Bioorganic & Medicinal Chemistry Letters 27 (2017) 5485–5489

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Protective effect of *Opuntia ficus-indica* L. cladodes against UVA-induced oxidative stress in normal human keratinocytes

Ganna Petruk [a,d], Flaviana Di Lorenzo [a,d], Paola Imbimbo [a], Alba Silipo [a], Andrea Bonina [b], Luisa Rizza [b], Renata Piccoli [a,c], Daria Maria Monti [a,c,*], Rosa Lanzetta [a,*]

[a] Department of Chemical Sciences, University of Naples Federico II, Complesso Universitario Monte Sant'Angelo, via Cinthia 4, 80126 Naples, Italy
[b] Bionap srl, R&D Contrada Fureria, ZIO 950125 Piano Tavola, Belpasso, Italy
[c] Istituto Nazionale di Biostrutture e Biosistemi (INBB), Rome, Italy

ARTICLE INFO

Article history:
Received 3 July 2017
Revised 5 October 2017
Accepted 19 October 2017
Available online 20 October 2017

Keywords:
Opuntia ficus-indica L.
Cladode water extracts
Antioxidants
UVA-induced oxidative stress
Eucomic acid
Piscidic acid

ABSTRACT

Opuntia ficus-indica L. is known for its beneficial effects on human health, but still little is known on cladodes as a potent source of antioxidants. Here, a direct, economic and safe method was set up to obtain water extracts from *Opuntia ficus-indica* cladodes rich in antioxidant compounds. When human keratinocytes were pre-treated with the extract before being exposed to UVA radiations, a clear protective effect against UVA-induced stress was evidenced, as indicated by the inhibition of stress-induced processes, such as free radicals production, lipid peroxidation and GSH depletion. Moreover, a clear protective effect against apoptosis in pre-treated irradiated cells was evidenced. We found that eucomic and piscidic acids were responsible for the anti-oxidative stress action of cladode extract. In conclusion, a bioactive, safe, low-cost and high value-added extract from *Opuntia* cladodes was obtained to be used for skin health/protection.

Natural products are receiving a great deal of interest by scientists and pharmacologists for their use in the prevention of oxidative stress-related pathologies, which include obesity, atherosclerosis, diabetes, cancer, neurodegenerative diseases, and aging.[1]

Opuntia is widely distributed in Mexico and in all American hemispheres, as well as in Africa and in the Mediterranean basin.[2] Among all the species, *Opuntia ficus-indica* (referred to here on as *Opuntia*) is the most widely distributed. The multiplicity of health-promoting properties of *Opuntia* are well known. Indeed, in traditional medicine it has been recognized as a source of prebiotics and phytochemicals.[3] Most of the studies report the protective effect of fruits and stems; for example, extracts from *Opuntia ficus-indica* var. *saboten* have been reported to protect against neuronal damage produced under oxidant conditions,[4] or against renal and hepatic alterations caused by mycotoxins.[5] On the other side, only few studies have been performed on the antioxidant, anti-inflammatory, wound healing, hypoglycemic and antimicrobial activities of *Opuntia* cladodes.[6,7] Lee and colleagues showed that an ethanol extract of cladodes decreased the oxidation of linoleic acid and DNA.[8] Recently, Avila demonstrated an increased antioxidant activity in plasma and blood in subjects consuming cladodes (300 g/day for 3 days).[9]

Given these premises, and taking into account the high annual productivity of biomass per hectare (10–40 tones dry weight), it is undeniable that *Opuntia* cladodes represent an economic and suitable substrate for isolation of antioxidants. However, it has to be considered that all the above studies have a common drawback, namely the use of organic solvents to extract bioactive compounds. Indeed, it was previously demonstrated that the extraction procedure, as well as the extraction solvent, notably affect the yield of natural products, their content as well as their antioxidant

Abbreviations: ABTS, 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); CASP-3, caspase-3; CASP-7, caspase-7; DCF, 2′,7′-dichlorofluorescein; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; HAA, hydrophilic antioxidant activity; H_2-DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ORE, *Opuntia* raw extract; OSMF, *Opuntia* small molecular weight fraction; P-p38, phosphorylated p38 MAP kinase; P-MAPKAPK-2, phosphorylated MAP kinase-activated protein kinase; ROS, reactive oxygen species; SPF, sun protection factor; TEAC, Trolox equivalent antioxidant capacity; TBA, thiobarbituric acid; TBARS, TBA reactive substances; TNB, 5-thio-2-nitrobenzoic acid.

* Corresponding authors at: Department of Chemical Sciences, University of Naples Federico II, Complesso Universitario Monte Sant'Angelo, via Cinthia 4, 80126 Naples, Italy (R. Lanzetta, D.M. Monti).

E-mail addresses: mdmonti@unina.it (D.M. Monti), lanzetta@unina.it (R. Lanzetta).

[d] These authors contributed equally to the paper.

https://doi.org/10.1016/j.bmcl.2017.10.043
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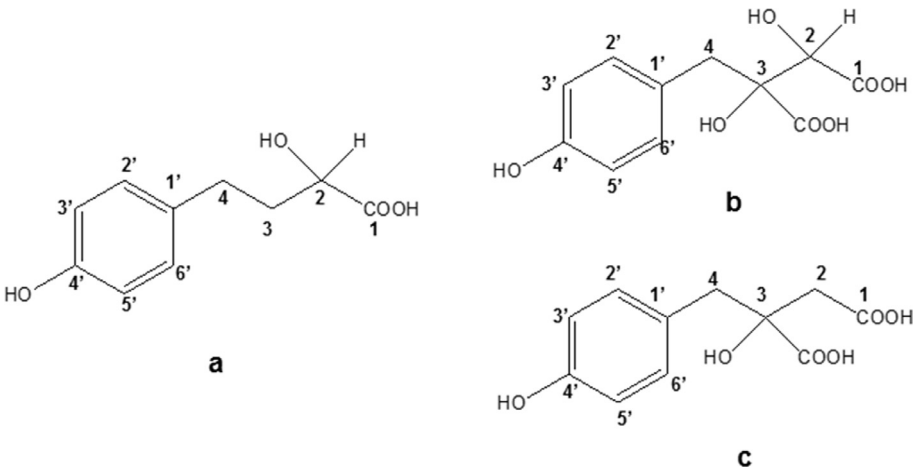

Fig. 1. Structure of 2-hydroxy-4-(4′-hydroxyphenyl)-butanoic acid (a) found in OSMF C, and of piscidic (b) and eucomic (c) acids found in OSMF D.

activity.[10] Furthermore, the use of organic solvents has elevated costs with the risk of contamination of the extract by solvent residues.

We previously reported that *Opuntia* extracts can be obtained by simple mechanical press and that the *Opuntia* raw extract (ORE) was constituted by two main components: i) a high molecular weight constituent consisting in two polysaccharide entities: a linear β-(1→4)-galactose polymer and a highly branched xyloarabinan; ii) a low molecular weight component consisting in lactic acid, D-mannitol and three phenolic derivatives, i.e. piscidic, eucomic and 2-hydroxy-4-(4′-hydroxyphenyl)-butanoic acids (Fig. 1).[6]

Phenolic compounds are used in several applications due to their proved antioxidant and potentially health-promoting properties.[11–13] Importantly, the chemical structure of phenolic compounds, in terms of their reducing properties as electron or hydrogen-donating agents, determines their potential for action as antioxidants.[14]

In this context, we investigated whether ORE is able to protect human keratinocytes against UVA-induced oxidative stress. UVA radiations are known to increase reactive oxygen species (ROS) production, thus causing oxidative damage of proteins, lipids and nucleic acids. These damages result in different detrimental effects on the skin, such as inflammation, premature aging and development of cancer.[15,16] Keratinocytes are essential components of skin and connective tissue, normally present in the outermost layer of the skin.[17] This prompted us to select cultured human keratinocytes (HaCaT cell line) as an excellent experimental model to test the protective role of *Opuntia* extracts against UVA radiations.

We first tested the *in vitro* antioxidant activity of the whole ORE and of each isolated fraction (OSMF A-D), obtained following the procedure described in Di Lorenzo et al.[6] and reported in Supplementary material.

By the ABTS colorimetric assay, we found that ORE is endowed with a significant antioxidant activity, as a low IC_{50} value was obtained (0.52 ± 0.01 mg/mL). Fraction OSMF A, namely the lactic acid component,[6] showed the highest IC_{50} value (1.4 ± 0.01 mg/mL), in agreement with findings obtained in a different experimental system by Lampe, who reported that lactic acid is able to scavenge free radicals.[18] The IC_{50} values of OSMF C (namely 2-hydroxy-4-(4′-hydroxyphenyl)butanoic acid, Fig. 1a) and D (namely piscidic and eucomic acids, Fig. 1b and c, respectively)[6] were found to be much lower (0.09 ± 0.02 and 0.03 ± 0.01, respectively) than those obtained for the whole extract and for OSMF B (D-mannitol,[6]) (IC_{50} = 0.79 ± 0.37 mg/mL). These results were confirmed by the TEAC (Trolox equivalent antioxidant capacity) test, from which much higher TEAC values were obtained for OSMF C and D, with respect to OSMF A and B, indicating a high content in antioxidants in the former two fractions (Table 1).

Table 1
Opuntia raw extract (ORE) and its fractions (OSMF A-D) were tested for their *in vitro* antioxidant properties. The antioxidant activity is expressed as the concentration required to scavenge 50% of free radical $ABTS^{\cdot+}$ (IC_{50}), Trolox equivalent antioxidant capacity (TEAC), and the ability to counteract UV radiations, expressed as sun protecting factor (SPF). Values are normalized to the concentration of each sample.

Sample	IC_{50} (mg/mL)	TEAC (μM/mg)	SPF
ORE	0.52 ± 0.01	43.2 ± 4.53	2.25 ± 0.5
OSMF A	1.4 ± 0.01	10.6 ± 3.39	1.4 ± 0.1
OSMF B	0.79 ± 0.37	24.08 ± 0.34	0.94 ± 0.11
OSMF C	0.09 ± 0.02	225.79 ± 15.85	0.2 ± 0.01
OSMF D	0.03 ± 0.01	749.65 ± 11.81	2.23 ± 0.41

We also tested the UV-protective properties of ORE and its OSMF fractions by measuring their sun protection factor (SPF) *in vitro*, according to a spectrophotometric method.[19] As shown in Table 1, while OSMF B and C did not show any significant protective effect, a value of about 2 was obtained when ORE or OSMF D

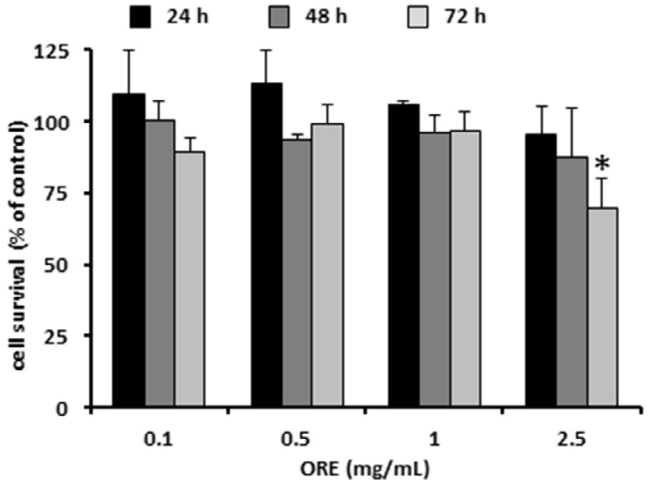

Fig. 2. Effect of ORE on the viability of human keratinocytes. Dose-response curve of HaCaT cells after 24 h (black bars), 48 h (dark grey bars) and 72 h (light grey bars) incubation in the presence of increasing concentrations of ORE. Cell viability was assessed by the MTT assay; the cell survival percentage was defined as described in Supplementary material. Values are given as means ± S.D. ($n \geq 3$). * indicates $p < 0.05$ with respect to untreated cells.

was tested; this value has been reported to correspond to 50% UV blockage. Therefore, these samples can be considered as having good sunscreen activity.[20] OSMF A showed an *in vitro* SPF of 1.4, in agreement with the results obtained by Kornhauser, who reported that lactic acid is commonly used in cosmetics for the improvement of photoaged skin.[21]

The biocompatibility of ORE on HaCaT cells was tested by performing a dose-response experiment, in which cells were exposed for different lengths of time to the extract within the range 0.1–2.5 mg/mL. We found that cell viability was not affected up to 1 mg/mL, even after 72 h incubation ($p > 0.05$) (Fig. 2), while after 72 h, at the highest concentration tested (2.5 mg/mL), about 30% cell death was observed. On the basis of these results and on previously reported data,[6] further experiments were carried out at 1 mg/mL ORE concentration.

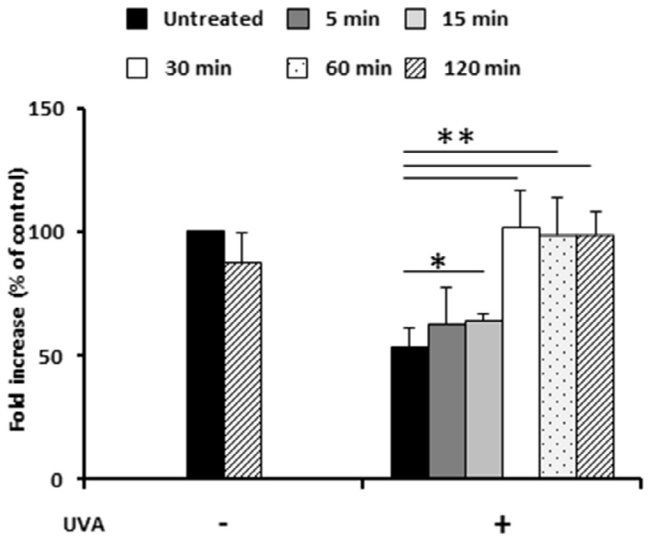

Fig. 3. GSH levels in HaCaT cells irradiated by UVA in the presence of ORE. Cells were pre-incubated in the presence of 1 mg/mL ORE (grey bars) for different lengths of time, irradiated by UVA (100 J/cm^2) and intracellular GSH levels determined by DTNB assay. Control cells, black bars; cells pre-treated with ORE are indicated by: dark grey bars (5 min of pre-incubation), light grey bars (15 min of pre-incubation), white bars (30 min of pre-incubation), spotted bars (60 min of pre-incubation) and dashed bars (120 min of pre-incubation). Values are expressed as fold increase with respect to control (i.e. untreated) cells. Data shown are the means ± S.D. of three independent experiments. * indicates $p < 0.05$; ** indicates $p < 0.001$.

To test if ORE was able to protect keratinocytes from oxidative stress, we performed a time course experiment in which cells were pre-treated with ORE (1 mg/mL) for different lengths of time (from 5 min to 2 h) before inducing oxidative stress by UVA irradiation (100 J/cm^2). Immediately after irradiation, intracellular GSH was evaluated by the DTNB assay (Fig. 3). As expected, UVA treatment significantly decreased intracellular GSH when compared to the non-irradiated samples (50% decrease), whereas ORE had no effect on intracellular GSH level. Interestingly, a pretreatment of cells with ORE, prior to UVA exposure, resulted in an inhibition of GSH oxidation in a time-dependent manner. In particular, we found that ORE was able to protect cells from GSH depletion after just 30 min incubation, and no difference was observed up to 2 h incubation. The efficacy of ORE at 1 mg/mL well correlates with the *in vitro* antioxidant assay (IC_{50} 0.52 mg/mL).

In order to identify the low molecular weight fraction from *Opuntia* extract responsible for the antioxidant activity of the whole extract, each fraction was tested by DTNB and DCFDA assays. For a direct comparison between the whole extract and its fractions, a suitable volume of each fraction was tested to reach the concentration of the single component in the whole extract tested at 1 mg/mL.[6] As shown in Fig. 4A, when cells were pre-incubated either with ORE or with the isolated fractions prior to be irradiated, ORE (dashed bars) and OSMF D (dotted bars) were found to be able to contrast the detrimental effects of UVA irradiation on GSH (GSH depletion), whereas the other fractions had no protective effect. In the absence of irradiation, instead, no effect on the intracellular GSH level was observed for any fraction. These results were also confirmed by the DCFDA assay (Fig. 4B), which showed no alteration in the redox state upon treatment with *Opuntia* samples. On the other hand, a significant increase in ROS production was observed when keratinocytes were irradiated by UVA (4-fold increase). Interestingly, the alteration in the redox state was fully counteracted when cells were pre-incubated either with ORE (dashed bars) or with OSMF D (dotted bars) prior to UVA

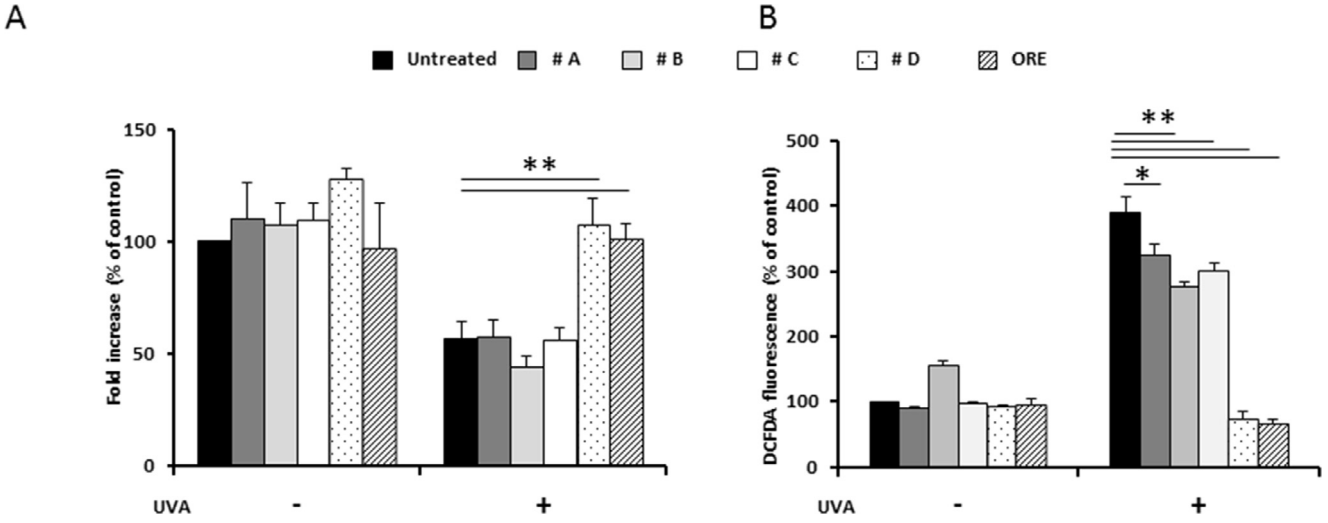

Fig. 4. GSH oxidation and ROS production in HaCaT cells irradiated by UVA in the presence of ORE and isolated fractions. A-B, Cells were pre-incubated in the presence of ORE (1 mg/mL) or equivalent amount of each *Opuntia* fraction for 30 min and then irradiated by UVA (100 J/cm^2). A, changes in intracellular GSH levels of cells before (−) and after (+) UVA treatment; B, intracellular ROS levels determined by DCFDA assay. Black bars, untreated cells; dark grey bars, fraction A (OSMF A, # A); light grey bars, fraction B (OSMF B, # B); white bars, fraction C (OSMF C, # C); spotted bars, fraction D (OSMF D, # D); dashed bars, ORE. Values are expressed as fold increase with respect to control (i.e. untreated) cells. Data shown are the means ± S.D. of three independent experiments. * indicates $p < 0.01$, ** indicates $p < 0.001$.

irradiation. A small, although significant, protective effect was observed with the other fractions. The slight antioxidant activity of lactic acid (present in OSMF A) is in line with Lampe and colleagues who found that lactic acid was effective in counteracting free radicals produced by a photoinitiator, but no effect was observed in the presence of hydrogen peroxide radicals, which are more reactive and able to enter the cell.[18] As for OSMF C, it is well known that the antioxidant activity of a phenolic compound must be related to the amount, position and number of the hydroxyl groups in the molecule.[22] In this context, piscidic and eucomic acids are richer in hydroxyl groups than 2-hydroxy-4-(4-hydroxyphenyl)-butanoic acid, the component of OSMF C (Fig. 1).

Based on these results, ORE and OSMF D were used to deeply analyze their antioxidant protective effect.

To this purpose, measurement of lipid peroxidation levels was performed (Fig. 5A).

We found that UVA treatment significantly increased lipid peroxidation levels when compared to a non-irradiated sample.

Noteworthy, a strong protective effect was observed when cells were pre-treated with ORE or OSMF D, since both values were comparable to those obtained in the absence of any treatment.

This result is in line with those obtained by Hfaiedh, who studied the effects of a water *Opuntia* extract in counteracting the negative effects of $NiCl_2$ in rats.[23]

The protective effect of ORE and OSMF D was further confirmed by Western blot experiments, in which the phosphorylation levels of p38 and its direct target, MAPKAPK-2, were analyzed (Fig. 5B). These proteins are directly involved in signaling stress pathways induced by UVA.[24,25] When cells were UVA irradiated, we observed a significant increase in the phosphorylation levels of the analyzed markers (Fig. 5B-C). On the contrary, when cells were exposed to ORE or OSMF D prior to UVA treatment, the phosphorylation levels of p38 and MAPKAPK-2 were similar to those observed in non-irradiated cells. These results are in agreement with those obtained by Zourgui and colleagues in mice using an ethanol:water extract.[5]

Furthermore, we verified whether ORE and OSMF D were able to protect cells against UVA-induced cell death. As shown in Fig. 6, a cell treatment with ORE did not induce apoptosis, since no cleavage of caspase-3 or caspase-7 was observed, while, as expected, upon UVA exposure, the activation of caspase-3 and -7 was remarkably increased, as indicated by the presence of the cleaved form of both proteins (Fig. 6, third lane). These results

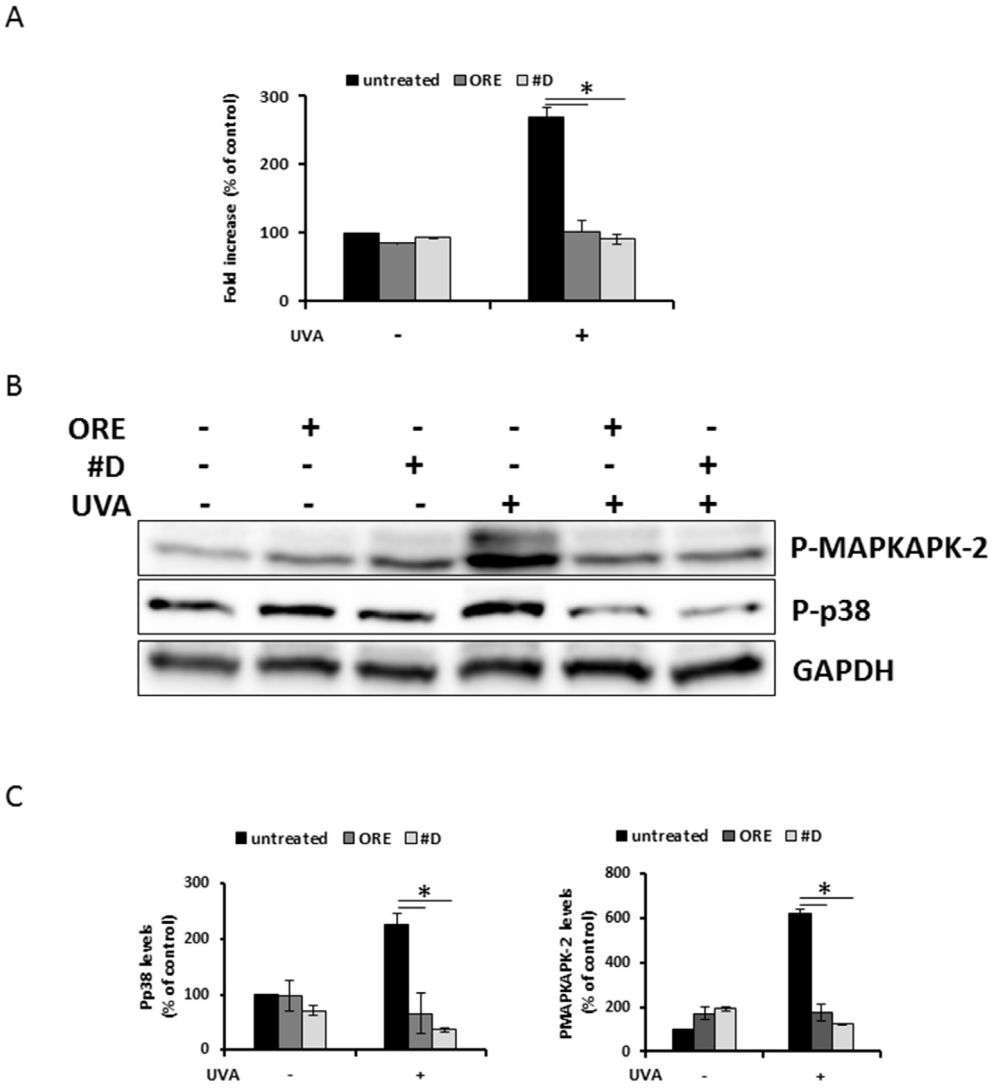

Fig. 5. Effect of ORE extract and OSMF D on UVA-induced oxidative stress markers in human keratinocytes. Cells were incubated with 1 mg/mL ORE or 0.15 mg/mL OSMF D for 30 min prior to UVA irradiation (100 J/cm^2) and then incubated for 90 min. A, lipid peroxidation levels determined by TBARS assay; B, Western blots. In Western blots the phosphorylation level of P-p38 and P-MAPKAPK-2 is reported, with the relative densitometric analysis (C) in the absence (black bars) or in the presence of ORE (dark grey bars) or OSMF D (light grey bars). GAPDH was used as internal standard. Data shown are the means ± S.D. of three independent experiments. * indicates $p < .005$.

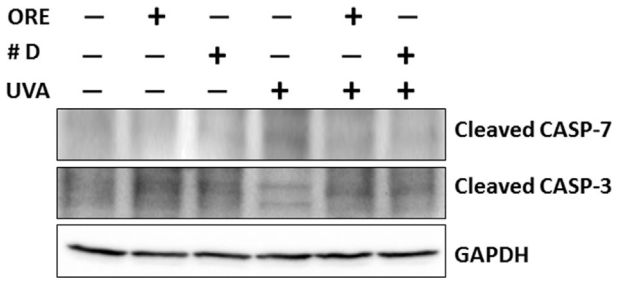

Fig. 6. ORE extract and OSMF D protect HaCaT cells from UVA-induced apoptosis. Cells were incubated with 1 mg/mL ORE or 0.15 mg/mL OSMF D for 30 min, irradiated with UVA (100 J/cm^2) and then grown for 24 h. Western blots were performed using anti-caspase-3 and -7, which recognize the activated forms of the proteins. GAPDH was used as loading control.

are in agreement with those reported by others, who demonstrated that many epidermal skin cells, including keratinocytes, undergo apoptosis following UVA exposure as a result of DNA strand breaks.[24,26] When, instead, keratinocytes were pre-incubated in the presence of ORE or OSMF D, and then exposed to UVA, no caspase-3 and -7 activation was observed, thus indicating a protective role of piscidic and eucomic acids from UVA-induced cellular stress.

The beneficial properties of *Opuntia* mucilage in producing a physical barrier on the cutis, favoring cutaneous reparative processes, is historically well known. Nowadays, given the enormous potential benefits on health of natural bioactive compounds isolated from *Opuntia* cladode extracts, intensive investigation is ongoing attracting the interest of food scientists and clinical pharmacologists. Some of the beneficial effects of *Opuntia* extracts have been attributed to fibers and polyphenols isolated from cladodes. However, all the studies published so far have been performed on extracts treated with organic solvents, which may not be completely removed with a potential danger for human health.

Here, we report the discover of an additional and intriguing beneficial property of water extracts from cladodes, i.e. a protective effect against UVA-induced oxidative stress and apoptosis, which has never been investigated so far. Altogether, our results demonstrate that ORE water extract has strong antioxidant properties, being able to counteract the negative effects induced in human keratinocytes by UVA radiations, and to protect cells from this common and pernicious source of stress. We demonstrated that the anti-stress effect of cladode extracts has to be ascribed to eucomic and piscidic acids, as these phenolic compounds are the sole components of fraction OSMF D, which fully retains the antioxidant activity of the whole extract. As widely known, the antioxidant potency of phenolic acids strongly depends on their structural features. Indeed, it has been demonstrated that the main structural characteristic responsible for the antioxidant activity of phenol derivatives is the number and location of the hydroxyl groups present in the molecule.[27] Two main mechanisms by which antioxidants can play their protective role have been proposed. Due to the presence of such hydroxy functions, phenols are able to easily donate hydrogen atoms to the free radicals thus blocking the chain propagation step occurring in the oxidation process (H-atom-transfer mechanisms).[14,28] Alternatively, the antioxidant can give an electron to the free radical becoming itself a radical cation (single-electron-transfer mechanisms). In addition, deprotonated carboxyl groups behave as electron-donating groups thus favoring the hydrogen atom transfer and electron-donating based radical scavenging.[29] Therefore, the potent antioxidant effect observed for both the piscidic and eucomic acids is clearly reflected in their structural features which promote the hydrogen atoms transfer to free radicals. The slight structural differences, as example, between the piscidic and the 2-hydroxy-4-(4′-hydroxyphenyl)-butanoic acid, namely the absence, in the latter, of one hydroxyl and one carboxyl group, could be reflected in the lower antioxidative power observed for OSMF C.

In our opinion the present study, shedding light on bioactive compounds responsible for *Opuntia* cladode antioxidant activity, represents an important step towards the therapeutic values of *Opuntia* properties.

It is important to underline that aqueous *Opuntia* antioxidant extracts can be obtained by simple mechanical press of cladodes, thus avoiding the use of expensive materials and of organic solvents, responsible for environmental pollution and persistence of residues dangerous for human health. Therefore, we obtained a bioactive, safe, low-cost and high value-added extract to be used in pharmaceutical and/or cosmetics applications for skin health/protection.

Conflict of interest

The authors declare no competing financial interest.

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2017.10.043.

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