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In vitro and in vivo evaluation of caffeic and ferulic acids as topical photoprotective agents

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Abstract

Topically-applied antioxidant drugs represent a successful strategy for protecting the skin against UV-mediated oxidative damage. However, they can afford to the skin a satisfactory photoprotection only if able to permeate through the stratum corneum and thus to reach deeper cutaneous layers. Caffeic and ferulic acids, dissolved in saturated aqueous solutions at pH 3 or 7.2, have been tested for their capability to permeate through excised human skin mounted in Franz cells. At both pH values, ferulic and, at a lower degree, caffeic acids appeared able to permeate through the stratum corneum. The known higher lipophilicity of ferulic acid may explain the fact that it permeates through the stratum corneum better than caffeic acid. However, vehicle pH values proved to have no influence on biophenol skin permeation profile; this observed lack of pH effect may reflect the drug higher concentration attainable in saturated solutions at high pH. On the basis of the findings obtained in these in vitro experiments, we designed the schedule of a series of in vivo experiments, carried out to evaluate the ability of caffeic and ferulic acids to reduce, in healthy human volunteers, UVB-induced skin erythema, monitored by means of reflectance spectrophotometry. Caffeic and ferulic acids, dissolved in saturated aqueous solution pH 7.2, proved to afford a significant protection to the skin against UVB-induced erythema. To conclude, we have confirmed, by means of in vitro and in vivo experiments, that caffeic and ferulic acids may be successfully employed as topical protective agents against UV radiation-induced skin damage; however their skin absorption is not influenced by the pH of the formulation.

Keywords: Caffeic acid; Ferulic acid; Antioxidant; Skin permeation; Photooxidative damage

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1. Introduction

The skin is exposed to a broad variety of biological, chemical and physical attacks. Ultraviolet light, the most described physical attack, is well-known to cause skin damage, resulting in both precancerous and cancerous skin lesions and acceleration of skin ageing (Dalle Carbonare and Pathak, 1992; Darr and Fridovich, 1994). In fact, UVB and UVA radiation (the biologically relevant solar radiation) has been shown to significantly reach epidermal and dermal layers, where they can provoke damage and degradation of both cellular components (lipids, proteins and DNA) and noncellular elements (like collagen and elastin fibres).

Reactive oxygen species (ROS) are believed to be largely responsible for some of the deleterious effects of UV light upon skin (Fuchs and Packer, 1991). Particularly, prolonged skin exposure to UV light results in a severe decrease of its antioxidant content (Shindo et al., 1994; Podda et al., 1998); overproduction of nitric oxide from keratinocytes seems to play a major role in the integrated response leading to erythema production and inflammation process following UV radiation exposure (Deliconstantinos et al., 1996; Romero-Graillet et al., 1997).

Endogenous antioxidant capacity of the skin is a major determinant in its response to oxidative stress-mediated damage. In fact, because it provides an efficient way to enrich the endogenous cutaneous protection system, topical administration of antioxidants has recently proved to represent a successful strategy for protecting the skin against UV-mediated oxidative damage (Bonina and Montenegro, 1996; Weber et al., 1997; Saija et al., 1998; Saliou et al., 1999).

Caffeic and ferulic acids are two hydroxycinnamic acids largely present in plants and also in vegetable foods, such as olives and olive oil. Their biological properties and, especially, their antioxidant activity are well recognized (Castelluccio et al., 1995; Rice-Evans et al., 1996; Chen and Ho, 1997). In particular, ferulic acid is employed as photoprotective ingredient in many skin lotions and sunscreens. However, literature reports little evidence concerning the utility of topically-applied hydroxycinnamic acids to protect against photooxidative skin damage. We have demonstrated previously that caffeic and ferulic acids are able, in vitro, to efficiently protect phosphatidylcholine liposomes from UV radiation-induced peroxidation and to react with nitrogen oxides produced from sodium nitroprusside (Saija et al., 1999). Furthermore, ferulic acid was shown to be a strong UV absorber (Graf, 1992). These findings represent an interesting background supporting a potentially successful employment of caffeic and ferulic acids as topical protective agents against UV radiation-induced skin damage.

Given that UV radiation penetrates deeply into the skin, topically applied antioxidant drugs can afford to the skin a satisfactory photoprotection only if they are able to reach deeper cutaneous layers, permeating through the stratum corneum (the main obstacle against the penetration of exogenous substances through the skin).

Our previous studies showed that ferulic and caffeic acids, dissolved in buffer pH 3 (where they exist, almost exclusively, as non-ionised species), are able to permeate through the stratum corneum of excised human skin mounted in Franz cells (Saija et al., 1999). The aim of the present paper was to determine the pH influence on in vitro percutaneous absorption of caffeic and ferulic acids. Thus the permeation profiles through excised human skin of these two drugs were evaluated at different pH values (3 and 7.2).

On the basis of the findings obtained in these in vitro experiments, we designed the schedule of a series of in vivo experiments, carried out to evaluate the ability of caffeic and ferulic acids to reduce, in healthy human volunteers, UVB-induced skin erythema. In fact, UVB erythema, whose extent may be monitored by means of reflectance spectrophotometry, is regarded as one of the most suitable models for studying in vivo skin damage after acute UV exposure (Dawson et al., 1980; Andersen et al., 1991) and provides a useful tool to assess radical scavenger activity of topically-applied compounds (Saija et al., 1998).

2. Materials and methods

2.1. In vitro skin permeation experiments

2.1.1. Protocol

The experiments were carried out according to the method described previously (Bonina and Montenegro, 1994; Saija et al., 1998). Adult human skin samples (mean age 35 ± 15 years) were obtained from breast reduction operations. Subcutaneous fat was trimmed and the skin samples were immersed in distilled water at 60 ± 1°C for 2 min; then stratum corneum and epidermis (SCE) were peeled off, since the dermis in vitro can act as a significant additional barrier to the absorption of lipophilic drugs. The SCE samples were dried at room temperature in a desiccator maintained at $\sim 25\%$ RH. The dried samples were wrapped in aluminum foil and stored at 4 ± 1°C until use. Samples of dried SCE were rehydrated by immersion in distilled water at room temperature for 1 h before being mounted in Franz diffusion cells (LGA, Berkeley, CA). The exposed skin surface area was 0.75 cm^2 and the receptor volume was 4.7 ml. The receiving compartment contained ethanol/water solution (1:1, v/v) to ensure sink conditions. The receiving solution was stirred and thermostated at 35°C during all the experiments. Skin barrier integrity of the SCE samples used in this study was assessed by determining their tritiated water permeability coefficient (K_p). K_p values were found to be $1.6 \pm 0.3 \times 10^{-3}$ cm h^{-1}, and were consistent with those reported previously (Bonina and Montenegro, 1994).

At the beginning of the experiment, 200 μl of a saturated solution of ferulic acid and caffeic acid in buffer solution, pH 3 or 7.2, were applied to the skin surface. Each experiment was run in duplicate on three different skin donors. Samples of the receiving solution were withdrawn at different times during the experimental period (24 h); the sample volumes were replaced with the same amounts of fresh solution. All samples were analyzed for caffeic or ferulic acid contents by means of an HPLC with UV/visible detection, as reported below.

2.1.2. Calculations

In vitro percutaneous fluxes (μg cm^{-2} per h) of ferulic and caffeic acids were calculated by plotting time versus the cumulative amount of active compound permeated through the skin and dividing the slope of the linear portion of the curve (steady-state) by the area of the skin surface through which diffusion took place. Data were analysed statistically by using Student's t-test.

2.1.3. HPLC analysis

The HPLC apparatus consisted of a Varian 5000 system (Varian, Walnut Creek, CA) equipped with a 20 μl loop and a Polychrom 3060 UV/vis detector (Varian). Integration of the chromatographic peaks was achieved with a 4290 integrator (Varian). Chromatography was performed on a Hypersil ODS column (particle size: 5 μm; 25 cm × 4.0 mm I.D.; Perkin–Elmer, Norwalk, CT). The mobile phase was acetonitrile–water (19:81) containing 2% acetic acid for ferulic acid and acetonitrile–water (18:82) containing 2% acetic acid for caffeic acid. The flow-rate was set at 1.0 ml min^{-1}. Each sample was filtered prior to injection using a Millex HV13 filter (Waters-Millipore Corporation, Milford, MA) and an aliquot (20 μl) was injected into the HPLC apparatus. Detection was effected at 302 nm.

2.2. In vivo evaluation of the photoprotective effect

UVB induced skin erythema was monitored by means of a reflectance visible spectrophotometer X-Rite mod. 968, having 0° illumination and 45° viewing angle. 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 a personal computer, which performed all color calculations from the spectral data by means of the Spectrostart 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 6 healthy volunteers (both sexes) of skin types II and III, with a mean age of 31 ± 9 years. All the

volunteers were fully informed of the nature of the study and the procedures involved and gave their written consent. The subjects did not suffer from any ailment, were not on medication at the time of the study and were rested for 15 min prior to the experiments. Room conditions were set at 22 ± 2°C and 40–50% relative humidity.

Skin erythema was induced by UVB irradiation using an ultraviolet lamp mod. 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 preliminarily and an irradiation dose corresponding to the double of the MED was used throughout the study.

For each subject, six sites on the ventral surface of one forearm were defined using a circular template (1 cm^2) and demarcated with permanent ink. Freshly prepared saturated aqueous solutions, pH 7.2, of caffeic or ferulic acids were employed. Skin sites were exposed to UV-B irradiation and then 200 μl of caffeic or ferulic acid solution were immediately applied to irradiated sites for three hours using a Hill Top chamber (Hill Top Research Inc., Cincinnati, OH). For each subject two skin sites were left untreated but exposed to UVB radiation (control).

After this period, the chambers were removed, the skin surfaces were gently washed with water to remove the solutions; after which the 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 the following equation (Dawson et al., 1980):

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

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 UVB irradiation and 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:

$$\text{Inhibition } \% \text{ (PIE)} = \frac{\mathrm{AUC_{(C)}} - \mathrm{AUC_{(T)}}}{\mathrm{AUC_{(C)}}} \times 100$$

where $AUC_{(C)}$ is the area under the response–time curve of sites which 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 was performed by using Student's t-test.

2.3. Drugs

Caffeic acid, ferulic acid, acetonitrile, acetic acid and ethanol were purchased from Sigma–Aldrich srl (Milan, Italy).

3. Results and discussion

UV radiation penetrates deeply into the skin and topically applied drugs can afford a satisfactory photoprotection only if able to permeate through the stratum corneum.

In the first part of the present study, we monitored the skin permeation profile of caffeic and ferulic acids in vitro, to evaluate if it may be influenced by vehicle pH.

Typical trends of cumulative drug amounts permeated through excised human skin from different buffered solutions (pH 3 and 7.2) are shown in Fig. 1. Furthermore, Table 1 reports the drug fluxes at steady-state, calculated for both experimental conditions. As clearly evidenced by permeation profiles and flux values, our findings demonstrate that, at both pH values, ferulic and caffeic acids are able to permeate through the stratum corneum. However, ferulic acid possesses a better percutaneous absorption than caffeic acid.

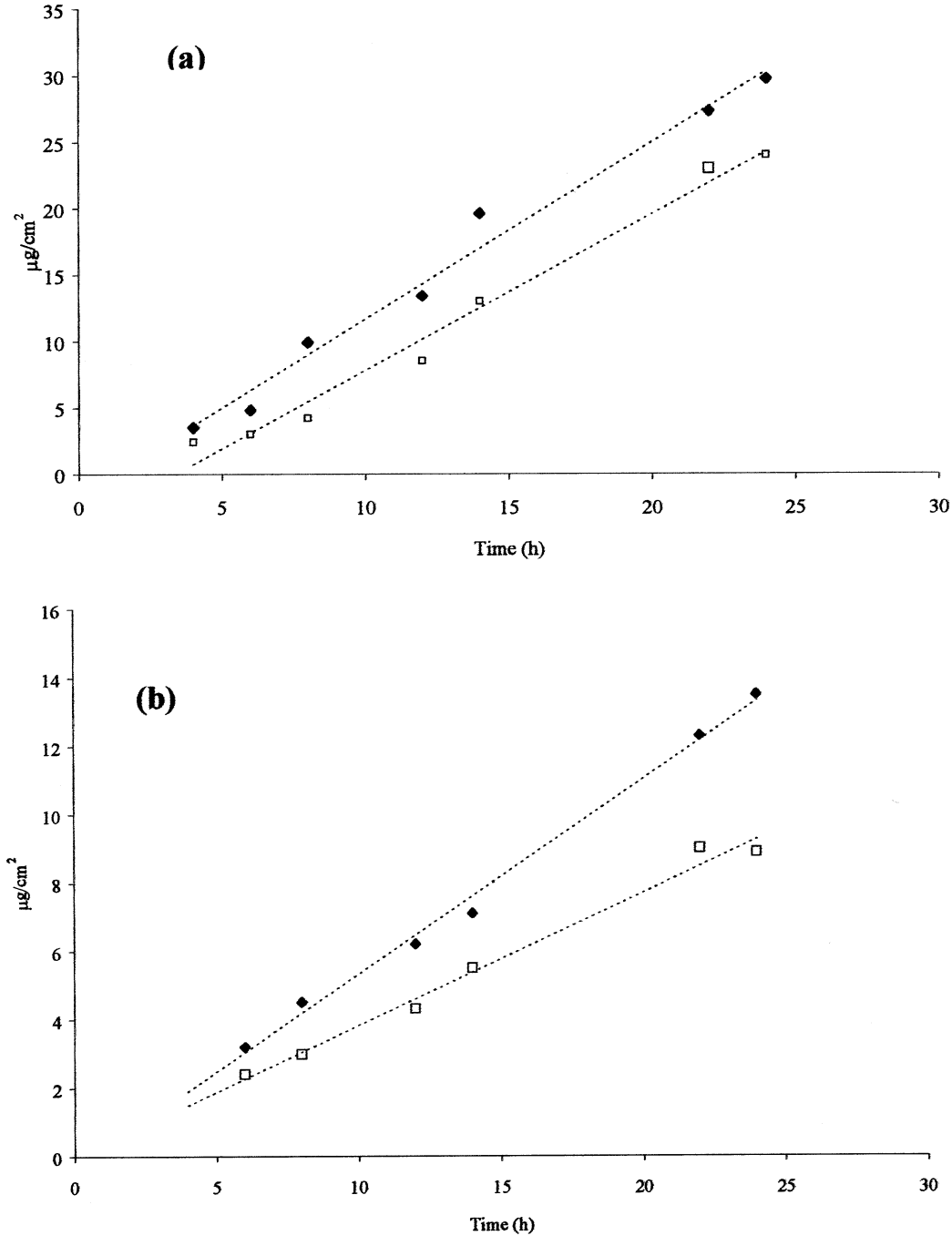

Fig. 1. Cumulative amounts (µg cm^{-2}) of caffeic acid (a) and ferulic acid (b) permeated through excised human skin from different buffered solutions (□, pH 3; ◆, pH 7.2).

Table 1
Values, evaluated at pH 7.2 and 3, of: (A) water solubility (mg ml^{-1}) of caffeic and ferulic acids; (B) flux (μg cm^{-2} per h) at steady-state of caffeic and ferulic acids permeated through excised human skin

Parameters	pH	Biophenols	
		Caffeic acid	Ferulic acid
(A) Solubility (mg ml^{-1})	7.2	6.51	6.63
	3	0.42	0.71
(B) Flux[a] (μg cm^{-2} per h)	7.2	0.56 ± 0.15	1.45 ± 0.32
	3	0.39 ± 0.12	1.18 ± 0.25

[a] Data are expressed as mean ± S.D. of three experiments at least.

Skin absorption of a compound is determined by its physicochemical properties; lipophilicity, in particular, plays an important role in the skin permeation process (Zatz, 1985). Thus, the known higher lipophilicity of ferulic acid may explain the fact that it permeates through the stratum corneum better than caffeic acid (Shahrzad and Bitsch, 1996).

Conversely, this concept does not justify the fact that at pH 7.2 and 3 caffeic and ferulic acids have a similar skin penetration, as shown by the calculated fluxes: when dissolved in water at pH 3, caffeic and ferulic acids exist, almost exclusively, as non-ionised, and thus more lipophilic, species.

Several papers concerning skin permeation of weak acidic drugs demonstrate that percutaneous absorption of these compounds is higher at lower pH values, since the skin seems to preferentially support a current of the molecule indissociate form (Hadgraft and Wotton, 1988; Lopez et al., 1996). However, conflicting findings about the effect of pH on skin permeability of weak acidic drugs are reported in literature. For example, no influence of vehicle pH on topical bioavailability and percutaneous absorption of indomethacin was found by De Vos et al. (1991); similarly Hughes et al. (1995) stated that pH does not affect in vitro percutaneous absorption of dimethylarsinic acid. In addition, an increase in in vitro indomethacin skin permeation at high pH was shown by Chien et al. (1988), probably due to the higher aqueous solubility of the drug.

The present findings point out that pH does not influence the skin penetration of the two biophenols tested. Because at higher pH both caffeic and ferulic acids are mainly in ionized form, the results suggest that also the corresponding anions are skin permeable. The lack of pH effect on skin permeation fluxes of caffeic and ferulic acids may reflect the higher concentration of these two drugs attainable in saturated solutions at high pH (see Table 1); this higher concentration of the tested compounds in the donor compartment at pH 7.2 should force more drug molecules to permeate through the skin, so balancing the concomitant decreased flux of the more lipophilic, non-ionised species.

Thus, on the basis of these in vitro findings, that show no difference in skin penetration of caffeic and ferulic acids at both vehicle pH values, the successive in vivo experimental phase was carried out to evaluate the photoprotective efficacy of caffeic and ferulic acids dissolved in buffered pH 7.2 solution. In fact, formulations with a neutral pH are generally more acceptable, for cosmetic use, in comparison with acidic formulations.

To assess the protective effect of caffeic and ferulic acids against UV-B-induced erythema, the extent of erythema in human volunteers was monitored by means of reflectance spectrophotometry. A brief methodological comment is needed before discussing the results. This reflectance method provides skin reflectance spectra, generally in the range of 400–700 nm, from which the values of different color space systems (CIELab, Lch, etc.) can be obtained using different CIE illuminants (C, D_{65}, D_{50}, A, etc.) and 2 or 10° illuminant observer. From spectral data it is possible to calculate, at different wavelengths, the relative reflectance or the logarithm of inverse reflectance (LIR), which indicates the absorption of skin chromophores (hemoglobin, melanin, etc.). The use of EI obtained from skin reflectance spectral values has been suggested for more accurate and reliable evaluations of skin erythema (Dawson et al., 1980; Andersen et al., 1991). Because skin erythema is due to increased hemoglobin content in skin vessels, EI values are calculated by sub-

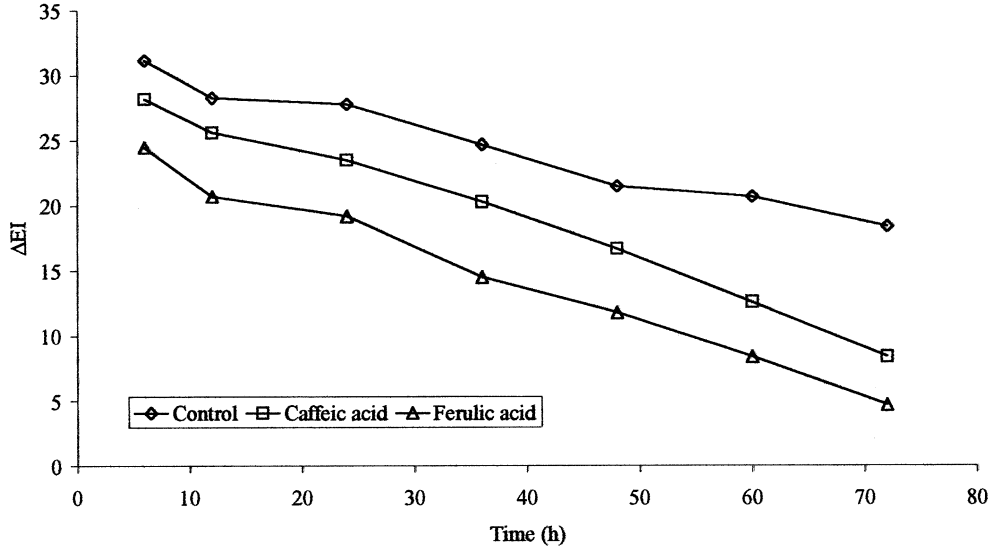

Fig. 2. Typical trend of erythema index variation (ΔEI) vs. time for one subject. Aqueous solutions were applied on skin sites after exposure to UV-B radiation and left for 3 h.

tracting LIR values at 510 and 610 nm (mainly due to melanin absorption) from the sum of hemoglobin LIR values at 540, 560 and 580 nm, which represent the wavelengths of hemoglobin absorption peak (Dawson et al., 1980).

The time course of erythema for skin sites treated with solutions of caffeic or ferulic acids after UVB irradiation is shown in Fig. 2. From ΔEI versus time plots, the area under the response–time curve (AUC_{0-72}) was computed using the trapezoidal rule. AUC_{0-72} values are reported in Table 2.

Caffeic acid and, at a higher degree, ferulic acid proved to afford a significant protection to the skin against UVB-induced erythema, since a significant difference was observed with controls. These hydroxycinnamic acids have been previously demonstrated to protect phospholipidic biomembranes from UV light-induced peroxidation (by inhibiting propagation of lipid peroxidative chain reaction) and to react with nitrogen oxides (Saija et al., 1999). Taking these data into account, one can speculate that the photoprotective effect of ferulic acid and caffeic acid proven in human volunteers is, very likely, correlated to their antioxidant/radical scavenging effectiveness.

PIE values of 26.31 and 47.85% were calculated, respectively, for caffeic and ferulic acids. The efficiency order of the two hydroxycinnamic acids tested (ferulic acid > caffeic acid) observed in in vivo experiments appears consistent with

Table 2
AUC_{0-72} values obtained, in healthy volunteers, treating, after skin exposure to UV-B radiation, the skin sites with saturated aqueous solution (pH 7.2) of caffeic or ferulic acids

Subject	AUC_{0-72}[a]		
	Control	Caffeic acid	Ferulic acid
A	1586	1231	924
B	1675	1044	824
C	1465	1192	850
D	1377	977	704
E	1450	1210	676
F	1390	934	686
Mean	1490	1098*	777**,***
S.D.	107	118	94
PIE[b]	–	26.31	47.85

[a] Each value represents the mean of two different sites in the same subject.

[b] Percentage inhibition of UVB skin erythema.

* $P<0.05$.

** $P<0.01$ compared with control.

*** $P<0.05$ compared with caffeic acid.

that obtained in vitro in skin permeation studies (see Table 1). Furthermore, a suggestive correlation might be hypothesized also between the better in vivo photoprotective activity of ferulic acid, in comparison with caffeic acid, and its higher effectiveness in scavenging nitric oxide (which is involved in the production of UV-induced skin erythema) (Saija et al., 1999).

In conclusion, we have confirmed, by means of in vitro and in vivo experiments, that caffeic and ferulic acids may be successfully employed as topical protective agents against UV radiation-induced skin damage; however their skin absorption is not influenced by the pH of the formulation.

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