Journal of Photochemistry and Photobiology B: Biology 132 (2014) 27–35

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

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

Protective effect of *Vaccinium myrtillus* extract against UVA- and UVB-induced damage in a human keratinocyte cell line (HaCaT cells)

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ARTICLE INFO

Article history:
Received 16 September 2013
Received in revised form 8 January 2014
Accepted 8 January 2014
Available online 8 February 2014

Keywords:
Vaccinium myrtillus
Genotoxicity
Oxidative stress
Apoptosis
Ultraviolet rays
Keratinocytes

ABSTRACT

Recently, the field of skin protection have shown a considerable interest in the use of botanicals. *Vaccinium myrtillus* contains several polyphenols and anthocyanins with multiple pharmacological properties. The purpose of our study was to examine whether a water-soluble *V. myrtillus* extract (dry matter 12.4%; total polyphenols 339.3 mg/100 g fw; total anthocyanins 297.4 mg/100 g fw) was able to reduce UVA- and UVB-induced damage using a human keratinocyte cell line (HaCaT). HaCaT cells were pretreated for 1 h with extract in a serum-free medium and then irradiated with UVA (8–40 J/cm^2) and UVB (0.008–0.72 J/cm^2) rays. All experiments were performed 24 h after the end of irradiation, except for oxidative stress tests. The extract was able to reduce the UVB-induced cytotoxicity and genotoxicity (studied by comet and micronucleous assays) at lower doses. *V. myrtillus* extract reduced lipid peroxidation UVB-induced, but had no effect against the ROS UVB-produced. With UVA-induced damage *V. myrtillus* reduced genotoxicity as well as the unbalance of redox intracellular status. Moreover our extract reduced the UVA-induced apoptosis, but had no effect against the UVB one. *V. myrtillus* extract showed its free radical scavenging properties reducing oxidative stress and apoptotic markers, especially in UVA-irradiated cells.

1. Introduction

Clinical and epidemiological studies suggest that over-exposure of human skin to environmental factors such as ultraviolet radiation (UV) has harmful effects and leads to different skin disorders like sunburn, erythema, skin aging, inflammation, oxidative damage, and immunosuppression, sometimes resulting in skin cancer [1–3].

The main source of UV exposure is sunlight, which is classified as UVA (400–315 nm), UVB (315–280 nm) and UVC (280–100 nm), depending on the wavelength. The UV rays that reach human skin are mainly UVA and UVB. UVB constitutes only 5% of total UV radiation but is considered the most damaging and genotoxic.

UVB-induced skin damage may be direct, due to UVB absorption by target molecules (lipid membrane, proteins and DNA), generating several types of pro-mutagenic lesions like cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4) pyrimidinone photoproducts ((6–4)PPs) [1–5], or indirect, in response to over-generation of oxygen reactive species (ROS) [6–9].

ROS are involved in some UVB-induced patho-physiological processes by activating mitogen-activated protein kinase (MAPK) and nuclear factor-kB (NF-kB) [6]. They induce apoptosis in human keratinocytes to protect against irreversible DNA damage, preventing the accumulation of abnormal cells, which can lead to skin malignancies [9–12].

UVB is also implicated in skin inflammatory processes that include infiltration of inflammatory blood leucocytes, increased production of prostaglandins and release of tumor necrosis factor-alpha, tumor necrosis factor-kappaB and inflammatory cytokines [13,14].

Much of the skin damage induced by UVA irradiation is associated with increased ROS levels, which may be linked to apoptosis and damage to cellular protein, lipids and polysaccharides. The most important biological effects caused by UVA are indirect DNA lesions which induce several oxidized bases, over-production of ROS [15] and LPO [3,16].

In view of the range of side effects, reducing the amount of UV penetrating the skin and reinforcing the skin's own protective mechanisms is an interesting strategy for the prevention of skin diseases, including cancer. Several herbs or herbal preparations have been proposed and investigated for this purpose, on account of their high antioxidant potential.

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http://dx.doi.org/10.1016/j.jphotobiol.2014.01.013
1011-1344/

The aim of this study was to investigate whether a water soluble extract of bilberry (*Vaccinium myrtillus*, dry matter 12.4%, total polyphenols 339.3 mg/100 g fresh weight (fw), total anthocyanins 297.4 mg/100 g (fw) [17] prevented UVA and UVB-induced damage to human keratinocytes (the HaCaT cell line). Several constituents from *V. myrtillus* berries including anthocyanoside flavonoids (anthocyanins), vitamins, sugar and pectins were isolated [18]. The anthocyanosides' antioxidant properties are well known. *In vitro* studies have also documented their anti-apoptotic, anti-inflammatory, and antibacterial effects. They can stabilize collagen fibers, promote collagen biosynthesis, reduce capillary fragility and inhibit platelet aggregation [19]. Moreover, Svobodova et al. observed the protective effect of the phenolic fraction of *V. myrtillus* against UVA and UVB damage [20,21]. *V. myrtillus* has also been reported to have pharmacological action against ophthalmologic disorders. It improves oxygen and blood delivery to the eye and scavenges free radicals which contribute to cataract and macular degeneration; it improves night-time visual acuity in high-fat diet mice and in individuals with retinitis pigmentosa and hemeralopia [22–25] protects against the development of glaucoma [26] and retards or blocks the development of cataract in mice and humans. *V. myrtillus* inhibited the formation of neovascular tufts during oxygen-induced retinopathy in mice [27] and alleviated pruritus in a mouse model of chronic allergic contact dermatitis [28].

Another important feature is its ability to stabilize DNA. DNA damage can increase the probability of mutations, and any bioactive food or component that protects against damage induced by different agents, reducing baseline DNA damage or increasing DNA repair, is a potential cancer-preventing agent [29]. Damage to DNA can result from many different processes and agents, but oxidation is thought to be a key culprit. Diet is therefore an important source of risk-limiting components. Some epidemiological studies have focused on the anticancer potential of mixed fruits and vegetables, others on the protection provided by bilberry either alone or in combination with other berries. *In vitro* studies have demonstrated the cancer-preventive or -suppressive activity through antioxidant activity, antiproliferative, apoptotic, antiangiogenic and anti-inflammatory action, inducing the antioxidant response element (ARE) with consequent phase II enzyme induction and other cytoprotective effects [19,27,30]. There are also some direct geno-protective mechanisms: anthocyanins intercalate with DNA, forming a DNA co-pigmentation complex that regulates gene expression while also protecting DNA against oxidative damage [19,31,32].

Many studies have confirmed the clinical efficacy of *V. myrtillus*. A diet with high doses of bilberry extract can reduce intestinal adenoma by 15–30% [33]. An extract of bilberry dose-dependently inhibited cell growth and promoted the induction of apoptosis in cultured breast cells (MCF-7), arrested cell cycling to G(2)/M phase and inhibited microtubule polymerization [19,34].

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM), penicillin–streptomycin solution, non-animal L-glutamine, MEM vitamin solution 100×, non-essential amino acid solution 100×, 3-(4-5-dimethyl thiazol-2-11)-2,5-diphenyl tetrazolium bromide (MTT), tert-butyl hydroperoxide solution (TBHP), dimethyl sulfoxide (DMSO), 2′,7′-dichlorofluorescin diacetate (DCFDA), trypsin-EDTA solution 1× (trypsin), low-melting-point agarose (LMA), agarose for routine use, propidium iodide (PI), sodium chloride (NaCl), tris(hydroxymethyl)aminomethane, sodium hydroxide (NaOH), potassium chloride (KCl), Hepes, Triton ×-100, trichloroacetic acid (TCA), thiobarbituric acid (TBA), hydrochloric acid (HCl), sodium-citrate, ethidium bromide, citric acid and sucrose were obtained from Sigma Aldrich, Milan, Italy. Fetal bovine serum (FBS) was purchased from Biochrom, Italy.

2.2. V. myrtillus (bilberry) extract

Freeze-dried bilberry extract was kindly donated by Dr. R. Lo Scalzo, Agricultural Research Council (CRA-IAA), Food Technology Research Unit, Milan. The bilberry extract (dry matter 12.4%, total polyphenols 339.3 mg/100 g fw; total anthocyanins 297.4 mg/100 g fw) was prepared using the protocol described by [17].

2.3. Cell culture

HaCaT keratinocytes, a spontaneously transformed human epithelial cell line, were purchased from Istituto Zooprofilattico di Brescia (Brescia, Italy). Cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 4 mM non-animal L-glutamine, 1% penicillin–streptomycin solution, 1% MEM vitamin solution 100× and 1% non-essential amino acid solution 100×, in a humidified incubator aerated with 5% CO_2, at 37 °C. Culture medium was changed twice a week.

2.4. Keratinocyte treatment with extract

HaCaT cells were exposed for 1 h to *V. myrtillus* extract (40–640 μg/mL, 37 °C) in serum-free medium. Cytotoxicity was assessed immediately after the end of exposure and 24 h after.

Then, to assess the protective effect of the extract, HaCaT cells were pretreated (1 h, 37 °C) with only one concentration of *V. myrtillus* (320 μg/mL) in serum-free medium, irradiated, then incubated for another 24 h in culture medium before genotoxic analysis or immediately collected to examine oxidative stress.

2.5. UV irradiation system

The illuminator system consisted of four UVA lamps (peak 365 nm) closed two-by-two, and two closed UVB lamps (peak 312 nm) (TRIWOOD 31/36, Helios Italquartz, Milan, Italy). The irradiances of the UVA and UVB lamps were respectively 2.25 mW/cm^2 and 0.8 mW/cm^2. The emitted intensity was measured using the HD 2302.0 radiometer (Delta OHM, Italy). Before irradiation, culture medium was removed, cells were rinsed once with phosphate-buffered saline (PBS 1×), covered with a thin PBS layer, and irradiated. To prevent the PBS overheating during irradiation, plates were kept on ice. Control cells were treated the same way as the experimental ones but were not exposed to UV rays.

2.6. Cell viability

To assess the effect of *V. myrtillus* extract on cell viability and its protection against UVA- and UVB-induced cytotoxicity we used the MTT test, according to [7] with some modifications. This colorimetric test measures the ability of mitochondrial dehydrogenases of viable cells to cleave the tetrazolium ring and yield purple formazan crystals which are insoluble in aqueous solutions. An increase or decrease in viable cells results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by UV rays.

HaCaT cells were seeded in 96-well plates and at 80% confluence were exposed to the extract (40–640 μg/mL) for 1 h, 37 °C. Its effect on cell viability was studied immediately and 24 h after the end of treatment.

In order to evaluate *V. myrtillus* protection against UVA- and UVB-induced cytotoxicity we pretreated cells with extract

(320 μg/mL) for 1 h at 37 °C and then irradiated them with different doses of UVA (8–24 J/cm^2) or UVB (0.008–0.24 J/cm^2). Cell viability was assessed immediately and 24 h after irradiation. To evaluate cytotoxicity we exposed the cells to the MTT solution (final concentration 0.5 mg/mL) and incubated them for 4 h at 37 °C. The formazan crystals were then dissolved with DMSO and measured spectrophotometrically on a microplate reader at 550 nm (Multilabel counter Victor Wallac 1420, Perkin–Elmer, Monza, Italy). Viable cells were calculated as a percentage of the negative control cells setted at 100%.

2.7. Oxidative stress

Overproduction of ROS or reduction in the ability of endogenous antioxidants to neutralize them results in oxidative stress which in turn can lead to damage to lipids, proteins and DNA. This situation is traceable by measuring ROS, LPO, protein oxidation and DNA fragmentation or cell death.

2.7.1. ROS generation

ROS formation was quantified according to [35], with some modifications, using the cell-permeable, non-fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFDA) which is de-esterified intracellularly and turns to highly fluorescent 2′,7′-dichlorofluorescein upon oxidation. Cells were seeded in black 96-well plates, pretreated with bilberry extract for 1 h, then exposed to UVA (8–40 J/cm^2) or UVB (0.064–0.72 J/cm^2). The cells were then incubated with 25 μM DCFDA at 37 °C for 30 min. Fluorescence was measured using a microplate reader (Multilabel counter Victor Wallac 1420, Perkin–Elmer, Monza, Italy) at 485 nm$_{ex}$/530 nm$_{em}$. The results were calculated as fluorescence units (FU)/mg of cell protein, according to [36].

2.7.2. Lipid peroxidation (MDA formation)

Lipid peroxidation is a well-established mechanism of cellular injury, an autocatalytic and a free radical chain reaction triggered by hydroxyl radical. It can lead to oxidative destruction of cell membranes and can cause cell death and the production of toxic and reactive aldehyde metabolites, MDA being the final, and most important, product of polyunsaturated fatty acid peroxidation. An increase in free radicals causes overproduction of MDA. We fluorimetrically quantified MDA levels (515$_{exc}$/553$_{em}$ nm), as a marker of oxidative stress, according to [3], with some modifications. HaCaT cells were plated in 100-mm culture dishes (Corning) and at 80% confluence were pretreated with bilberry extract. Cells were then irradiated with UVA (8–40 J/cm^2) or UVB (0.024–0.72 J/cm^2) and immediately scraped off to measure MDA. We added 100 μL TCA 50% (final concentration 0.5%) to the homogenate and centrifuged the samples for 20 min at 1500 g. Then 1.1 mL TBA 0.6% was added to 1 mL of supernatant and they were incubated in a water-bath at 100 °C for 1 h. After this, the samples were cooled on ice and 3 mL of isobutanol (Sigma) were added to each tube. Samples were vortexed for 2 min and centrifuged again (5 min, 5000 rpm) to extract the pre-formed MDA completely. MDA levels were quantified fluorimetrically and calculated as MDA/mg of cell proteins according to [36], against the predetermined MDA standard curve.

2.8. Genotoxicity

2.8.1. Alkaline single-cell gel electrophoresis (SCGE, comet assay, pH > 13)

DNA alkali-labile sites, single- and double-strand breaks were monitored using the alkaline form of the comet assay. This detects DNA lesions in individual cells. It was carried out according to [37–39], with minor modifications. Cells were plated in 28-cm^2 culture dishes (Corning) and after pretreatment were exposed to UVA (16–32 J/cm^2) or UVB (0.016–0.032 J/cm^2). After 24 h in culture, cells were collected with trypsin. Trypsinization was stopped with 1 mL of culture medium and the cell suspension was centrifuged for 5 min at 2000 rpm. Supernatant was removed and the pellet resuspended in 1 mL of culture medium and kept on ice. A total of 2×10^4 cells/mL (counted by the trypan blue method) were resuspended in 200 μL of 0.5% low-melting-point agarose (LMA) in PBS then transferred onto pre-coated microscope slides with 1% agarose for routine use in PBS, and covered with a coverglass. Slides were prepared with a first layer of cell suspension in 0.5% LMA and a second layer only of 0.5% LMA, then stored at 4 °C for 10 min to allow solidification. The coverglasses were gently removed and slides were immersed in lysis buffer (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 250 mM NaOH, pH 10) at 4 °C for 1 h. Slides were then rinsed with neutralization solution (0.4 M Tris, pH 7.5) and placed in a horizontal gel electrophoresis tank (PBI) filled with ice-cold electrophoresis buffer (0.3 M NaOH, 1 mM Na-EDTA, pH 13) for 20 min, on ice in the dark, to allow DNA to unwind. Electrophoresis was done at 25 V and 300 mA for 30 min, followed by 5 min neutralization with 2 mL of neutralization solution, then fixation with ethanol (Fluka) at −20 °C for 5 min. Once the slides were dry, they were stained with 500 μL PI (20 μg/mL) and analyzed using a fluorescence microscope (Axioplan 2, Zeiss, Milan, Italy) at 25-fold magnification.

For each sample, 50–60 randomly selected nucleoids were acquired. Images of the fluorescently stained cell nuclei were analyzed using TriTek Comet Score Imaging Software 1.5. Nucleoids were first classified in five categories on the basis of the area (data not shown) and intensity of the tail staining, then DNA damage was quantified as the percentage DNA in the tail.

2.8.2. Micronucleus formation

The protective effects of *V. myrtillus* were also tested against UVA- and UVB-induced micronucleus (MN) formation. A micronucleus is formed during the metaphase/anaphase transition of mitosis. It may arise from a whole lagging chromosome (aneugenic event leading to chromosome loss) or an accentric chromosome fragment detaching from a chromosome after breakage (clastogenic event) which does not integrate in the daughter nuclei. Experiments were done according to [40], with some modifications.

Cells were seeded in 28-cm^2 culture dishes (Corning), and pretreated with bilberry extract; 24 h after the UVA (8 J/cm^2) and UVB irradiation (0.008 J/cm^2) they were collected with trypsin to assess MN formation. Cells were centrifuged for 5 min at 1000 rpm, and the supernatant was removed; cells were then resuspended in 1 mL of PBS + 5% FBS, counted by the trypan blue method (1×10^6 cells/mL) and centrifuged again. Supernatant was aspirated, leaving approximately 50 μL per sample. Cells were gently resuspended by tapping them, and maintained at room temperature for 1 h. 500 μL of Solution I (584 mg/L NaCl, 1000 mg/L Na-citrate, 25 mg/L ethidium bromide, 10 mg/L RNAse, 0.3 mL Nonidet P-40) were added for each sample. After 1 h, 500 μL of Solution II (1.5% citric acid, 0.25 M sucrose, 40 mg/L ethidium bromide) were added for 30 min. Samples were then stored at 4 °C until flow cytometry analysis, measuring the fluorescence at 530$_{exc}$/575$_{em}$ nm (FACScalibur Becton Dickinson).

2.9. Mitochondrial membrane potential (JC-1)

The loss of mitochondrial potential ($\Delta\Psi$) is a hallmark of apoptosis. It is an early event preceding phosphatidylserine externalization and coinciding with caspase activation. MitoProbe™ JC-1 Assay Kit for Flow CytometryJC-1 (Molecular Probes) was used to assessed protection of Bilberry pretreatment against depolarization of mitochondrial membrane potential (MMP) UV-induced.

JC-1 (5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) is a dye cationic dye, that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~529 nm) to red (~590 nm). Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. The potential-sensitive color shift is due to concentration-dependent formation of red fluorescent J-aggregates. Experiments were conducted according to the manufacturer's instructions, with some modifications.

Cells were seeded in 28-cm^2 culture dishes (Corning), and pretreated with bilberry extract; 24 h after the end of UVA (8–24 J/cm^2) and UVB irradiation (0.008–0.032 J/cm^2) they were collected with trypsin to evaluate $\Delta\Psi$ disruption. Cells were centrifuged for 5 min at 2000 rpm, and resuspended in 1 mL of PBS + 5% FBS, counted by the trypan blue method (1×10^6 cells/mL) and centrifuged again. Supernatant was discarded and 500 μL of staining solution (1 μM JC-1 in 0.9% NaCl solution) were added to each sample for 30 min at 37 °C. After, cells were centrifuged 5 min at 2000 rpm, pellets resuspended in 1 mL of PBS + 5% FBS and centrifuged again. Supernatants were discarded and 1 mL of 0.9% NaCl solution was added to each sample for flow cytometry analysis (FACScalibur Becton Dickinson).

2.10. Annexin V-FITC detection of apoptosis

In apoptotic cells, phosphatidyl serine (PS) is translocated from the inner to the outer leaflet of the plasma membrane. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for PS. We studied the protective effect of *V. myrtillus* extract against UVA- and UVB-induced apoptosis using the Alexa Fluor 488 annexin V/Dead cell apoptosis kit (Molecular Probes), according to the manufacturer's instructions, with some modifications. The kit contains Annexin V conjugated with the bright fluorophore Alexa Fluor 488 and the red-fluorescent PI nucleic acid binding dye which is impermeant to live and apoptotic cells so it only stains dead cells.

Briefly, cells were seeded in 28-cm^2 culture dishes (Corning), exposed to bilberry extract and 24 h after UVA- (8–24 J/cm^2) or UVB irradiation (0.008–0.032 J/cm^2) washed with PBS and collected with trypsin to assess apoptosis. Cells were centrifuged for 5 min at 1000 rpm and resuspended in 1 mL of PBS + 5% FBS, counted by the trypan blue method (1×10^6 cells/mL) and centrifuged again. Supernatant was discarded and 100 μL of Annexin Binding Buffer (1:10 in sodium citrate 0.1%) were added to each sample (except for control cells and only PI stained cells). HaCaT were double stained with 5 μL of Alexa Fluor 488 Annexin V and 1 μL of PI (100 μg/mL) and kept for 15 min at room temperature in the dark. After the incubation, 400 μL of Annexin Binding Buffer 5× (1:10 in sodium citrate 0.1%) were added to each sample and the samples were analyzed by flow cytometry, measuring the fluorescence emission at 530 nm and 575 nm with 488 nm excitation (FACScalibur Becton Dickinson).

3. Results

3.1. Cell viability

V. myrtillus cytotoxicity was quantified by MTT test immediately after the end of treatment and 24 h after. Results showed that our extract (40–640 μg/mL) had a low cytotoxicity both immediately after the end of exposure and 24 h after the end of it (Fig. 1a and b).

UVA and UVB cytotoxic effects were evaluated by the MTT test, immediately after irradiation or 24 h later (Fig. 2a and b). Immediately after UV exposure, UVA (8–24 J/cm^2) and UVB (0.008–0.24 J/cm^2) did not cause any significant decrease in cell viability. However, 24 h after the irradiation the situation was different. UVA significantly reduced HaCaT viability by 40% starting from 16 J/cm^2 ($p < 0.05$). UVB also significantly lowered cell viability in a dose-dependent manner, starting from 0.032 J/cm^2 ($p < 0.01$). Pretreatment with *V. myrtillus* slightly increased UVA-induced cytotoxicity at 24 J/cm^2 and significantly reduced it only in UVB-irradiated cells (0.032–0.064 J/cm^2, $p < 0.01$, $p < 0.001$, respectively).

3.2. Oxidative stress

To investigate the protective effects of *V. myrtillus* against oxidative stress in UVA- and UVB-irradiated cells, we recorded intracellular ROS production and peroxidation of cellular lipids immediately after irradiation.

3.2.1. ROS generation

In HaCaT exposed to UVA (8–40 J/cm^2) and UVB (0.064–0.72 J/cm^2) there was a significant, dose-related increase in ROS generation (Fig. 3a and b). *V. myrtillus* pretreatment significantly reduced the increase after UVA 24–40 J/cm^2 ($p < 0.001$, $p < 0.01$, respectively) so there was less protective effect at higher UVA doses. Pretreatment did not affect UVB-induced ROS.

3.2.2. Lipid peroxidation (MDA formation)

Intracellular levels of MDA rose significantly in response to UVA irradiation (8–40 J/cm^2, $p < 0.001$) (Fig. 4a). The UVA-induced LPO was reduced by *V. myrtillus*, from the lower to the higher UVA doses, but it had less protective effect at the higher doses (40 J/cm^2).

UVB also raised MDA levels (Fig. 4b), but the increase was significant only at 0.72 J/cm^2 ($p < 0.01$). *V. myrtillus* lowered

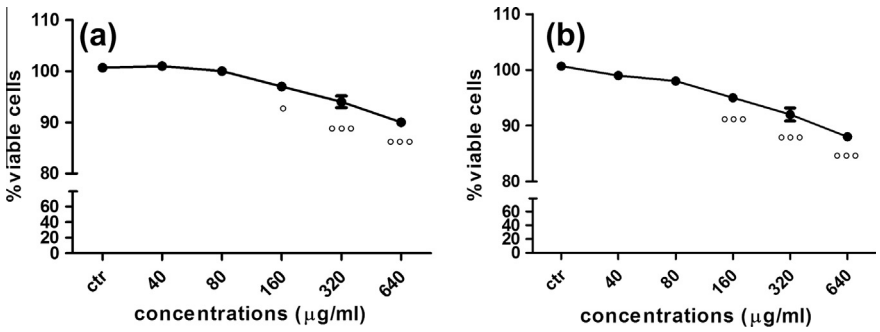

Fig. 1. (a and b) Evaluation of *Vaccinium myrtillus* extract effect on cells viability by MTT test. HaCaT were exposed to different concentrations of the extract (40–640 μg/ml) for 1 h at 37 °C, 5% CO_2. Cytotoxicity was assessed immediately after the end of exposure and 24 h after the end. Results are shown as percentage of viable cells (% cell viability) compared to negative control cells (ctr) set on 100%. Each data point is the mean of three independently reproduced experiments. °$p < 0.05$, °°°$p < 0.001$ vs. ctr (One-Way ANOVA test, Dunnett's Multiple Comparison Test).

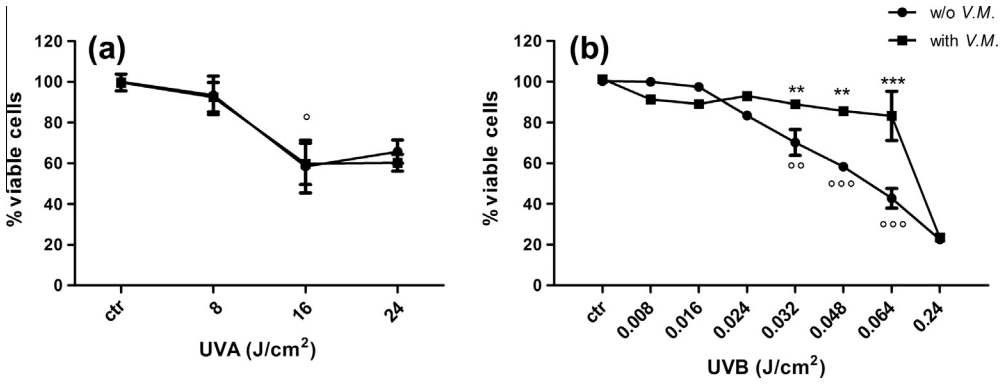

Fig. 2. (a and b) Effect of *Vaccinium myrtillus* extract on cytotoxicity of irradiated cells evaluated by MTT test. HaCaT were pretreated 1 h with *Vaccinium myrtillus* (320 μg/ml), exposed to UVA (8–24 J/cm^2) and UVB (0.008–0.24 J/cm^2) and after incubated in fresh cell culture medium at 37 °C, 5% CO_2 for other 24 h. Results are shown as percentage of viable cells (% cell viability) compared to negative control cells (ctr) set on 100%. Each data point is the mean of three independently reproduced experiments. °$p < 0.05$, °°$p < 0.01$, °°°$p < 0.001$ vs. ctr (One-Way ANOVA test, Dunnett's Multiple Comparison Test); **$p < 0.01$, ***$p < 0.001$ vs. the same treatment w/o *Vaccinium myrtillus* extract (Two-Way ANOVA test, Bonferroni post test).

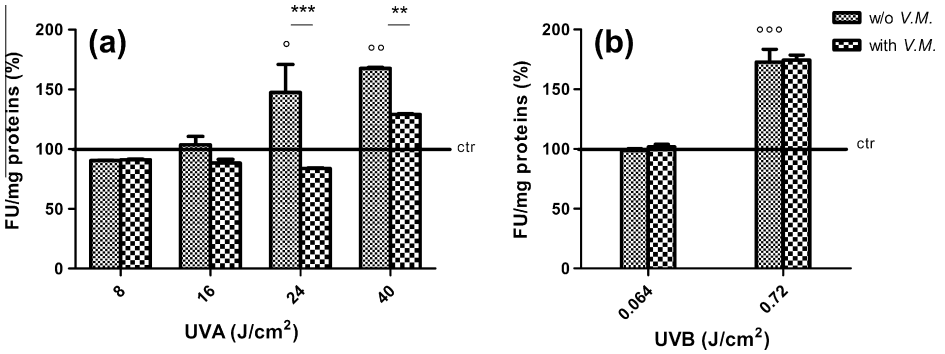

Fig. 3. (a and b) Protective effect of *Vaccinium myrtillus* extract pretreatment (1 h, 320 μg/ml) on ROS formation immediately after the end of UVA (8–40 J/cm^2) and UVB (0.064–0.72 J/cm^2) irradiation. Results are shown as UF/mg proteins (%) and are mean values of three independently reproduced experiments ± SD. °$p < 0.05$, °°$p < 0.01$, °°°$p < 0.001$ vs. ctr (One-Way ANOVA test, Dunnett's Multiple Comparison Test); **$p < 0.01$, ***$p < 0.001$ vs. the same treatment w/o *Vaccinium myrtillus* extract (Two-Way ANOVA test, Bonferroni post test).

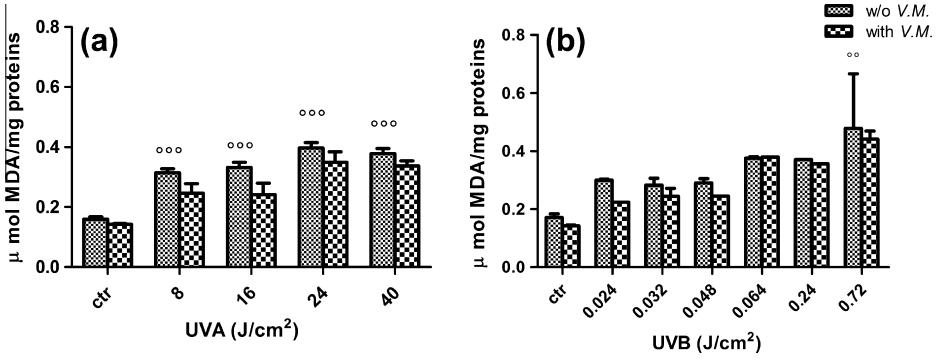

Fig. 4. (a and b) Protective effect of *Vaccinium myrtillus* extract pretreatment (1 h, 320 μg/ml) on lipid peroxidation (MDA/mg proteins) immediately after the end of UVA (8–40 J/cm^2) and UVB (0.024–0.72 J/cm^2) irradiation. Results are expressed as μmoli MDA/mg proteins and are means ± SD of three independent experiments. °°$p < 0.01$, °°°$p < 0.001$ vs. ctr (One-Way ANOVA test, Dunnett's Multiple Comparison Test).

UVB-induced MDA levels, though not significantly, at lower doses (0.024–0.048 J/cm^2) but again it had less protective effect at higher doses (0.064–0.72 J/cm^2).

3.3. Genotoxicity

3.3.1. Alkaline single-cell gel electrophoresis (SCGE, comet assay, pH > 13)

DNA strand breakages are one of the most frequent types of damage induced by ultraviolet rays. UVA (16–32 J/cm^2) and especially UVB (0.016–0.032 J/cm^2) induced significant dose-related DNA damage in HaCaT cells (Fig. 5a and b). *V. myrtillus* pretreatment significantly reduced the UVA-induced DNA damage only at 16 J/cm^2 ($p < 0.05$), but reduced the UVB adverse effects at all irradiation doses, though not significantly.

3.3.2. Micronucleus formation

V. myrtillus pretreatment significantly reduced micronucleus formation after the lower doses of UVA (8 J/cm^2, $p < 0.05$) and UVB (0.008 J/cm^2, $p < 0.001$) (Fig. 6).

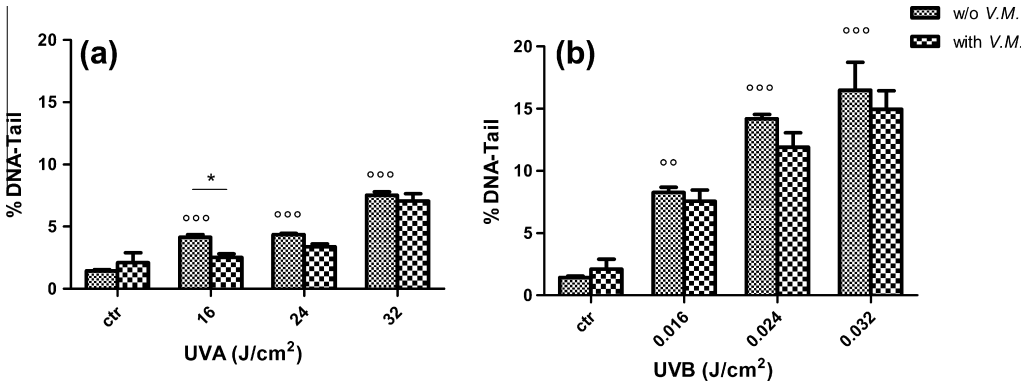

Fig. 5. (a and b) Protective effect of *Vaccinium myrtillus* extract pretreatment (1 h, 320 μg/ml) on UVA (16–32 J/cm^2) and UVB (0.016–0.032 J/cm^2)-induced DNA damage. Genotoxicity was assessed by alkaline comet assay (pH > 13) 24 h after the end of irradiation. Migration of DNA is reported as percentage of DNA in the tail (% DNA-Tail). Results are means ± SD of three independent experiments. °°$p < 0.01$, °°°$p < 0.001$ vs. ctr (One-Way ANOVA test, Dunnett's Multiple Comparison Test). *$p < 0.05$ vs. the same treatment w/o *Vaccinium myrtillus* extract (Two-Way ANOVA test, Bonferroni post test).

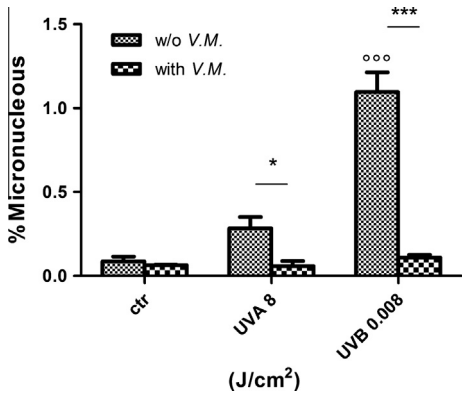

Fig. 6. Protective effect of *Vaccinium myrtillus* extract pretreatment (1 h, 320 μg/ml) on UVA (8 J/cm^2) and UVB (0.008 J/cm^2)-induced DNA damage. Genotoxicity was evaluated quantifying the percentage of micronucleus UV-formed 24 h after the end of irradiation. Results are means ± SD of three independent experiments. °°°$p < 0.001$ vs. ctr (One-Way ANOVA test, Dunnett's Multiple Comparison Test); *$p < 0.05$, ***$p < 0.001$ vs. the same treatment w/o *Vaccinium myrtillus* extract (Two-Way ANOVA test, Bonferroni post test).

3.4. Apoptosis

3.4.1. Mitochondrial membrane potential (JC-1)

UVA (8–24 J/cm^2, $p < 0.001$) and UVB (0.008–0.032 J/cm^2, $p < 0.001$) both significantly and dose-dependently increased the depolarization of membrane potential (Fig. 7a and b). Pretreatment with *V. myrtillus* significantly reduced the $\Delta\Psi$ UVA-induced ($p < 0.001$), but had no effect on the UVB-induced one.

3.4.2. Annexin V assay

UVA (8–24 J/cm^2, $p < 0.001$) and UVB (0.008–0.032 J/cm^2, $p < 0.001$) both significantly and dose-dependently raised the percentages of apoptotic cells (Fig. 8a and b). Pretreatment with *V. myrtillus* significantly decreased the amount of UVA-induced apoptotic cells at 24 J/cm^2 ($p < 0.001$), but had no effect on the UVB-induced ones.

4. Discussion

Interest has been growing recently in new sun-protective dermatological preparations containing either lower concentrations of chemical and physical UV filters or combinations of different plant-derived photoprotective agents – or both – in order to limit the side effects of these filters. The natural products have antioxidant, anti-inflammatory, immunomodulatory and antitumor properties, so they could be very important for UV skin protection [41]. Anthocyanins appear to be useful against the main acute or chronic effects of UV light.

A natural plant thought to be one of the richest sources of antioxidant phytonutrients and anthocyanins is *V. myrtillus* (bilberry) [42]. Bilberry, sometimes known as European blueberry or huckleberry, is a shrub belonging to the *Ericaceae* family [21]. Experimental studies have described the molecular effects of *V. myrtillus* and its clinical applications. Bilberry improves neurocommunication,

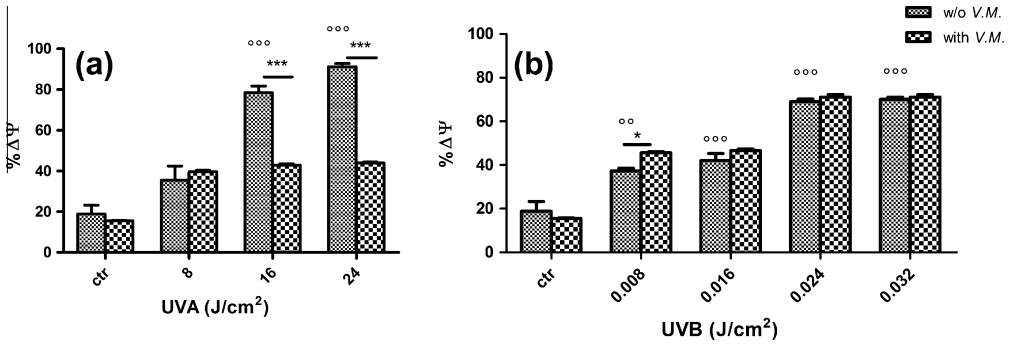

Fig. 7. (a and b) Protective effect of *Vaccinium myrtillus* extract pretreatment (1 h, 320 μg/ml) on UVA (8–24 J/cm^2) and UVB (0.008–0.032 J/cm^2)-induced mitochondrial membrane potential disruption ($\Delta\Psi$). After irradiation cells were incubated in fresh cell culture medium for other 24 h. Results are shown as % of mitochondrial membrane depolarization compared to negative control cells (ctr). Results are means ± SD of three independent experiments. °°$p < 0.01$, °°°$p < 0.001$ vs. ctr (One-Way ANOVA test, Dunnett's Multiple Comparison Test); *$p < 0.05$, ***$p < 0.001$ vs. the same treatment w/o *Vaccinium myrtillus* extract (Two-Way ANOVA test, Bonferroni post test).

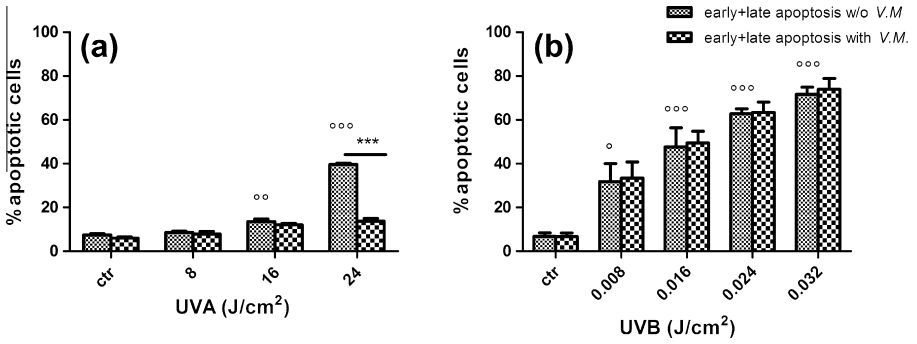

Fig. 8. (a and b) Effect of *Vaccinium myrtillus* extract pretreatment (1 h, 320 μg/ml) on UVA (8–24 J/cm^2) and UVB (0.008–0.032 J/cm^2)-induced apoptosis. After irradiation cells were incubated in fresh cell culture medium for other 24 h. Results are shown as percentage of apoptotic cells compared to negative control cells (ctr). Results are means ± SD of three independent experiments. °$p < 0.05$, °°$p < 0.01$, °°°$p < 0.001$ vs. ctr (One-Way ANOVA test, Dunnett's Multiple Comparison Test); ***$p < 0.001$ vs. the same treatment w/o *Vaccinium myrtillus* extract (Two-Way ANOVA test, Bonferroni post test).

increasing T3 transport to the brain; it protects retinal cells from oxidative stress, increases insulin secretion, lowering blood glucose, has lipid-lowering effects, stabilizes and protects DNA-inducing phase II enzymes and promoting apoptosis in cancer cells, has antimicrobial activity and, finally, promotes antioxidant defenses and lowers oxidative stress [19].

Many of the pharmacological properties of *V. myrtillus* seem related to its antioxidant activities. Moreover, bilberry acts as a superoxide scavenger, reducing hydrogen peroxide-induced radicals and inhibiting LPO in *in vitro* and *in vivo* models [20].

To examine the protective effect of *V. myrtillus* extract, we treated HaCaT cells before irradiation and then investigated its ability to reduce oxidative and genotoxic damage. The cells were exposed to UV irradiation doses that correspond to real sun exposure. The 10 J/cm^2 dose of UVA corresponds to about 30 min exposure [43], while the UVB dose, 0.032 J/cm^2, corresponds to 1–2 min sunbathing at sea level, since the amount of solar UVB reaching the surface of the earth is 0.018–0.030 J/cm^2 [44,45].

The first aspect we investigated was the protection *V. myrtillus* extract offered against UVA- and UVB-induced effects on intracellular oxidative status. So we examined the UV rays' ability to induce the formation of ROS and alter membrane lipids (LPO). Oxygen is the main cause of ROS formation, so despite its importance in normal intracellular activity, oxygen can lead to overproduction of ROS that would overwhelm the efficiency of enzymatic and non-enzymatic antioxidant defense systems, which are highly developed in keratinocytes, and could cause tissue damage [20,46].

Our experimental data obtained using the fluorescent probe DCFDA confirmed reports about the UV rays' ability to cause ROS overproduction [46] depending on their wavelength. UVA raised intracellular ROS generation after low doses, but UVB significantly increased ROS only at high irradiation doses. *V. myrtillus* pretreatment significantly reduced the UVA-induced ROS, progressively from the lowest to the highest doses used. The reduction – hence also the antioxidant activity – was more evident at lower UVA doses than higher ones. No changes were seen in pretreated UVB-irradiated cells.

We wondered whether we had used too short, too weak a pretreatment with *V. myrtillus* extract to protect against UV damage. Antioxidant activity was weaker at higher UVA doses and had no effect against UVB-induced ROS. Many studies have reported the scavenger activity of *V. myrtillus* extract *in vitro* and *in vivo* [19].

Massive ROS production could cause oxidation of cellular molecules such as lipids, proteins and DNA, and may be implicated in the pathogenesis of several diseases. Membrane oxidation mainly affects polyunsaturated fatty acids, forming pro-oxidant molecules that can cause loss of membrane fluidity, destruction of membrane enzymatic activity, increased membrane permeability to ions, and finally rupture and release of organelles [20,47]. Our data indicated a significant increase in MDA levels (a marker of LPO) only after low UVA and UVB doses – lower than those that induced ROS. Probably in our conditions LPO was not directly ROS-related, but was due to the photo-activation, hence also photo-oxidation of endogenous photosensitive molecules of the membrane [46,48]. Pretreatment with *V. myrtillus* extract reduced UVA-induced LPO at all doses, but there was less protective effect at more powerful UVA doses, as in the ROS experiments.

The pretreatment had different effects against UVB-induced LPO. The decrease in MDA levels was more evident at low irradiation doses and disappeared at higher ones. These findings confirm other studies' results about *V. myrtillus* protection against LPO, even though the treatment with the extract was very different: before and after treatment with *V. myrtillus* extract (DMSO solution, anthocyanins 25% w/w) there were marked reductions of both ROS generation and LPO levels [20].

UV rays can directly or indirectly alter DNA bases. DNA bases show different effects when exposed to different UV wavelengths. UVA rays penetrate deep into the dermis of the skin, also affecting epidermal cells. So they cause oxidative DNA damage through excessive production of ROS that may oxidize cellular components such as lipids and DNA bases, and finally could lead to photo-aging and skin carcinogenesis [15,49,50].

UVB act mainly on the epidermal basal layer of the skin and damage DNA directly through base modification and formation of dimers such as cyclobutane–pyrimidine dimers and pyrimidine (6–4) pyrimidone photoproducts [41]. UVB-induced DNA damage is very complex because this wavelength can cause genotoxicity not only directly but also indirectly (through excessive ROS formation) especially at higher doses [35,39,45]. UVB irradiation can also activate inflammatory pathways, through the transcription and release of cytokines and chemokines from skin keratinocytes, leading to skin damage [19].

The two genotoxic tests we used to evaluate DNA damage were the comet test to quantify single- and double-strand breaks and the micronucleus test to study chromosomal alterations. The damage was greater in UVB- than in UVA-irradiated cells, so UVB can be considered the more genotoxic component of the spectrum. Pretreatment with *V. myrtillus* extract slightly reduced the genotoxic damage only at low UVA doses, but there was an evident reduction of UVB-induced damage at all doses. *V. myrtillus* extract significantly reduced micronucleus formation after UVA and UVB irradiation.

Another aspect we investigated was the effect of *V. myrtillus* extract on apoptosis of irradiated cells. Apoptosis is a strictly regulated cell death process that is useful during the development of an organism or to eliminate cells that have been badly impaired

by exogenous agents or viral infections. The anti-apoptotic [21] and pro-apoptotic properties of *V. myrtillus* extract in normal and cancer cells are amply documented [34,51].

JC-1 and Annexin V analysis could hypothesize that UVA and UVB rays activate apoptosis inducing different pathways. Particularly, the decrease in % $\Delta\Psi$ and in % of apoptotic cells could suggest that UVA require activation of mitochondrial (ROS-related) pathway to induce apoptosis. So, *V. myrtillus*, exerting its free radical scavenging properties reduced these apoptotic parameters. On the other hand, we probably did not see any variation in % $\Delta\Psi$ and in % of apoptotic cells because UVB activate mainly the cell death receptor pathway to induce apoptosis.

In conclusion, our results suggest that *V. myrtillus* extract reduced UV-induced damage in the HaCaT cell line. This might be due to its ability to directly eliminate ROS, which can trigger an oxidative chain reaction in cell membrane lipids, leading to LPO. The end-products of LPO (MDA) are mutagenic and carcinogenic and react with deoxyguanosine and deoxyadenosine in DNA, forming DNA adducts [52]. The ability of *V. myrtillus* extract to reduce DNA damage, detected in the comet and micronucleus assays, may also be linked to the reduced amount of ROS and probably also to the enhanced expression of some NER genes in order to better repair UV-induced damage, as demonstrated for other compounds [53–56].

Finally, *V. myrtillus* extract can be considered a good chemopreventive agent on account of its ability to reduced UV-induced damage. Bilberry extract could therefore be a candidate ingredient in broad-spectrum sun protective dermatological preparations. In the future, we could probably boost the protection provided by this extract by changing our model (e.g. pre- or post-treating for a longer time).

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