

***In vitro* antioxidant activity and *in vivo* photoprotective effect of a red orange extract**

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Synopsis

Ultraviolet radiation causes damage to the skin, which may result in both precancerous and cancerous skin lesions and acceleration of skin ageing. Topical administration of enzymatic and non-enzymatic antioxidants is an effective strategy for protecting the skin against UV-mediated oxidative damage. Hence, a systematic study to evaluate the *in vitro* antioxidant activity and *in vivo* photoprotective effect of a standardized red orange extract (ROE) has been undertaken, where the main active ingredients are anthocyanins, hydroxycinnamic acids, flavanones and ascorbic acid. For the *in vitro* experiments, the ROE was tested in three models: (1) bleaching of the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH test); (2) peroxidation, induced by the water-soluble radical initiator 2,2'-azobis(2-amidinopropane) hydrochloride, of mixed dipalmitoylphosphatidylcholine/linoleic acid unilamellar vesicles (LUVs) (LP-LUV test); and (3) UV-induced peroxidation of phosphatidylcholine multilamellar vesicles (UV-IP test). The *in vivo* antioxidant/radical scavenger activity was assessed by determining the ability of topically applied ROE to reduce UVB-induced skin erythema in healthy human volunteers. The results obtained in the DPPH, LP-LUV and UV-IP tests demonstrated the strong antioxidant properties of ROE, with a clear relationship between ROE scavenger efficiency and its content in antioxidant compounds. In particular, the findings obtained in the UV-IP test provide a strong rationale for using this extract as a photoprotective agent. During *in vivo* experiments, ROE provided to efficiently protect against photooxidative skin damage when topically applied immediately after skin exposure to UVB radiations. Interestingly, the protective effect of ROE appears higher than that elicited by another natural antioxidant (tocopherol) commonly employed in cosmetic formulations. In conclusion, the present findings demonstrate that ROE affords excellent skin photoprotection, which is very likely a result of the antioxidant/radical scavenger activity of its active ingredients. Thus, ROE might have interesting applications in both anti-photoageing and after-sun cosmetic products.

Résumé

Les radiations ultraviolettes causent des dommages à la peau, qui se manifestent avec lésions cutanées précancéreuses et cancéreuses et avec une accélération du vieillissement cutané. L'administration topique des antioxydantes enzymatiques et non-enzymatiques c'est une stratégie efficace pour protéger la peau du dommage

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oxydative UV-médiat. Nous avons donc conduit une étude systématique pour évaluer l'activité antioxydante *in vitro* et l'effet photoprotecteur d'un extrait standardisé d'orange rouge (ROE), les principaux composants actifs sont des anthocyanines, des acides hydroxycinnamiques, des flavonones et de l'acide ascorbique. En ce qui concerne les recherches *in vitro*, on a testé le ROE en suivant trois modèles: (1) la décoloration du radical stable 1,1-diphényl-2-picryl-hydrazyle (test DPPH); (2) la peroxydation provoquée par l'initiateur radical hydrosoluble 2,2'-azobis(2-amidinopropane) chlorure sur des vésicules unilamellaires obtenues en mélangeant de la dipalmitoyl-phosphatidylcholine et de l'acide linoléique (test LP-LUV); et (3) la peroxydation provoquée par les radiations UV sur les vésicules multilamellaires de phosphatidylcholine (test UV-IP). *In vivo* l'activité radical scavenger/antioxydant a été déterminée en vérifiant la capacité de la ROE, appliquée topiquement, de réduire l'érythème cutané UV-induit sur des volontaires sains. Nos résultats obtenus selon les tests DPPH, LP-LUV et UV-IP, ils ont démontré les grosses propriétés antioxydantes de l'extrait, il y a une claire relation entre la capacité scavenger de la ROE et son contenu en composants antioxydants; en particulier les résultats obtenus selon le test UV-IP garantissent une forte raison pour l'employer comme agent photoprotecteur. En ce qui concerne les expériences *in vivo*, le ROE protège du dommage photooxydative cutané s'il est appliqué aussitôt après exposition aux radiations UVB. C'est vraiment intéressant de remarquer que l'effet protecteur de la ROE est plus grand qu'un autre antioxydant naturel (tocophérol) qui est souvent employé dans les formulations cosmétiques. En conclusion, ces résultats démontrent que l'extrait d'orange rouge est capable d'exercer une excellente photoprotection cutanée, qui est probablement déterminée par l'activité radical scavenger/antioxydant des composants actifs, et il pourrait donc avoir des applications intéressantes dans les produits cosmétiques après-soleil et anti-âge de la peau.

Introduction

It is well established that ultraviolet radiation causes damage to the skin, which may result in both precancerous and cancerous skin lesions, and acceleration of skin ageing. The use of antioxidants to prevent UV-induced skin damage has recently been met with considerable interest. Topical administration of enzymatic and non-enzymatic antioxidants provides an excellent way of enriching the endogenous cutaneous protection system and therefore is an effective strategy for protecting the skin against UV-mediated oxidative damage [1–5].

Certain biological effects of many plant extracts may be ascribed to the presence of polyphenolic compounds, especially flavonoids and phenolcarboxylic acids. The opinion prevails that the broad biological profile of plant polyphenols is related to their capability to protect against the damaging action of free radicals. In fact, biophenols have been shown to interfere not only with the propagation reaction, but also with the formation of free radicals, either by chelating the transition metal or by inhibiting the enzymes involved in the initiation reaction [6].

Anthocyanins, the water soluble pigments responsible for the cyanic colour of fruits such as cranberry and strawberry, are a group of flavonoids whose exceptionally good antioxidant properties have been clearly demonstrated in several experimental models [6–10].

A substantial accumulation of flavones and hydroxycinnamic acids (apart from ascorbic acid) is typical of *Citrus sinensis* fruits (sweet oranges). In particular, red orange varieties (*Citrus sinensis* var. Moro, Tarocco, Sanguinello) are characterized by high levels of anthocyanins, together with a particularly large content of the antioxidant compounds mentioned before [11]. This antioxidant pool acts as a fruit protective system against extreme climatic fluctuations that occur in Sicily's Etna area, where these orange cultivars grow almost exclusively. Hence, a systematic study to evaluate the *in vitro* antioxidant activity and *in vivo* photoprotective effect of a standardized red orange extract (ROE) has

been undertaken. The main active ingredients are anthocyanins, hydroxycinnamic acids and flavanones. During *in vitro* experiments, the ROE was firstly tested in two models: (1) in homogenous solution and (2) in a membranous system, given that the concomitant use of different tests is needed to determine the actual effectiveness and suitability of a potential antioxidant. Then the protective effect of the ROE against UV-induced peroxidation of phosphatidylcholine (PC) multilamellar vesicles, as a model membrane, was evaluated. In fact, UV exposure has been shown to significantly increase malondialdehyde (MDA) production in PC liposomal membranes [12].

In the second part of the study, *in vivo* antioxidant/radical scavenger activity was assessed by determining the ability of ROE to reduce UVB-induced skin erythema in healthy human volunteers. This test is regarded as one of the most suitable models for studying *in vivo* skin damage after acute UV exposure [4,5]. With this aim a solution of ROE or of tocopherol (TOC) (used as a reference compound) was applied topically before and after skin exposure to UVB radiation and the subsequent skin erythema was monitored by reflectance spectrophotometry.

Materials and methods

Drugs used

The extract of red oranges (ROE) employed in this study was a kind gift of ICI Surfactants (Everberg, Belgium), and was obtained by a patented extraction process from oranges of three pigmented *Citrus sinensis* varieties (Moro, Sanguinello, Tarocco). The extract had the following composition: anthocyanins (cyanidin-3-glucoside) 3.1%, hydroxycinnamic acids (caffeic, cumaric, ferulic, sinapic acid) 2.07%, flavanone glycosides (naringin, hesperidin) 8.1% and ascorbic acid 5.0%. Cyanidin-3-glucoside was purchased from Extrasynthèse (Genay, France) and tocopherol from Sigma-Aldrich (Milan, Italy).

Quenching of DPPH (DPPH test)

The free radical-scavenging capacity of ROE was tested by its ability to bleach the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). The reaction mixture contained 86 μM DPPH and different concentrations of ROE or cyanidin-3-glucoside (dissolved in ethanol) in 3.5 ml of ethanol. After 10 min at room temperature, the absorbance was recorded at 517 nm.

Linoleate peroxidation in LA/DPPC LUVs (LP-LUV test)

The method consisted of the spectrophotometric determination of the peroxidation products (conjugated dienes, LOOH) that occur upon reaction between the water-soluble radical initiator 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) and mixed dipalmitoylphosphatidylcholine/linoleic acid (DPPC/LA, molar ratio 1:0.125) unilamellar vesicles (LUVs) [13]. ROE or cyanidin-3-glucoside were dissolved in saline solution and an aliquot (5 μl) was added to 1.2 ml of LUV suspension (21 mg DPPC/ml). The mixture was incubated for 20 min at 37°C in a shaking water bath. Then, AAPH was added to the suspension to obtain a final concentration of 10 μM . Oxidation was carried out at 37°C under air. At given time points (5–90 min) 120 μl aliquots of the reaction mixture were withdrawn and added to 1 ml methanol. The accumulation of LOOH formed from LA was evaluated by measuring the absorbance of the samples at 234 nm. The ratio of oxidation-

induced change in absorbance with and without antioxidant addition was used to calculate a percent inhibition of oxidation according to the following equations:

$$[\text{LOOH}]_{\text{contr}} = A_{90} - A_5/ET_{\text{sec}}$$

$$[\text{LOOH}]_{\text{antiox}} = A'_{90} - A'_5/ET_{\text{sec}}$$

$$\% \text{ inhibition} = ([\text{LOOH}]_{\text{contr}} - [\text{LOOH}]_{\text{antiox}}/[\text{LOOH}]_{\text{contr}}) \times 100$$

where A_{90} and A'_{90} = absorbance at 90 min, without and with antioxidant addition respectively; A_5 and A'_5 = absorbance at 5 min, without and with antioxidant addition respectively; E (molar extinction coefficient of LOOH) = $26\,100 \pm 400 \text{ M}^{-1} \text{ cm}^{-1}$; T_{sec} (time, in seconds) = 5100; $[\text{LOOH}]_{\text{contr}}$ and $[\text{LOOH}]_{\text{antiox}}$ = hydroperoxide concentration without and with antioxidant addition, respectively.

UV-induced peroxidation in liposomal membranes (UV-IP test)

The protective effect of ROE and cyanidin-3-glucoside against UV-induced peroxidation was evaluated on phosphatidylcholine (PC) multilamellar vesicles [14]. Briefly, 1.0 ml of liposome suspension (in a glass flask with a 3 cm² exposure surface area) was exposed to UV radiation from a 15 W Philips germicidal lamp (254 nm) for 1.5 h. Exposure was given at 10 cm from the lamp, at room temperature. Different concentrations of ROE or cyanidin-3-glucoside, dissolved in water, were added to the system. An equal volume (50 μl) of vehicle only was added to control tubes. Malondialdehyde (MDA) concentration in the mixture was measured by using a colorimetric assay kit (Calbiochem–Novabiochem Corp., La Jolla, CA, USA).

All *in vitro* experiments were carried out in triplicate and repeated at least three times. Results were expressed as percentage decrease with respect to control values, and mean scavenging (SC_{50}) or mean inhibitory concentrations (IC_{50}) were calculated by using the Litchfield & Wilcoxon test.

In vivo evaluation of the photoprotective effect

UVB induced skin erythema was monitored by using a reflectance visible spectrophotometer X-Rite model 968, having 0° illumination and 45° viewing angle, as reported previously [5]. The instrument was calibrated with a supplied white standard traceable to the National Bureau of Standard's perfect white diffuser. The spectrophotometer was connected to an IBM PS2 50 computer, which performed all colour calculations from the spectral data by means of the Spectostart program supplied with the instrument. Reflectance spectra were obtained over the wavelength range 400–700 nm using illuminant C and 2° standard observer.

In vivo experiments were performed on six healthy volunteers (both sexes) of skin types II and III with an average age of 31 ± 9 years. All the volunteers were fully informed of the nature of the study and the procedures involved, and they gave their written consent. The subjects did not suffer from any illness and were not receiving medication at the time of the study. They were rested for 15 min prior to the experiments, and room conditions were set at $22 \pm 2^\circ\text{C}$ and 40–50% relative humidity.

Skin erythema was induced by UVB irradiation using an ultraviolet lamp, model UVM-57 (UVP, San Gabriel, CA) which emitted in the range 290–320 nm with an output peak at 302 nm. The flux rate measured at the skin surface was 0.80 mW cm^{-2} . For each subject, the minimal erythema dose (MED) was determined firstly and then an irradiation dose corresponding to $2 \times \text{MED}$ was used throughout the study.

For each subject, six sites on the ventral surface of one forearm and four sites on the other were defined using a circular template (1 cm^2) and demarcated with permanent ink. Two freshly prepared solutions were used: (1) a saturated buffered ROE aqueous solution (pH 3.5); and (2) a saturated ROE solution in water/ethanol 50:50. For each subject two skin sites were left untreated but exposed to UVB radiation (control).

The protocol consisted of two series of experiments [5]. In the first series (pretreatment protocol), the test solutions were applied randomly for 3 h on the skin sites of one forearm using a Hill Top chamber (Hill Top Research Inc., Cincinnati, OH) in which a cotton pad was saturated with $150 \mu\text{l}$ of the formulation being tested. After 3 h, the chambers were removed, the skin surfaces were gently washed with water to remove the formulation and each pretreated site was exposed to UVB irradiation.

The second series of experiments was performed simultaneously on the other forearm of the same subjects. Skin sites were exposed to UVB irradiation and then each solution tested ($150 \mu\text{l}$) was immediately applied to the irradiated sites (using the same Hill Top chamber described above) for 6 h. After this period, the chambers and the formulations were removed. For both experimental protocols, UVB induced erythema was monitored for 72 h using the reflectance spectrophotometer described above. From the skin spectral data obtained, the erythema index (EI) was calculated using Equation 1, as given by Dawson *et al.* [15]:

$$\text{EI} = 100 \left[\log \frac{1}{R_{560}} + 1.5 \left(\log \frac{1}{R_{540}} + \text{Log} \frac{1}{R_{580}} \right) - 2 \left(\log \frac{1}{R_{510}} + \log \frac{1}{R_{610}} \right) \right] \quad (1)$$

where $1/R$ is the inverse reflectance at a specific wavelength (560, 540, 580, 510, 610 nm). EI baseline values were taken at each designated site before application of the formulations tested (pretreatment protocol) or before UVB irradiation (post-treatment protocol) and they were subtracted from the EI values obtained at each time point, to determine ΔEI values following UVB exposure. For each site, the area under the response (ΔEI)–time curve (AUC) was computed using the trapezoidal rule.

AUC values were inversely related to the ability of the formulations tested to inhibit UVB skin erythema. To better compare the efficacy of the different products tested the percentage inhibition of UVB skin erythema (PIE) was calculated from AUC values, using the following equation:

$$\text{Inhibition \% (PIE)} = \frac{\text{AUC}_C - \text{AUC}_T}{\text{AUC}_C} \times 100 \quad (2)$$

where AUC_C is the area under the response–time curve of sites that received no treatment (control); AUC_T is the area under the response–time curve of the sites treated with the solutions being tested. Statistical analysis of the results was carried out by using Student's *t*-test.

Results and discussion

One of the major targets for oxygen radicals is undoubtedly tissue macromolecules. There is evidence that antioxidants may be of benefit in preventing the deleterious consequences of oxidative stress [16,17]. Our results obtained in the DPPH and LP-LUV tests have demonstrated the strong antioxidant properties of ROE, with a clear relationship between ROE scavenger efficiency and its content in antioxidant compounds. In fact, in the DPPH test, the addition of ROE or cyanidin-3-glucoside showed a good concentration-dependent scavenging effect on DPPH radicals, allowing the calculation of the half-scavenging concentrations (SC_{50}) shown in Table 1. In the LP-LUV test, incubation of DPPC/LA LUVs in presence of AAPH induced a large increase in the accumulation of LOOH formed from LA peroxidation (Fig. 1). The addition of ROE or cyanidin-3-glucoside to the reaction mixture reduced the amount of LH formed in a concentration-dependent manner, as shown by half-inhibition concentration (IC_{50}) reported in Table I.

In the UV-IP test, ROE appeared able to efficiently protect PC within liposomal bilayers from UV-induced peroxidation. In fact, exposure of PC liposomes to UV radiation for 1.5 h caused a large increase in MDA production; the addition of ROE reduced the amount of formed MDA in a dose-dependent manner, allowing IC_{50} calculations (Table I). Since there is considerable evidence relating radical oxygen species with UV light-induced phospholipid degradation, the ROE protective effect should very likely be due to the scavenger activity of its active ingredients against hydroxyl and peroxy radicals and superoxide anions, so inhibiting propagation of lipid peroxidative chain reaction.

Taken together, besides clearly demonstrating the strong antioxidant/radical scavenger activity of ROE, the findings obtained in these three *in vitro* tests allow us to emphasize three points. Firstly, it is evident that ROE is more effective in the membranous system (LP-LUV test) than in homogenous solution (DPPH test); thus the antioxidant activity of ROE appears to be very likely dictated not only by structural features of its active principles, but also by their capability to interact with biomembranes. In AAPH-induced peroxidation of LUVs, the chain-initiating radical is generated in aqueous phase and chain-

Table I. Scavenger activity and antioxidant effect of ROE or cyanidin-3-glucoside (cyan-3-gl).

<i>Test</i>	<i>ROE</i> <i>as extract</i> in toto ($\mu\text{g/ml}$)	<i>ROE</i> <i>as cyan-3-gl</i> (μM)	Cyan-3-gl (μM)
DPPH			
SC_{50}	134.51	8.60	26.87
95% CL	118.19–153.07	7.55–9.78	24.35–29.65
LP-LUV			
IC_{50}	36.12	2.31	20.03
95% CL	31.85–40.85	2.03–2.61	15.61–25.70
UV-IP			
IC_{50}	883.41	56.49	429.41
95% CL	748.12–1043.25	47.84–66.71	386.28–477.35

SC, scavenger concentration, IC, inhibitory concentration, CL, confidence limits.

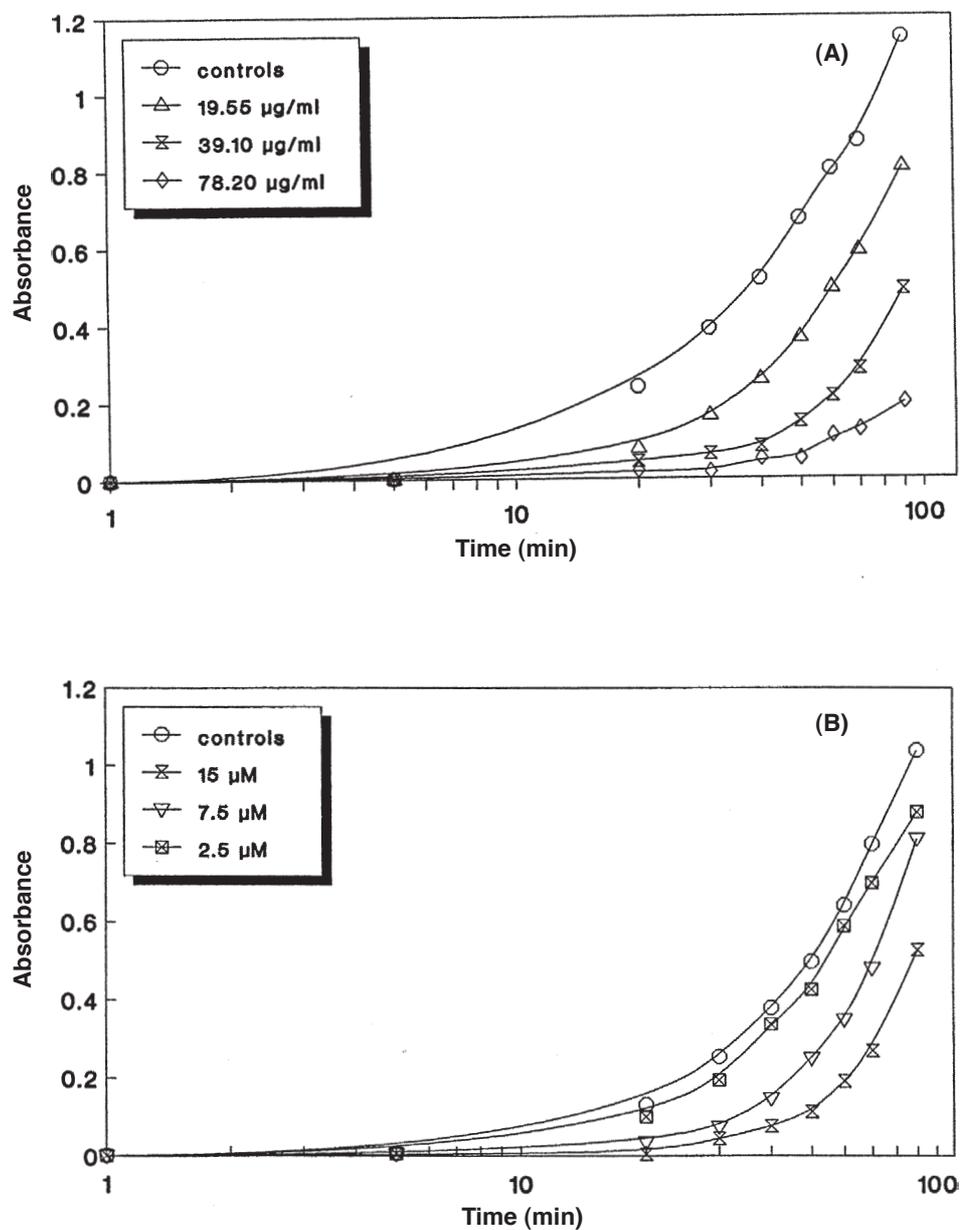


Figure 1. Antioxidant activity against LA peroxidation in DPPC/LA LUVs by increasing concentrations of ROE (A) or cyanidin-3-glucoside (B). Experiments were carried out as described in Materials and methods.

propagating lipid peroxy radicals are located within membranes. Scavenging of aqueous peroxy radicals at the surface of membranes, as well as scavenging of lipid peroxy

radicals within the membranes, seems to play a considerable part in antioxidant activity of lipophilic antioxidants. The data suggest that active ingredients of ROE can act as scavengers not only of aqueous peroxy radicals near the membrane surface but also of chain-propagating lipid peroxy radicals within the membranes.

Secondly, the half-effective concentrations of ROE calculated in the DPPH, LP-LUV and UV-P tests are lower than those measured for pure cyanidin-3-glucoside. Among the active compounds contained in red orange juices (anthocyanins, flavanone glycosides, cinnamic acid derivatives, ascorbic acid) this anthocyanin contributes mostly to their antioxidant activity [10,18]. We obtained similar results also when the other polyphenolic ingredients contained in ROE were used (data not shown). Thus, the antioxidant activity of ROE seems to be related to an overall synergistic effect of the active ingredients of the extract.

Thirdly, the findings obtained in the UV-IP test provide a strong rationale for using this extract as a photoprotective agent. *In vivo* and *in vitro* studies have shown that activated oxygen species and oxygen radicals are involved both in the inflammatory response elicited by acute UV skin exposure (skin erythema) [19] and in photoageing and carcinogenesis processes induced by chronic UV skin irradiation [20]. It has therefore been suggested that the evaluation of the photoprotective effect [21,22] against ultraviolet light-mediated cutaneous damage may provide a useful tool for assessing antioxidant activity of topically applied products. In our study the protective effect of ROE against UVB-induced skin erythema in human volunteers was assessed by using reflectance spectrophotometry to monitor the extent of erythema. Moreover, the activity of ROE was compared with that of TOC, an antioxidant compound used in cosmetic formulations. We used two different protocols for evaluating ROE and TOC ability in inhibiting UVB skin erythema: (1) skin sites were pretreated with ROE and TOC solutions and then, after removal of the solutions, they were exposed to UVB radiation; and (2) skin sites were irradiated with UVB, and then the same solutions used in the pretreatment protocol were applied.

Initially, in order to evaluate a possible effect of the vehicles used in our study, the influence of buffered aqueous solution (pH 3.5) and of aqueous alcohol solution (water/ethanol, 50:50) on UVB-induced skin erythema, was monitored in both the pretreatment and posttreatment protocols. However, we found no significant difference between vehicle treatments and controls (data not shown).

The time course of erythema for skin sites treated with ROE and TOC solutions before and after UVB irradiation is shown in Fig. 2. From ΔEI vs time plots, the area under the response (ΔEI)–time curve (AUC) was computed using the trapezoidal rule, and AUC values are reported in Table II. As may be noted, both TOC and ROE were unable to inhibit UVB-induced skin erythema using the pre-treatment protocol, because AUC values for skin sites treated with TOC and ROE solutions were not significantly different ($P > 0.05$) from those of the control (untreated sites).

In agreement with these findings, natural antioxidants, such as tocopherol, SOD and glutathione, have been shown to be unable to protect the skin against UVB erythema when topically applied before skin exposure to UV radiation [5]. Conversely, topical synthetic compounds lacking absorbance in the 290–400 nm range of the UV spectrum (unlike ROE active ingredients) are effective also when applied before UVB irradiation of the skin [4]. Several hypotheses may be put forward to explain our results. Firstly, rapid depletion of active natural compounds within the skin should be caused by UVB irradiation [23] or by quick metabolism within a viable epidermis or by reabsorption through microcirculation in

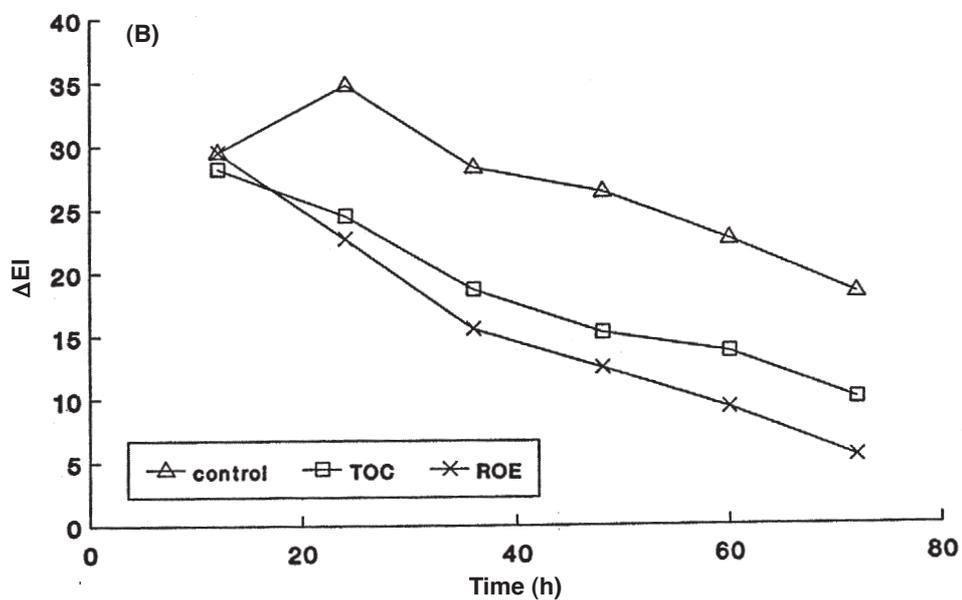
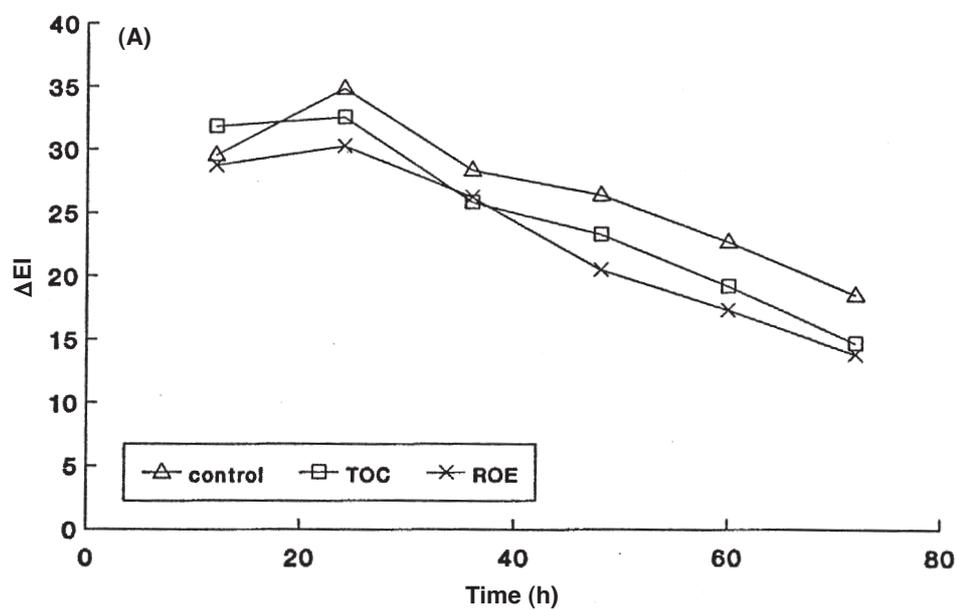


Figure 2. Typical trend of erythema index variation (ΔEI) vs time for one subject.. ROE or TOC solutions were applied to skin sites 3 h before (pre-treatment protocol) (A) or immediately after (post-treatment protocol) (B) exposure to UV-B radiation.

Table II. AUC values obtained after applying ROE or TOC solutions to the skin sites 3 h before (pre-treatment protocol) or immediately after (post-treatment protocol) exposure to UV-B radiation

Subject	Control	Pre-treatment		Post-treatment	
		TOC	ROE	TOC	ROE
A	1634.4	1488.6	1385.2	1097.4	930.6
B	1458.6	1257.6	1244.4	1084.2	793.2
C	1172.0	1086.6	1070.4	970.8	827.4
D	1533.6	1402.8	1299.6	1093.8	970.8
E	1176.6	1093.8	983.4	876.0	756.6
F	1170.5	1102.8	1050.6	826.2	776.4
Mean	1357.6	1238.7	1173.2	991.4*	842.5**
± SD	209.8	174.5	158.8	119.5	87.9
PIE (%)	–	–	–	26.9	37.9

* $P < 0.05$ ** $P < 0.01$.

the dermis. Secondly, a long occlusion period could increase skin hydration and therefore skin permeability to UV radiation, causing an inflammation process which overwhelms antioxidant photoprotective ability [24]. However, further studies are planned to investigate the influence of different experimental conditions (occlusive and non-occlusive conditions, time of occlusion, time interval from formulation removal and UVB exposure intensity) in the pretreatment protocol.

Since antioxidant topical activity is often evaluated applying the active compound after exposure to UVB, we also used a post-treatment protocol. As shown in Table II, both ROE and TOC protected the skin against UVB-induced erythema, with ROE being more efficient (PIE 37.9%) than TOC (PIE 26.9%).

These results clearly demonstrated the protective action of ROE against photo-oxidative skin damage and are in agreement with those observed in the *in vitro* UV-P test (showing that ROE is able to prevent UV radiation-induced lipid damage). Interestingly the ROE protective effort appears greater than that elicited by another natural antioxidant (TOC) commonly employed in cosmetic formulations.

In conclusion, the present findings demonstrate that ROE gives excellent skin photo-protection, very likely as a result of the antioxidant/radical scavenger activity of its active ingredients, and thus it might have interesting applications in both anti-photoageing and after-sun cosmetic products.

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