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Flavonoids as potential protective agents against photo-oxidative skin damage

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Abstract

Flavonoids, a group of phenolic compounds widely occurring in the plant kingdom, have been reported to possess strong antioxidant activity. This preliminary study was designed to estimate the potential utility of topically applied flavonoids to prevent photooxidative stress in the skin. With this aim we have evaluated the protective effect of three flavonoids (quercetin, hesperetin and naringenin), chosen according to their structural characteristics, against UV radiation-induced peroxidation on phosphatidylcholine (PC) vesicles as a model membrane. Furthermore 'in vitro' human skin permeation of these flavonoids was measured, given that a suitable percutaneous absorption is an essential requirement for satisfactory topically applied photoprotective agents. The flavonoids tested in our study proved to protect efficiently PC liposomes from UV radiation-induced peroxidation, probably by scavenging oxygen free radicals generated by UV irradiations; their antilipoperoxidative activity can be classified as follows: quercetin > hesperetin > naringenin. In addition, naringenin, hesperetin and, at a very lower degree, quercetin were able to permeate through the stratum corneum (which is the main barrier against the penetration of exogenous substances through the skin) and, so, to penetrate into deeper skin layers. Taken together, these findings suggest that topically applied flavonoids could be excellent candidates for successful employment as protective agents in certain skin diseases caused, initiated or exacerbated by sunlight irradiation. Copyright © 1996 Elsevier Science B.V.

Keywords: Flavonoids; Skin; UV radiations

1. Introduction

Flavonoids are a group of polyphenolic compounds broadly distributed as secondary metabo-

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lites in the plant kingdom and represent one of the most important and interesting classes of biologically active compounds. As evidenced in the most recent literature, flavonoids have been reviewed for their wide biological properties, including hepatoprotective, antithrombotic, antibacterial, antiviral, anticancer and immunostimulant activities (Middleton and Kandswami, 1986). The capability to interact with protein phosphorylation and the antioxidant, iron chelating and free-radical scavenging activity may account for the wide pharmacological profile of flavonoids (Morel et al., 1993; Saija et al., 1995a; van Acker et al., 1996). Interestingly, flavonoid intake has been recently demonstrated to exert beneficial effects on some human diseases involving uncontrolled lipid oxidation (Hertog et al., 1995).

Furthermore, flavonoids are known to possess good anti-inflammatory activity both in humans and animals (Middleton and Kandswami, 1986), and recently their topical application has met with considerable interest (Hörmann and Korting, 1994). For example, a rutin derivative prevents acute hind limb limphedema in rats (Casley-Smith et al., 1993), hamamelis distillate suppresses human UV irradiation-induced erythema (Korting et al., 1993) and various flavonoids show good inhibitory activity against croton oil-induced mouse ear or paw edema (Della Loggia et al., 1986; Kim et al., 1993a).

Oxygen radicals are responsible for some of the deleterious effects of UV light upon skin (Tyrrell, 1991; Fuchs and Packar, 1991). Orally and topically administered natural antioxidants, such as carotenoids, ferulic acid and SOD, provide protection against UV irradiation-induced erythema and cytotoxicity (Graf, 1992; Fuchs and Packar, 1991; Montenegro et al., 1995). Thus topical administration of flavonoids might be efficacious in the prevention of UV radiation-induced skin damage.

The present study was designed to estimate the potential utility of topically applied flavonoids to prevent photooxidative stress in the skin. With this aim, we evaluated the protective effect of three flavonoids (quercetin, hesperetin and naringenin) against UV radiation-induced peroxidation on phosphatidylcholine (PC) vesicles as a model membrane. Furthermore 'in vitro' human skin permeation of these flavonoids was measured, given that a suitable percutaneous absorption is an essential requirement for successful photoprotective agents.

2. Materials and methods

2.1. UV radiation-induced peroxidation in liposomal membranes

The protective effect of quercetin, hesperetin and naringenin (chosen according their structural features) against UV-induced peroxidation was evaluated on PC liposomes. In fact, UV exposure has been demonstrated to significantly increase malondialdehyde (MDA) production in PC liposomal membranes (Mandal and Chatterji, 1980).

Liposomes were prepared according to the method described by Perrett et al. (1991). Briefly, 100 mg of PC, dissolved in chloroform, were transferred to a small stoppered tube. The lipid was thoroughly dried under nitrogen. It was then dissolved in warm ethanol (80 mg) and 25 mM Tris-HCl, pH 7.4 (200 mg) was added to yield a (100:80:200 w/w/w) lipid:ethanol:water mixture. This mixture was heated to 60°C for a few minutes and then allowed to cool to room temperature (20°C) yielding a proliposome mixture. The proliposome mixture was finally converted to a liposome suspension by the dropwise addition of 25 mM Tris-HCl, pH 7.4, to a final volume of 10 ml. The suspension was vortex-mixed throughout this last stage.

Usually 1.0 ml of liposome suspension (in a glass flask with a 3 cm^2 exposure surface area) was exposed to UV-radiation from a 15 W Philips germicidal lamp (254 nm) for 1.5 h. Exposure was given 10 cm from the lamp, at room temperature (De and Ghosh, 1993). Five concentrations of each flavonoid tested, dissolved in ethanol (EtOH), were added to the system; an equal volume (50 μl) of only EtOH

was added to control tubes. Then the reaction was halted by the addition of 200 μl of 35% $HClO_4$ and MDA concentration in the supernatant was measured (Saija et al., 1995a). One ml of the supernatant was heated with 0.5 ml of 0.5% thiobarbituric acid (50% acetic acid solution) at 90°C for 60 min. The samples were filtered on a Millex-GS filter (0.22 μm) and the absorbance of the mixture was read at 532 nm. All experiments were carried out in duplicate and repeated at least three times. Results were expressed as percentage decrease with respect to control values and mean inhibitory concentrations (IC_{50}) were calculated by using the Litchfield and Wilcoxon test.

Furthermore, an aqueous solution of quercetin (6.24 μM) was exposed to UV radiation under the same experimental conditions described above and the spectra were recorded at different times (0.5, 1 and 1.5 h) from the beginning of the incubation.

2.2. 'in vitro' skin permeation experiments

The experiments were carried out according to the previously described method (Bonina and Montenegro, 1992). Briefly, adult human skin samples (mean age 33 ± 6 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 (Bronaugh and Stewart, 1984). The SCE samples were dried at room temperature in a desiccator maintained at approximately 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. 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), which was stirred and thermostatted 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 $1.5 \pm 0.3 \times 10^{-3}$ cm/h and were consistent with those previously reported (Bonina and Montenegro, 1992; Bronaugh et al., 1986; Scott et al., 1986).

To evaluate flavonoid 'in vitro' skin permeation, quercetin, naringenin and hesperetin were dissolved in acetone (4 mg/ml) and 50 μl were applied to the skin surface; after which the solvent was allowed to evaporate. We dissolved flavonoids in acetone due to their poor solubility in aqueous medium; however, acetone has been demonstrated not to affect human skin permeability (Bond and Barry, 1988). Each experiment was run in duplicate on three different skin donors. After 24 h all the receiving solution was withdrawn and analyzed for flavonoid content by means of a HPLC with UV/visible detection, to calculate drug cumulative amounts permeated through the skin during 24 h (Q_{24}).

The HPLC apparatus consisted of a Varian 5000 system (Varian, Walnut Creek, CA, USA) 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 Pecosphere HS-5 HC ODS column (particle size: 10 μm; 15 cm × 4.6 mm I.D.; Perkin-Elmer, Norwalk, Connecticut, USA). The mobile phase was methanol–water (45:55) containing 1% acetic acid. The flow-rate was set at 1.0 ml/min. Each sample was filtered prior to injection using a Millex HV13 filter (Waters-Millipore Corporation, Milford, MA, USA) and an aliquot (20 μl) was injected into the HPLC apparatus. Detection was effected at 254 nm (quercetin), 282 nm (hesperetin) or 287 nm (naringenin).

2.3. Log capacity factor (log K')

Reverse-phase chromatographic retention times can be used to estimate oil/water partition coefficients; a good correlation is found between log octanol/water partition coefficients and log K' using octadecyl silica columns (Saija et al., 1995a).

Log K' values for flavonoids were determined by HPLC with UV/visible detection, as previously

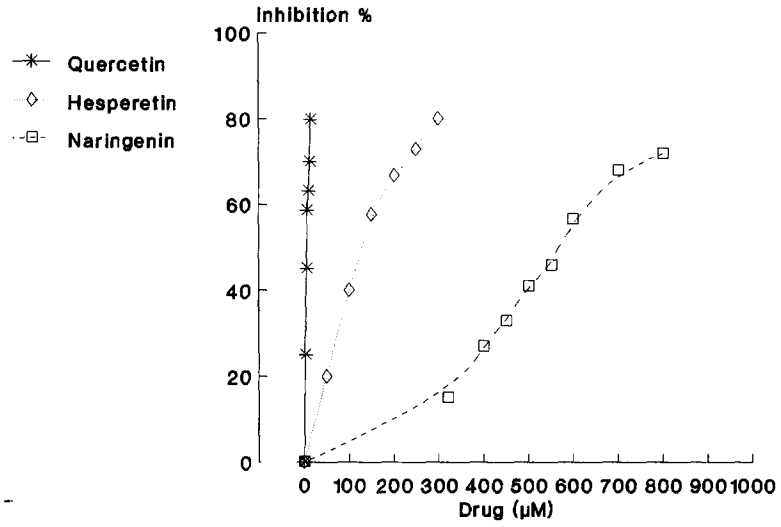

Fig. 1. Inhibition of UV radiation-induced peroxidation on phosphatidylcholine liposomes by increasing concentrations of quercetin, hesperetin and naringenin. Experiments were carried out as described under Materials and Methods.

described. Each flavonoid was dissolved in 100 ml of absolute methanol to give a final concentration of 10 μg/ml; samples were filtered prior to injection using a Millex HV13 filter (Waters-Millipore) and an aliquot (20 μl) was analyzed by HPLC. Detection was effected at 220 nm (non-retained solvent peak), 254 nm (quercetin), 282 nm (hesperetin) or 287 nm (naringenin).

Log K' values were calculated from the following relationship:

$$\log K = \log \frac{T_r - T_o}{T_o}$$

where T_r is the retention time of the flavonoid peak and T_o denotes the retention time of the non-retained solvent peak.

2.4. Drugs

Quercetin dihydrate, hesperetin and naringenin (analytical grade) were purchased from Extrasynthèse (Genay, France), phosphatidylcholine, thiobarbituric acid, perchloric acid, TRIS-HCl, ethanol, methanol, chloroform, dimethylsulphoxide, acetone and acetic acid from Sigma-Aldrich S.r.l. (Milan, Italy).

3. Results and discussion

Flavonoids tested in our study proved to protect efficiently PC within liposomal bilayers from UV-induced peroxidation. In fact, exposition of PC liposomes to UV radiation for 1.5 h elicited a large increase in MDA production; the addition of quercetin, hesperetin or naringenin reduced the amount of formed MDA in a dose-dependent manner which allowed IC_{50} calculation (Fig. 1). The effectiveness of the flavonoids tested could be classified as follows: quercetin > hesperetin > naringenin (Table 1).

Consistently with these findings, a standardized *Gingko biloba* extract (a complex mixture composed of terpenes and flavonoids, the latter being

Table 1
Protective effect of quercetin, hesperetin and naringenin against UV radiation-induced peroxidation on phosphatidylcholine liposomes (experiments were carried out as described under Materials and Methods)

Flavonoids	IC_{50} (μM)	95% Confidence limits
Quercetin	6.24	5.31–7.33
Hesperetin	122.85	104.90–143.85
Naringenin	562.59	522.36–605.92

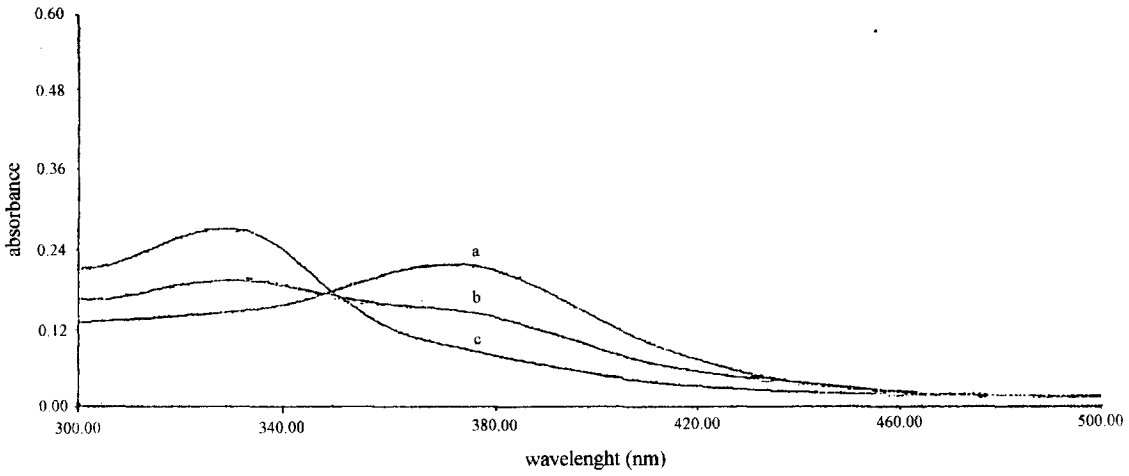

Fig. 2. Spectral changes of an aqueous solution of quercetin (6.24 μM), exposed to UV radiation (see the text for more details). The spectra were recorded at different times (a, 0.5 h; b, 1 h; c, 1.5 h) from the beginning of the incubation.

responsible for the antioxidant properties of this extract) was demonstrated able to protect microsomal fatty acids and proteins against UV-irradiation peroxidative degradation (Dumont et al., 1992). Since there is considerable evidence relating radical oxygen species with UV light-induced phospholipid degradation, the observed protective effect of flavonoids is very likely due to their well-known scavenger activity against hydroxyl and peroxyl radicals and superoxide anions. Quercetin, which showed the strongest effectiveness in our study, is reported to possess the highest antiradical property; this predominance was attributed to its structural characteristics (a cathecol moiety, a C_2–C_3 double bond and a 3-OH group). Furthermore, our findings confirm the favorable antioxidant activity of flavonoids carrying only one OH in ring B, such as hesperetin (a 3′OH,4′OMe compound) and naringenin (a 4′OH compound).

When the spectra of an aqueous solution of quercetin exposed to UV radiation were recorded at different times during a 1.5 h experimental period, an excellent isosbestic point was obtained (see Fig. 2). Similar changes were present also in the spectra of hesperetin and naringenin (data not shown). These spectral modifications indicate the conversion of quercetin under another form, which might be (due to the high degree of conjugated unsaturation of the drug) a stable UV-generated radical. This UV absorption has been demonstrated to inhibit other free radical reactions (Fenton et al., 1978). Thus, besides scavenging UV-induced radicals and so inhibiting propagation of lipid peroxidative chain reactions, flavonoids might provide their protective effect against UV radiation by acting as strong UV-absorbing screens.

Wavelengths in the UV region are believed to be largely responsible for the most damaging skin effects of sunlight; in fact UV light exposure of the skin gives rise to the formation of active oxygen intermediates (Mefferth et al., 1976; Fuchs and Packar, 1991) and products of lipid peroxidation are elevated in chronically sun-exposed human skin (Mefferth et al., 1976). Furthermore, several natural antioxidants are known to provide protection against UV irradiation-induced cytotoxicity (Graf, 1992; Fuchs and Packar, 1991; Montenegro et al., 1995). Thus one tempting speculation is that the flavonoids tested in our experiments might be successfully employed to prevent UV light-induced skin damage.

UV radiation penetrates deeply into the skin (Tyrrell, 1991); thus, after topical application, antioxidant drugs can afford to the skin a satisfactory photoprotection only if they are able to permeate through the stratum corneum and, thus,

to reach deeper skin layers. The permeability barrier of the skin is localized in the stratum corneum, which is viewed as the main obstacle against the penetration of exogenous substances through the skin (Zatz, 1985). As clearly shown by drug Q_{24} values (Table 2), our findings show that hesperetin, naringenin and (at a much lower degree) quercetin are able to permeate through the stratum corneum. Similarly, Merfort et al. (1994) demonstrated, by means of an 'in vivo' human skin penetration study, that, after cutaneous application, the flavonoids apigenin, luteolin and apigenin 7-*O*-β-glucoside penetrate into deeper skin layers. However, since naringenin and hesperetin have proved to have, in our study, a significantly higher percutaneous absorption than quercetin (see Table 2), they should be better candidates for successful employment as protective agents against UV radiation-induced skin damage.

Multilamellar vesicles of dipalmitoylphosphatidylcholine (DPPC) have been used as a simple model representing the lipid bilayers of the stratum corneum (Rolland et al., 1991). Since analysis of the phase transition of phospholipid vesicles by differential scanning calorimetry (DSC) is an excellent tool for evaluating the capability of a drug to penetrate lipid bilayers (Rolland et al., 1991; Blume et al., 1993; Kim et al., 1993b; Saija et al., 1995b), it may help to predict a similar effect at stratum corneum lipids. Previously we investigated, by DSC, flavonoid interactions with DPPC liposomes (Saija et al., 1995b); naringenin showed an interaction with DPPC bilayers similar to that of hesperetin, but significantly deeper than that of quercetin. These data are consistent with those obtained in the present 'in vitro' percutaneous absorption study and confirm the good capability of flavonoids to interact with human stratum corneum lipids.

Table 2
Cumulative amounts permeated after 24 h through excised human skin (Q_{24}), percentage of the dose absorbed (%D) and log K' values of quercetin, naringenin and hesperetin (data are expressed as mean ± S.D. of three experiments

Flavonoids	Q_{24} (μg cm^{-2})	%D	log K'
Quercetin	1.82 ± 0.34	0.91 ± 0.15	0.510
Naringenin	16.20 ± 3.65	8.13 ± 1.70	0.458
Hesperetin	20.57 ± 3.46	10.28 ± 1.96	0.530

Although lipid-soluble substances are usually considered to penetrate the stratum corneum fairly rapidly, lipophilicity does not appear, in the present study, to be the key parameter in determining flavonoid skin permeation. In fact, no relationship was observed between skin permeation values and liposolubility of the drugs tested, since the log K' values calculated for naringenin, quercetin and hesperetin were respectively 0.458, 0.510 and 0.530 (Table 2). However, skin absorption of a drug is determined not only by its partition coefficient, but also by other physicochemical properties, including water solubility, molecular size and diffusivity (Zatz, 1985). For example, the absolute water-insolubility of quercetin might justify its poor capability to permeate through excised human skin.

Flavonoids possess an interesting antiinflammatory profile, related to their capability to interfere with a variety of processes involved in mediator release (such as the arachidonic acid metabolism, the histamine release from mast cells and basophils, Ca^{2+}-regulated events and the respiratory burst of neutrophils) (Kahl, 1991). These flavonoid properties might contribute to their possible protective effect against UV light-induced skin inflammation, given that lipid peroxidation products exert acute inflammatory effects in mammalian skin (Fuchs and Packar, 1991; Ohsawa et al., 1984). Besides, flavonoids are claimed to be free of toxicity and side effects and, particularly, harmless to the skin (Jager et al., 1988; Benezra, 1988); this would be an additional advantage in the possible employment of flavonoids as skin photoprotective agents.

In conclusion, our findings demonstrate that flavonoids are able to prevent UV radiation-induced lipoperoxidation, probably by scavenging oxygen free radicals generated by UV irradiation. Furthermore, one can suggest that, also by virtue of their capability to permeate through human skin, topically applied flavonoids (particularly naringenin and hesperetin) might afford excellent photoprotection to the skin. The present results

must be taken into consideration in further developments of these promising compounds, which could have important therapeutic applications in certain skin diseases caused, initiated or exacerbated by sunlight irradiation.

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