skin DNA photodamage and its biological consequences, J. Am. Acad. Dermatol. 58 (2008) S139–S148.
[36] D.E. Heck, A.M. Vetrano, T.M. Mariano, J.D. Laskin, Uvb light stimulates production of reactive oxygen species: unexpected role for catalase, J. Biol. Chem. 278 (2003) 22432–22436.
[37] J.K. Leach, G. Van Tuyle, P.S. Lin, R. Schmidt-Ullrich, R.B. Mikkelsen, Ionizing radiation-induced, mitochondria-dependent generation of reactive oxygen/nitrogen, Cancer Res. 61 (2001) 3894–3901.
[38] M.M. Mathews-Roth, M.A. Pathak, J. Parrish, T.B. Fitzpatrick, E.H. Kass, K. Toda, W. Clemens, A clinical trial of the effects of oral beta-carotene on the responses of human skin to solar radiation, J. Invest. Dermatol. 59 (1972) 349–353.
[39] J. Lee, S. Jiang, N. Levine, R.R. Watson, Carotenoid supplementation reduces erythema in human skin after simulated solar radiation exposure, Proc. Soc. Exp. Biol. Med. 223 (2000) 170–174.
[40] B. Eberlein-Konig, M. Placzek, B. Przybilla, Protective effect against sunburn of combined systemic ascorbic acid (vitamin c) and d-alpha-tocopherol (vitamin e), J. Am. Acad. Dermatol. 38 (1998) 45–48.
[41] J. Fuchs, H. Kern, Modulation of uv-light-induced skin inflammation by d-alpha-tocopherol and l-ascorbic acid: a clinical study using solar simulated radiation, Free Radic. Biol. Med. 25 (1998) 1006–1012.
[42] D. Bouilly-Gauthier, C. Jeannes, Y. Maubert, L. Duteil, C. Queille-Roussel, N. Piccardi, C. Montastier, P. Manissier, G. Pierard, J.P. Ortonne, Clinical evidence of benefits of a dietary supplement containing probiotic and carotenoids on ultraviolet-induced skin damage, Br J. Dermatol. 163 (2010) 536–543.
[43] S. Cho, C.W. Choi, D.H. Lee, C.H. Won, S.M. Kim, S. Lee, M.J. Lee, J.H. Chung, High-dose squalene ingestion increases type i procollagen and decreases ultraviolet-induced DNA damage in human skin in vivo but is associated with transient adverse effects, Clin. Exp. Dermatol. 34 (2009) 500–508.
[44] A.K. Greul, J.U. Grundmann, F. Heinrich, I. Pfitzner, J. Bernhardt, A. Ambach, H.K. Biesalski, H. Gollnick, Photoprotection of uv-irradiated human skin: an antioxidative combination of vitamins e and c, carotenoids, selenium and proanthocyanidins, Skin. Pharmacol. Appl. Skin Phisiol. 15 (2002) 307–315.