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Short Communication

Aqueous and polyphenol-rich *Larrea divaricata* Cav. extracts as potential skin care agents

[Extractos acuoso y rico en polifenoles de *Larrea divaricata* Cav. Como potenciales agentes protectores de la piel]

Carla Marrassini[1]*, Renzo Martino[1], María Laura Barreiro Arcos[2], Elina Malén Saint Martin[1], Laura Cogoi[1], María Rosario Alonso[1] & Claudia Anesini[1]

[1]Facultad de Farmacia y Bioquímica, Instituto de la Química y Metabolismo del Fármaco (IQUIMEFA), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad de Buenos Aires. Buenos Aires, Argentina
[2]Instituto de Investigación Biomédica (BIOMED), Consejo de Investigaciones Científicas y Técnicas (CONICET), Universidad Católica Argentina (UCA). Buenos Aires, Argentina
*Correspondence to: cmarra@ffyb.uba.ar

Abstract: Skin cells are affected by UV-induced oxidative stress resulting in photoaging and diseases. The objective of this work was to assess the effect of an aqueous extract of *Larrea divaricata* (Aq) and a flavonoid rich fraction (EA), obtained by liquid/liquid fractionation, on the proliferation of keratinocytes and fibroblasts by the tritiated thymidine uptake assay and on cell viability by the trypan blue exclusion assay. The in vitro sun protection factor (SPF), peroxidase (Px)-like and superoxide dismutase (SOD)-like activities were determined by kinetic spectrophotometric assays, and the phytochemical composition was determined by HPLC-UV and HPLC MS/MS. Aq and EA induced keratinocytes and fibroblast proliferation, protected fibroblasts from H_2O_2-induced apoptosis and exerted SOD-like and Px-like activities. Aq and EA had a high sun UVB protection factor. So, *L. divaricata* could be used in a future for the development of new dermocosmetic or phytotherapy adjuvant in skin oxidative damage.

Keywords: *Larrea divaricata* Cav; Skin protection; UVB radiation; Antioxidant activity; Fibroblast; Keratinocytes.

Resumen: Las células de la piel se ven afectadas por el estrés oxidativo inducido por los rayos ultravioleta resultando en foto-envejecimiento y enfermedades. El objetivo de este trabajo fue evaluar el efecto de un extracto acuoso de *Larrea divaricata* (Aq) y una fracción rica en flavonoides (EA), obtenida por fraccionamiento líquido/líquido, sobre la proliferación de queratinocitos y fibroblastos y sobre la viabilidad celular. Se determinó también el factor de protección solar (SPF) y las actividades simil peroxidasa (Px) y superóxido dismutasa (SOD) y la composición fitoquímica por HPLC-UV y MS/MS. Aq y EA indujeron la proliferación de queratinocitos y fibroblastos, protegieron a los fibroblastos de la apoptosis inducida por H_2O_2 y ejercieron actividades de tipo SOD y de tipo Px con un factor de protección solar UVB alto. Por lo tanto, *L. divaricata* podría utilizarse para el desarrollo de nuevos dermocosméticos o adyuvantes fitoterápicos en enfermedades oxidativas de piel.

Palabras clave: *Larrea divaricata* Cav; Protección de la piel; Radiación UVB; Actividad antioxidante; Fibroblasto; Queratinocitos.

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INTRODUCTION

The skin keratinocytes and fibroblast exert a protective barrier through the secretion of extracellular **matrix proteins** and by the connection with immune cells, connective tissue cells and a variety of cytokine signals (Guaratini *et al*., 2007; Proksch *et al*., 2008).

UVB (290-320 nm) and UVA (320-400 nm) sunlight, by generating reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide anion (O_2^{-}) (Guaratini *et al*., 2007) induce skin cell damage, principally on fibroblasts, destroying the collagen and elastin fibers and impairing hydration, leading to changes in skin relief and wrinkles (Wulf *et al*., 2004). Cells, are known to have efficient non-enzymatic and enzymatic antioxidants but in some occasion the excessive production of ROS outbalance the capacity of the antioxidant mechanisms and lipid peroxidation, DNA damage and protein denaturation occur (Poljsak *et al*., 2012). In this case, exogenous antioxidants are necessary to avoid oxidative stress. However, as the synthetic agents cause dermic and ocular irritation, hypersensitivity, edema, erythema, photosensitivity, and acne, they need to be replaced by safe plant extracts rich in polyphenols, which are promising candidates for the prevention of skin damage, being capable of modulating skin cell proliferation (Stevanato *et al*., 2014; Tong & Young, 2014).

One plant with high content in polyphenols is *Larrea divaricata* (Zygophyllaceae), an autochthonous South American plant widely distributed in Argentina and used in popular medicine in inflammatory diseases. The aqueous extract (Aq) presents antioxidant and immunomodulatory activities reported (Anesini *et al*., 1996; Peralta *et al*., 2019). In addition, the extract of *L. divaricata* combined with *Coffea arabica* is commercially available under the registered name of ECOHAIR® for the prevention and treatment of hair, eyelashes and eyebrow loss (Alonso & Anesini, 2017; Alonso *et al*., 2019).

Therefore, the objectives of this work was to study: the effects of Aq in the proliferation of skin cells such as keratinocytes and fibroblasts, the protective effects against apoptosis induced by H_2O_2 in fibroblasts, and the participation of polyphenols (flavonoids) in its action. The final objective was to find new dermocosmetics or phytomedicines for the prevention or treatment of skin oxidative damage.

MATERIALS AND METHODS

Plant material and preparation of extracts

Leaves of *Larrea divaricata* Cav. (Zygophyllaceae) were obtained from the wild by Dr Renzo Martino, during autumn 2017, in the province of San Luis, Argentina and identified by botanists of the herbarium of Universidad Nacional de San Luis, Argentina (voucher N° 467 UNSL). After collection, the plant material (leaves) was dried under air at room temperature (25°C) and then extracted with solvent. An aqueous extract of the leaves was prepared at 7.5%. Briefly, the air-dried leaves were extracted for 20 min with boiling distilled water. The extract was then filtered and lyophilized. The final yield was 26.6 g/100 g of plant material. A fraction enriched in flavonoids was obtained by liquid-liquid partition of the Aq with ethyl acetate (EA). The aqueous extract was treated twice with EA overnight. The EA extracts were collected by decantation and joined. The original aqueous extract Aq and the extracted with the ethyl acetate named EA were then lyophilized or evaporated under rotavapor and stored at -20°C until use (Davicino *et al*., 2011). Aq and EA were used for subsequent studies.

Phytochemical study of Aq and EA: Determination of polyphenols and Q3ME levels by HPLC-UV

The HPLC analysis was performed in a Varian Pro Star instrument equipped with a Rheodyne injection valve (20 µL) and a photodiode array detector set at 280 nm (NDGA) and 260 nm (Q3ME). A Phenomenex-Kinetex (250 mm x 4.6 mm and 5 µm pd) reversed-phase column was used. For the identification and quantification of NDGA, samples were eluted with a gradient of solution A (water and acetic acid, 98:2), and solution B (methanol and acetic acid, 98:2). The gradient was from 15% B to 40% B in 30 min; 40% B to 75% B in 10 min; 75% B to 85% B in 5 min and 100% B in 5 min. The mobile phase B was kept at 100% for 10 min before restoring the initial conditions. Mobile phases were delivered with a flow rate of 1.2 mL/min. The chromatographic procedure was performed at room temperature (18-25ºC). Pure standards of NDGA, 4-hydroxybenzoic acid (4-HBA) and Q3ME (Sigma, USA)) were used for identification by comparing retention times and by plotting peak areas, respectively. Data were analyzed with a Varian Star 5.5 program (USA). Lyophilized aqueous extracts at 10 mg/mL and the pure standard were dissolved in methanol: water (70:30). A calibration curve of NDGA was obtained by injecting 20 µL of solutions ranging from 2 µg/mL to 200 µg/mL. (Pearson's correlation coefficient: $\Gamma = 0.9988$). For the identification and quantification of Q3ME, the EA was

analyzed by HPLC using three chromatographic systems: System 1 was the same as that described for the quantification of NDGA. In system 2, a mixture of water/methanol/phosphoric acid (100:100:1) was used as mobile phase. In system 3, two mobile phases were used: solution A was a water-phosphoric acid (0.5%) mixture and solution B was a methanol-phosphoric acid (0.5%) mixture. The elution profile was 100% A to 25% A in 30 min, 25% A to 0% A in 2 min, and back to the initial conditions. Both the retention time in each method and the ultraviolet peak OD obtained were compared with those of a Q3ME standard (Sigma, USA).

The water employed to prepare working solutions was of ultrapure quality (Milliq). Methanol (J.T. Baker) and acetic acid (Merck, Argentine) were HPLC grade.

Quantification of total polyphenols and flavonoids

Polyphenols were determined by spectrophotometry by the Folin-Ciocalteu's method using gallic acid as standard (Hosseinzadeh *et al*., 2013). Briefly, the dried extracts were weighed and dissolved in distilled water. A sample of 1.0 mL of the extracts were transferred to separate tubes containing 7.0 mL of distilled water, 0.5 mL of Folin–Ciocalteu's reagent, and 1.5 mL of a 20% anhydrous sodium carbonate solution. Mixtures were then allowed to stand at room temperature for 60 min and then the absorbance at 765 nm was measured in a UV-vis spectrophotometer. The concentration of polyphenols in samples was calculated from a standard curve of gallic acid ranging from 10 to 50 μg/mL (Pearson's correlation coefficient: $\Gamma = 0.9996$). Results were expressed as mg GAE/g extract. Flavonoids were quantified by spectrophotometry. Briefly, the extracts were mixed with aluminum trichloride (10% w/v) and 1 M potassium acetate and incubated for 30 min. The absorbance was then read at 415 nm. Results were expressed as mg quercitrin/g extract and derived from a calibration curve obtained with known concentrations of the flavonoid quercitrin (Sigma) (Dantas Fernandes *et al*., 2012).

Cell suspensions, culture conditions and Proliferation and Viability assays

Fibroblasts (3T3, ATCC, a mouse fibroblast cell line) and keratinocytes (HeCaT, ATCC, a human immortalized keratinocyte cell-line) were cultured at 10^5 cells/mL in Dulbecco's modified Eagle's medium (DMEM, GIBCO-BRL, Grand Island, NY, USA) supplemented with a 10% fetal calf serum, 2 nM L-glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin, at 37ºC in a humidified atmosphere containing 5% CO_2.

The effect of the extracts and isolated compounds on cell proliferation was evaluated by the tritiated thymidine [3H]TdR uptake assay. Briefly, cells were pulsed with [3H]TdR (20 Ci/mmol) for the last 6 h of incubation as previously described (Anesini et al., 1996).

Fibroblasts were incubated in the presence of Aq and the EA from 0.1 μg/mL to 100 μg/mL, keratinocytes were incubated with Aq and EA from 0.1 μg/mL to 1000 μg/ml. Q3ME was assayed at 1 μg/mL, 10μg/mL and 20 μg/mL on all cell types. Fibroblasts and keratinocytes, were cultured for 24 h. Results were expressed in cpm. Data represent the mean ± SEM of three experiments performed in triplicate. The protective effect of the Aq and EA against oxidative stress was determined by incubating fibroblasts in the presence of Aq and EA at 0.1 μg/mL and 1 μg/mL in either the presence or the absence of 10^{-2} M H_2O_2 for 24 h. In both cases, cell viability was determined by the Trypan blue exclusion method. Briefly, the cell suspension was mixed with 0.4% Trypan blue in a 1: 9 ratio and the mixture was transferred to a Neubauer hemocytometer to determine the number of viable and non-viable cells, viability was expressed as % of viable cells, calculated according to the following formula:

%Viable cells = [number of viable cells/number of total cells] x 100

To assess nuclear morphology of apoptotic, fibroblasts incubated with either Aq or EA at 1 μg/mL in either the presence or the absence of 10^{-2} M H_2O_2 for 24 h were suspended in PBS at a concentration of $1x10^6$ cells/mL, plated onto slides and fixed with ethanol. Cells were stained with a 0.01 mg/mL Hoechst 33258 solution for 10 min at room temperature. Cells were then washed three times with PBS, and the nuclear morphology was examined by fluorescence microscope (Nikon Diaphou; Nikon Inc., Melville, NY) (Di Rosso *et al*., 2013).

Peroxidase-like and superoxide–like activities

The peroxidase-like activity of Aq and EA (tested from 0.01 μg/mL to 1000 μg/ml) was determined by the method described by Herzog and Fahimi (1973). Briefly, 200 μL of each sample were mixed with 25 μL of 0.03 M H_2O_2 and 775 μL of 10^{-4} M diaminobencidine tetrahydrochloride (DAB) (Sigma, St Louis, Mo, USA) prepared in Krebs-Henseleit buffer (125 mM NaCl; 4.0 mM KCl; 0.5 mM NaH_2PO4; 0.1 mM $MgCl_2$; 1.1 mM $CaCl_2$ and 5.0 mM glucose, pH 7.4). A DAB solution without H_2O_2 was used as reaction blank. The reaction was initiated by the addition of H_2O_2 and the change in optical density readings was recorded at 30 sec intervals for 5 min using a Shimadzu recording spectrophotometer UV-240 (graphic printer PR-1) set at 465 nm. Then, the Δabsorbance/min was calculated. A calibration curve of peroxidase concentration *vs.*Δ absorbance/min was constructed using a horseradish peroxidase solution of known concentration, obtaining a linear relationship in the range of 1.95×10^{-3} to 2.5×10^{-5} U/mL. The activity of samples was calculated by interpolation in the standard curve. The superoxide dismutase-like activity was determined through the capacity of extracts to inhibit the epinephrine auto-oxidation to adrenochrome in the presence of atmosphere oxygen (Carrillo et al., 1991). Briefly, 50 μL of the extracts (tested from 0.01 μg/mL to 1000 μg/mL), 910 μL phosphate buffer pH 10.7 and 40 μL 2 mM epinephrine (Sigma, St Louis, Mo, USA) were mixed and the absorbance was measured at 480 nm every 10 sec for 5 min. The Δabsorbance/min was calculated. The antioxidant activity of samples was evaluated as the percentage of epinephrine auto-oxidation inhibition using the following formula:

$$\% \text{ inhibition} = [(\Delta Abs/min_{\text{epinephrine}} - \Delta Abs/min_{\text{sample}})/ \Delta Abs/min_{\text{epinephrine}}] \times 100$$

In both reactions a blank with Aq or EA was done to corroborate that the extracts "per se" did no absorbed at 480 or 465 nm.

Determination of UV absorption and the sun protection factor (SPF)

The sun protection factor of Aq and EA (tested from 0.1 mg/mL to 2 mg/mL) was determined *in-vitro*. Briefly, extracts were dissolved in ethanol and the sample absorbance was determined over a range of 290-320 nm (at 5 nm intervals) in triplicate. The SPF was calculated according to Mansur et al., 1986:

$$SPF = CF \times \Sigma(320\text{-}290\ nm)\ EE \times I(\lambda) \times Abs(\lambda)$$

where EE: Erythemal effect spectrum; I(λ): Solar intensity spectrum; Abs(λ): absorbance of sunscreen product; CF is a correction factor (10.0). The values of EE × I are constant and were determined before as expressed below (Sayre *et al.*, 1979).

Normalization function used to calculate EE x I

Wavelength (nm)	*EEx I (normalized)*
290	**0.0150**
295	**0.0817**
300	**0.2874**
305	**0.3278**
310	**0.1864**
315	**0.0839**
320	**0.0180**

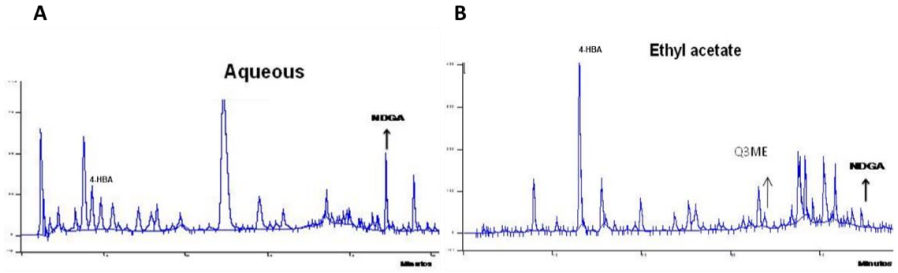
Statistical analysis

The Student's *t* test for unpaired measures was used to determine the level of significance of results. When multiple comparisons were necessary, one way ANOVA plus the Dunnett or Tukey's test was applied. Differences between means were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

In this work it was demonstrated that an aqueous extract and a polyphenol-rich extract from *L. divaricata* could modulate the proliferation of keratinocytes and fibroblasts at the same time that exerted antioxidant activity and absorbed UVB radiation.

The HPLC-UV analysis of Aq showed the presence of NDGA (retention time: 47 min) and 4-hydroxybenzoic acid (4-HBA) (retention time: 12 min) together with other compounds, of flavonoid nature, with retention time between 24-25 min, shown as an only peak (Figure No. 1A). By HPLC MS/MS working in positive mode, the flavonoids rutin, epicatechin, quercetin-3-O-arabinopiranoside and quercetin-3-O-galactoside were identified (Data not shown). As flavonoids represent the majority compounds present in Aq, their contribution to Aq effects was assessed. To do this, a liquid/liquid fractionation of Aq in ethyl acetate was done giving a fraction named EA that presented more polyphenols and flavonoids than Aq (Table inserted in Figure No. 1). The HPLC-UV of this fraction showed the presence of NDGA, 4-HBA and the flavonoid quercetin 3methyl ether (Q3ME) (Figure 1B). The presence of NDGA and Q3ME was previously reported in *L. divaricata* extracts (Martino *et al*., 2013; Martino *et al*., 2016).



Extracts	Yield Yield (%)	Q3ME g%	Flavonoids g%*	Polyphenols g%
Aqueous	26.6	-----	4.2 ± 0.56 [a]	11.7 ± 1.6[a]
Ethyl acetate	3.83	0.52 ± 0.03	7.3 ± 0.68 [b]	50.24 ± 2.13[b]

Figure No. 1
Phytochemical composition of Aq (A) and EA (B) by HPLC-UV. Table inserted: Quantification of total polyphenols and flavonoids and Q3ME
Flavonoids and polypohenols were determined by spectroscopic methods. Q3ME was quantified by HPLC. Values are expressed as mean ± SEM of three independent measures. *Quercitrin equivalent ** Gallic acid equivalent. [a] and [b] significant differences ($p < 0.001$, Student's *t* test.)

On cell proliferation Aq and EA increased keratinocytes proliferation at high concentrations, being EA more active than Aq (maximum stimulation percentage: 200%, Figure No. 2A). Both extracts increased fibroblasts proliferation at low concentrations but EA was more active than Aq (maximum stimulation percentage 270%) (Figure No. 2B). This phenomenon could be important in the case the extracts were applied on the skin, with the epidermis and the dermis being exposed to high and low extract concentrations, respectively. Q3ME appeared to be involved in these actions (Figure No. 2C), which is in line with previous reports indicating that quercetin can promote the proliferation of human oral keratinocytes and re-epithelialization *in vitro* (Hujiahemaiti *et al*., 2018).

Moreover, Aq and EA prevented the H_2O_2-induced decrease of cell viability of fibroblasts, at low concentrations, by inhibiting nuclear chromatin condensation, with the presence of apoptotic bodies and nuclear fragmentation, sings of apoptosis (Figure No. 2D and No. 2E).

This last effect could be related to the concentration-dependent SOD and Px-like activities (Figures No. 3A and No. 3B) but also to the capacity of absorbing principally UVB and partially UVA radiation by Aq and EA (Figure No. 3C and No. 3D). EA exerted a higher UV absorption capacity. The maximum absorption wavelength for both extracts was 275 nm (Figure No. 3E).

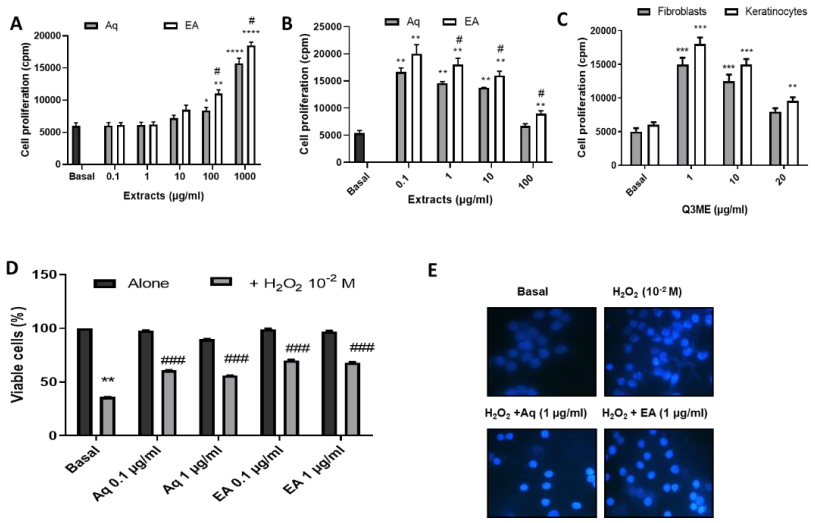


Figure No. 2

Effect of Aq (A), EA (B) and Q3ME (C) on the proliferation of normal cells

D. Effect of the extracts on the viability of cells incubated with 10^{-2} M H_2O_2. E: Analysis of nuclear morphology by Hoechst 33258 staining and fluorescence microscope. Results were expressed as mean ± SEM of three experiments carried out in triplicate. A, B and C *p<0.05; ** p<0.01; ***p<0.001: significant differences with respect to basal (One way ANOVA followed the Dunnett's multiple comparison test). #p<0.05: significant differences between Aq and EA at each concentration (Student's t test). D. **p<0.01 significant differences with respect to basal conditions and ###p<0.001 significant differences with respect to H_2O_2 alone (One way ANOVA followed the Dunnett's multiple comparison test)

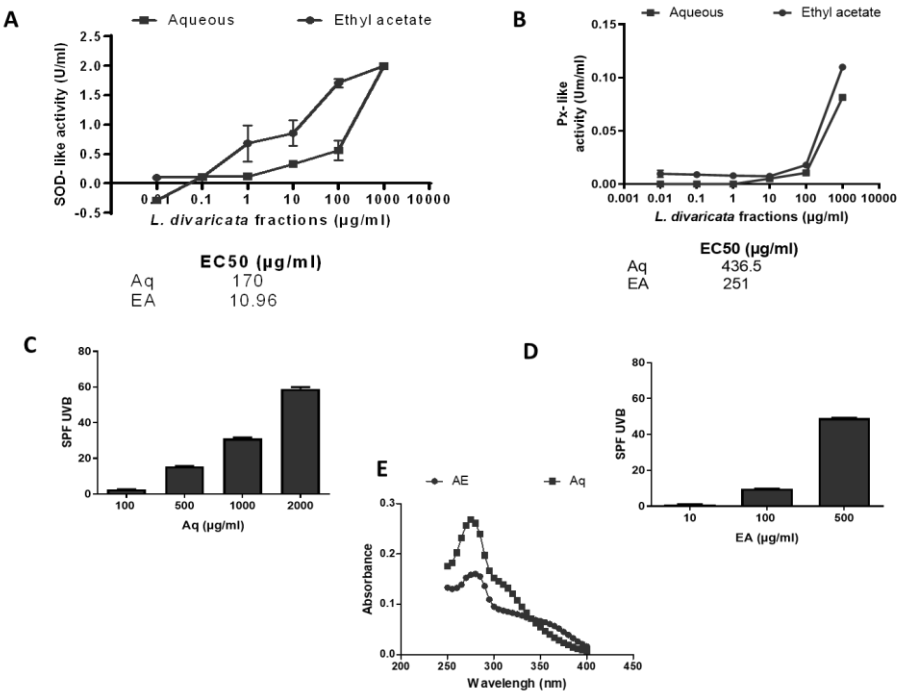


Figure No. 3

Antioxidant activity of Aq and EA and UVB absorbation

A: SOD-like activity and B: Px-like activity, embedded in the graphs: EC_{50} (µg/mL) calculated by the Alexander's method. Results are expressed as mean ± SEM of three experiments performed in triplicate. C and D: SPF UVB of Aq and EA, respectively. E: the maximum absorption wavelength. Extracts were scanned spectrophotometrically over a wavelength range of 250 to 400 nm. Results are expressed as mean ± SEM of three experiments performed in triplicate

CONCLUSIONS

The exposure of the skin to environmental UV radiation generate ROS, involved in the suppression of some immune responses in humans, and in an imbalance between apoptotic and anti-apoptotic events in keratinocytes and fibroblasts, that may result in the development of skin diseases (Gilhar *et al*., 2004; Tarozzi *et al*., 2005).

Both Aq and EA demonstrated to have pleiotropic effects: they modulated cell proliferation, exerted antioxidant effects protecting fibroblasts from the deleterious effect of hydrogen peroxide, and were capable of absorbing UVB and UVA radiation. So, they could be considered for "in vivo" researches and in a future for the development of new dermocosmetic or phytotherapy adjuvant to be used in the treatment or prevention of skin oxidative disseasses.

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