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In vitro evaluation of quercetin cutaneous absorption from topical formulations and its functional stability by antioxidant activity

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Abstract

Recently, it was demonstrated that two different formulations containing quercetin inhibit the UVB-induced cutaneous oxidative stress and inflammation. Therefore, in the present study it was evaluated the functional stability of those formulations by the antioxidant activity, the release of quercetin from the formulations, and its skin retention using modified Franz diffusion cells. Both formulations tested ((1) non-ionic emulsion with high lipid content and (2) anionic emulsion with low lipid content) remained functionally (hydrogen-donating ability) stable during 180 days. Furthermore, quercetin was released from both formulations as determined using nitrocellulose membrane. *In vitro* antioxidant activity of retained quercetin into the skin was observed for both formulations as detected by the inhibition of malondialdehyde formation. The effect of quercetin retention was time-dependent for formulation 1. Concluding, this study demonstrates that quercetin remains functionally stable in formulations, and measuring the antioxidant activity is an efficient approach to evaluate quercetin skin retention with minimal interference of the tissue products. Furthermore, the present results on skin retention explain the previous study on quercetin *in vivo* activities, and together, these data suggest that formulations containing quercetin may be used as topical active products to control UVB-mediated oxidative damage of the skin.

Keywords: Free radicals; Antioxidant; Flavonoid; Quercetin; Stability test; Cutaneous absorption

1. Introduction

Oxidative stress occurs in modern life as a result of excessive sun exposure and increased air pollution. Very few tissues in the body are subjected to the same elevated degree of oxidative stress as the skin. In fact, it is exposed to oxidative stress from both endogenous and exogenous sources (Kohen, 1999; Fuchs et al., 2001a). Topical administration of an antioxidant provides an efficient way of enriching the endogenous cutaneous protection system, and thus, may be a successful strategy for diminishing ultraviolet radiation-mediated oxidative damage of the skin (Saija et al., 1998; Röpke et al., 2002). Moreover, topical delivery of bioactive substances is certainly a powerful strategy to avoid possible systemic toxicity and, at the same time, to restrict therapeutic effects to specific tissues. Therefore, experimental systems to deliver an active agent to cutaneous or subcutaneous level may be of great interest as a therapeutic or a cosmetic approach for selective treatment and prevention of skin disorders (Bonina et al., 1995; Saija et al., 2000; Wagner et al., 2001).

Antioxidants from natural products provide novel possibilities for the treatment and prevention of oxidative stress-mediated skin diseases (Aquino et al., 2002). Some enzymes and secondary compounds of higher plants presented protective effects against oxidative damage by inhibiting or scavenging free radicals (Röpke et al., 2002). Researches have focused on the potential use of these drugs as free radical scavengers to prevent oxidative skin damage (Schoemaker et al., 1995; Mortimer,

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1997), and thus, their topical application has been of considerable interest (Saija et al., 1998).

In this context, flavonoids such as quercetin may delay oxidant injury and cell death by scavenging oxygen radicals, protecting against lipid peroxidation, and chelating metal ions (Formica and Regelson, 1995; Skaper et al., 1997; Inal and Kahraman, 2000; Pietta et al., 2003). Corroborating, it was recently demonstrated that formulations containing quercetin inhibit the UVB-induced cutaneous oxidative stress and inflammation (Casagrande et al., 2006).

Therefore, the evaluation of stability and percutaneous absorption of formulations with quercetin becomes a very important issue in the study of new pharmaceutical products that could be useful in the treatment of UVB-induced oxidative skin damage (Bonina et al., 1996; Wessels et al., 1997). In the present study it was demonstrated that different stable formulations vehicle quercetin, which is released from the formulations and retained in the skin. In addition to previous data (Casagrande et al., 2006), the present results explain and demonstrate the possible applicability of quercetin in topical formulations to prevent UVB-induced oxidative/inflammatory skin damages.

2. Materials and methods

2.1. Materials

Quercetin and DPPH• (2,2-diphenyl-1-picrylhydrazyl) were obtained from Acros (New Jersey, USA). Nitrocellulose membrane 0.45 μm, 47 mm, model HAWP04700, white HAWP from Millipore (Sao Paulo, SP, Brazil). Raw materials for formulations were obtained from Galena (Campinas, SP, Brazil) and are presented in Section 2.3. All other reagents used were of pharmaceutical grade or HPLC grade.

2.2. Hydrogen-donating ability by DPPH• assay

The DPPH• assay has been widely used to evaluate the ability of several free radical scavenger molecules (Dinis et al., 1994). Briefly, a stock solution of quercetin was prepared with the aid of propylene glycol (5000 μg/mL), and diluted five times in ethanol (1000 μg/mL). This ethanolic solution of quercetin was diluted 200, 50, 20, 10, 5 and 2 times in ethanol to obtain solutions containing 5, 20, 50, 100, 200 and 500 μg/mL, respectively. One milliliter of 100 mM acetate buffer (pH 5.5), 1 mL of ethanol, and 0.5 mL of 250 μM ethanolic solution of DPPH• were mixed, and 50 μL of each sample of the above prepared solutions were added, then after 10 min the absorbance was measured at 517 nm (Blois, 1958; Dinis et al., 1994). The positive control was prepared in the absence of the raw material, and it indicates the maximum odd electrons of DPPH•, which was considered 100% of free radicals in the solution to calculate the hydrogen-donating ability (%) of quercetin. The blank was prepared from the reaction mixture without DPPH• solution. All measurements were performed in triplicate.

2.3. Formulations

The non-ionic emulsion with high lipid content (formulation 1) was prepared with commercially available self-emulsifying wax 10.00% (Polawax®—cetostearyl alcohol and polyoxyethylene derived of a fatty acid ester of sorbitan 20E) and the anionic emulsion with low lipid content was prepared with Polawax® 2.00% (formulation 2) and the anionic hydrophilic colloid 0.18% (carboxypolymethylene, Carbopol® 940) was also added as a stabilizing agent and triethanolamine 0.20% as neutralized. Macadamia nut oil 2.50% and squalene 1.00% were added as emollients, and propylene glycol 6.00% as a moisturizer and solubilizer. The preservative used was a mixture of phenoxyethanol and parabens 0.40% (Phenova®). Deionized water qs 100.00% was used for the preparation of all formulations. Quercetin (1.00%) was solubilized in propylene glycol and then added to the formulations at room temperature. The control formulations did not contain the flavonoid. All formulations were allowed to equilibrate for 24 h prior to use in the study.

2.4. Accelerated functional stability of quercetin

The stability test was performed according to the World Health Organization. Firstly, the possible interferences of the formulations constituents were evaluated using the DPPH• assay (see Section 2.2). The raw material was diluted in propylene glycol (5000 μg/mL). This solution was diluted 100-fold in ethanol, to obtain quercetin concentration of 50 μg/mL of which 50 μL were utilized for the reaction (1 μg/mL). Formulations with and without quercetin were diluted in ethanol to obtain the same concentration (quercetin 1 μg/mL) used for the analysis of the raw material in the reaction medium. The following controls were included in the test: (i) one positive control was prepared in the absence of the raw material, and (ii) another by adding the formulations without quercetin. The positive control indicates the maximum odd electrons of DPPH•, which was considered 100% of free radicals in the solution to calculate the hydrogen-donating ability (%) of quercetin. The blank was prepared from the reaction mixture without DPPH• solution.

After, the raw material (quercetin) and the formulations with or without quercetin were stored at 4 °C, RT (room temperature), and 40 °C ±70% RH (relative humidity) for 180 days (6 months) (Singh, 1999). At pre-determined times (initial, 24 h, 30, 60, 90 and 180 days) samples were collected for the evaluation of the functional stability measuring the antioxidant activity by DPPH• assay as described above. One positive control for each storage condition was added. All measurements were performed in triplicate.

2.5. HPLC analysis

The HPLC analyses were used to further investigate the cutaneous absorption of quercetin, to identify a suitable receptor medium for quercetin, its release from formulations, permeation and retention in the skin. The HPLC apparatus consisted of a Shimadzu LC-10AT (Shimadzu Corporation, Tokyo, Japan) equipped with a 20 μL loop and SPD-10A UV/VIS detector

(Shimadzu). Integration of the chromatographic peaks was achieved with C-R6A Chromatopac integrator (Shimadzu). Chromatography was performed on a Hypersil® BDS-CPS column (particle size: 5 μm; 250 mm × 4.6 mm i.d.; Thermo Quest-Hypersil Division, Runcorn, UK) with methanol:2% of acetic acid water solution (35:65; v/v) mobile phase and flow rate. Each sample was filtered prior to injection using a Millex LCR filter (Millipore Corporation, Sao Paulo, SP, Brazil) and an aliquot (20 μL) was injected into the HPLC apparatus with detection at 254 nm (Saija et al., 1998).

2.6. Quercetin solubility in different receptor media

In order to identify a suitable receptor medium for *in vitro* experiments, the saturation solubility of the drug in different media was analyzed. Three media were tested: (i) 0.1 M phosphate buffer (pH 7.6), (ii) 0.1 M phosphate buffer (pH 7.6) plus 0.5% ethanol and (iii) 0.1 M Phosphate buffer (pH 7.6) plus 0.5% of polyoxyethylene (20) sorbitan monolaurate (Tween 20). An excess of quercetin was added to the media at 37 °C and stirred for 12 h. After filtering (Millex LCR filter 0.45 μm), the quercetin content was analysed by HPLC (Saija et al., 1998; Valenta and Janisch, 2003).

2.7. In vitro release studies

Quercetin release rates from the different formulations were measured through 0.45 μm nitrocellulose membranes using modified Franz diffusion cells with a diffusional area of 1.77 cm^2. Nitrocellulose membrane was sandwiched between the upper donor compartment and the lower receptor compartment; 1 g of the formulation containing 1% of the drug was placed on the membrane surface in the donor compartment while the receptor compartment was filled with 10 mL of receptor medium containing 0.1 M phosphate buffer (pH 7.6) with tween 20 (0.5%), which was in contact with the membrane. During the experiments, the receptor solution was continuously stirred at 100 rpm and kept at 37 ± 1 °C. At designated time intervals (3, 6, 9, 10 and 12 h) the receptor medium was removed, the amount of released quercetin was analysed by HPLC (see Section 2.5) and its diffusion coefficient (*D*) was calculated using the Higuchi equation (Higuchi, 1962; Larrucea et al., 2001). All measurements were performed in quintuplicate and formulations without quercetin were used as control.

2.8. Preparation of pig ear skin

In vivo studies in humans provide the most direct, relevant and therefore conclusive information on skin permeation. Nevertheless, the advantages of *in vitro* experiments are lower cost, and the ability to test large numbers of formulations in relatively short time. Moreover, *in vitro* data can also be used to identify the rate-limiting skin layer for a given compound (Zatz, 1993). In this regard, pig ear skin is considered an excellent skin model, because the histological characteristics of pig and human skins have been reported to be very similar in terms of epidermal thickness and composition, pelage density, epidermal lipid biochemistry and general morphology (Bhatia and Singh, 1996). Therefore, we used this pig ear skin model for *in vitro* permeation studies. Pig ears were obtained within 2 h after slaughter of the animals. The whole skin membrane was then carefully removed from the underlying cartilage with the help of a scalpel. The subcutaneous tissues were removed and the skin was stored at −4 °C for a maximum period of 30 days before use (Lopez et al., 2003).

2.9. In vitro permeation studies

The skin was mounted on modified Franz diffusion cell with the dermis facing the receptor compartment, and 1 g of the formulation containing 1% of the drug was placed in the donor compartment. The experiments were carried out under the same conditions described in the release studies (see Section 2.7). At designated time points (3, 6, 9 and 12 h) the receptor medium was removed and the amount of quercetin was analysed by HPLC (see Section 2.5) and antilipoperoxidative activity (see Section 2.11) (Bhatia and Singh, 1996; Bentley et al., 1999). All measurements were performed in quintuplicate and formulations without quercetin were used as control.

2.10. In vitro skin retention studies

After each time point (3, 6, 9 and 12 h) evaluated as described above in the permeation studies, the skin was removed from the diffusion cell, cleaned with cotton soaked in methanol, and homogenized in methanol. The solvent was evaporated with compressed air, and the precipitate was suspended in medium I containing 125 mM sucrose, 65 mM KCl and 10 mM Tris–HCl (pH 7.4). The obtained supernatant was used for the determination of the antilipoperoxidative activity (see Section 2.11) and analysed by HPLC (see Section 2.5). All measurements were performed in quintuplicate and the formulations without quercetin were used as control.

2.11. Fe^{2+}/citrate-mediated lipid peroxidation assay

For the skin retention study, the antilipoperoxidative activity of the samples obtained as described above (see Section 2.10) was estimated by the formation of malondialdehyde (MDA) based on its description (Buege and Aust, 1978). For 1.0 mL of medium I, 50 μL of each sample of the above prepared solutions, mitochondria in order to yield a final concentration of 1 mg of protein, plus 50 μM $(NH_4)_2Fe(SO_4)_2$ and 2 mM sodium citrate were added, and kept for 30 min at 37 °C. To determine MDA formation, 1 mL of 1% thiobarbituric acid (TBA), prepared in 50 mM of NaOH, plus 0.1 mL of 10 M NaOH and 0.5 mL of 20% H_3PO_4, were further added, followed by a 20 min incubation at 85 °C. The MDA–TBA complex was extracted with 2 mL of *n*-butanol, centrifuged at 1660 × *g* for 10 min, and the supernatantes read at 535 nm (Ohkawa et al., 1979; Afanas'ev et al., 1989; Kowaltowski et al., 1996; Santos et al., 1998; Rodrigues et al., 2002). The following controls were included in the test: (i) absence of sample, (ii) a positive control using the sample obtained (see Section 2.10) of the skin in the presence of for-

mulations without quercetin and, (iii) a negative control in the absence of iron. The blank was prepared from the medium I without mitochondria. Thus, the positive control indicates the maximum MDA formation, which was considered 100% of peroxidation to calculate the inhibition of lipid peroxidation by the quercetin retained in the skin. All measurements were performed in triplicate.

2.12. Statistical analysis

The concentration of quercetin that caused 50% of hydrogen-donation was considered the IC_{50}, which was determined using the GraphPad Prism® software. Data were statistically analyzed by one-way ANOVA, followed by Bonferroni's multiple comparisons *t*-test for evaluation of the formulations influence in the hydrogen-donating ability assay and the study of percutaneous absorption. Results were presented as mean ± S.E.M. (standard error mean) and considered significantly different when $P < 0.05$ was obtained.

3. Results

3.1. Hydrogen-donating ability by DPPH• assay

A recently described method for free radical scavenging was utilized. The reduction of DPPH• is monitored by decrease of the absorbance of its radical at 517 nm (Parejo et al., 2000). Firstly, it was investigated whether the DPPH• assay was effective to determine quercetin functional activity. Different concentrations of quercetin were used to determine the most adequate concentration to evaluate the functional stability of formulations containing quercetin. Quercetin presented concentration-dependent hydrogen-donating ability as shown in Fig. 1. These results allowed to calculated the IC_{50} (1.17 μg/mL). The maximum percentage of hydrogen-donating ability (89.0%) was obtained using 4 μg/mL of quercetin; at a higher concentration a plateau effect was observed (10 μg/mL).

3.2. Accelerated functional stability of quercetin

Before evaluating the functional stability of quercetin in formulations, possible interferences of the formulations components in the DPPH• assay were investigated (Georgetti et al., 2006). No significant statistical difference was detected in the hydrogen-donating ability of quercetin compared to formulations 1 and 2 (Fig. 2). This result further confirms the adequacy of the assay to evaluate the quercetin functional stability in the present formulations. The initial hydrogen-donating ability of the raw material quercetin, the formulation 1 and formulation 2 containing quercetin were 41.60%, 37.80%, and 38.50%, respectively.

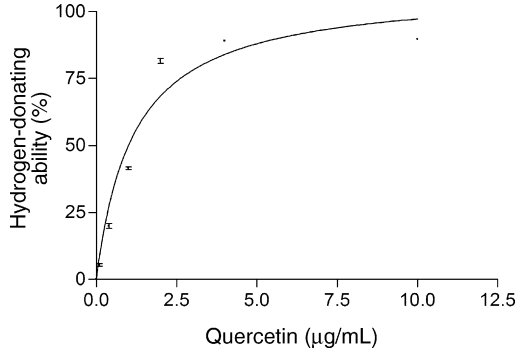

Fig. 1. Hydrogen-donating ability of quercetin. The quercetin used concentrations were 0.1, 0.4, 1.0, 2.0, 4.0 and 10 μg/mL. Results are represented by means ± S.E.M.

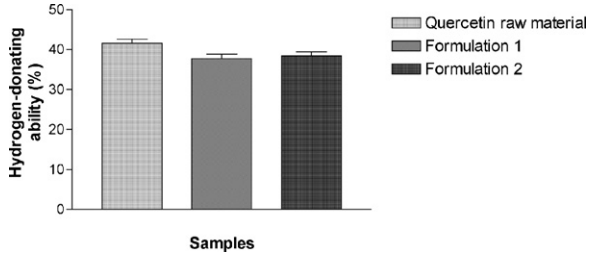

Fig. 2. Hydrogen-donating ability of the raw material (quercetin), formulations 1 and 2. The final concentration of quercetin used in the reaction medium was 1 μg/mL for all samples. DPPH• was determined as described in Section 2. Results are represented by means ± S.E.M. ($n = 3$). No statistical significant difference was detected. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's test of multiple comparisons ($P < 0.05$).

3.3. Quercetin solubility in different receptor media

In order to identify the optimal receptor medium, the solubility of quercetin in different possible solvents was analysed (Table 1). In this study, phosphate buffer (pH 7.6; 0.1 M) containing polyoxyethylene (20) sorbitan monolaurate (Tween 20) (0.5%) was chosen and used as receptor solution.

3.4. In vitro release studies

Quercetin release from the formulations was evaluated up to 12 h to determine its diffusion rate (D) in the emulsions. The calculated diffusion coefficient of quercetin of formulation 1 was $7.5 \pm 0.02 \times 10^{-12}$ cm^2/s. On the other hand, the release of the drug from the formulation 2 was about two times faster than formulation 1 rending a diffusion coefficient of $14.9 \pm 0.1 \times 10^{-12}$ cm^2/s (Fig. 3).

3.5. In vitro skin retention studies

Fig. 4 shows the antilipoperoxidative activity (%) over time ± S.E.M. of retained quercetin in the skin. It was detected that the pig ear skin with formulations without quercetin did not

Table 1
Saturation solubility of quercetin in different medium

Medium	Solubility at 37 °C (μg/mL)
0.1 M phosphate buffer (pH 7.6)	0.59
0.1 M phosphate buffer (pH 7.6) + 0.5% ethanol	1.37
0.1 M phosphate buffer (pH 7.6) + 0.5% tween 20	30.17

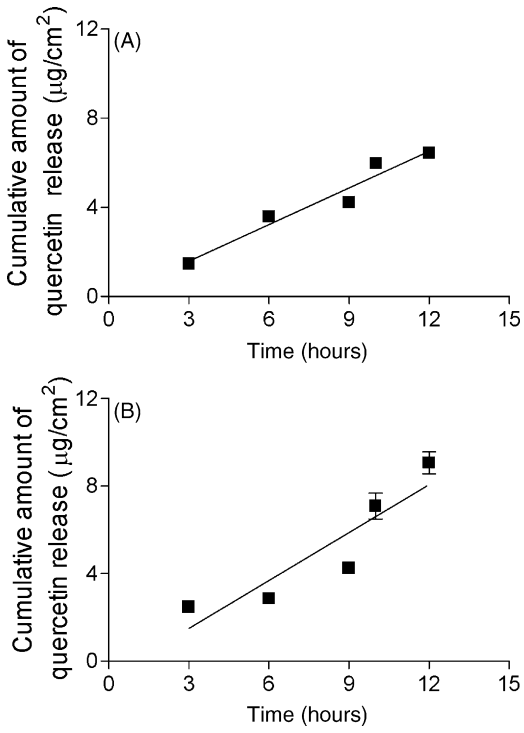

Fig. 3. Amount of quercetin release as a function of the time from formulation 1 (A) and formulation 2 (B). Quercetin release rates from the different formulations were measured through 0.45 μm nitrocellulose membranes using modified Franz diffusion cells with a diffusional area of 1.77 cm^2.

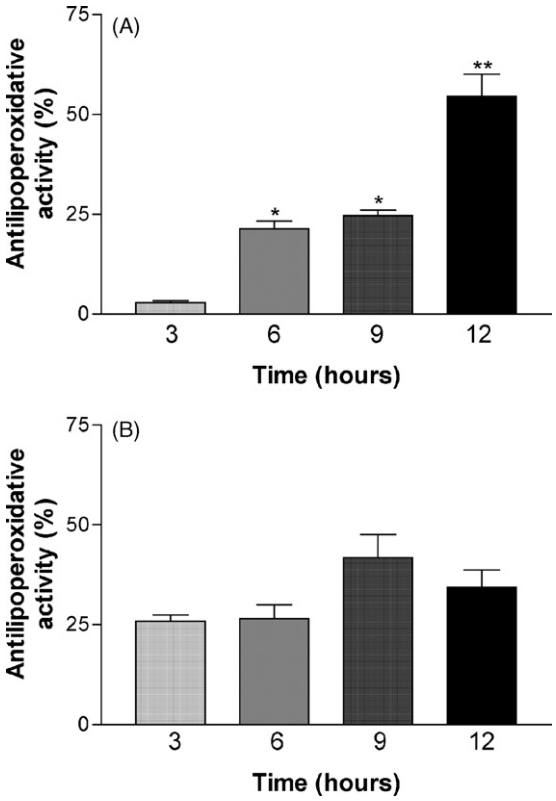

Fig. 4. Quercetin retention in pig ear skin as a function of the time from formulation 1 (A) and formulation 2 (B). The retention of quercetin was determined by antilipoperoxidative activity. Mitochondria (1 mg protein) were incubated in the medium I with concentrated extract of the skin, $(NH_4)_2Fe(SO)_4$ (50 μM) and sodium citrate (2 mM) for 30 min at 37 °C (1 mL final volume). MDA was determined as described in Section 2. Results are represented by means ± S.E.M., ($n = 5$). Statistical analysis was performed by one-way ANOVA followed by Bonferroni's test of multiple comparisons. *Significant statistical difference compared to 3 h ($P < 0.05$). **Significant statistical difference compared to 3, 6 and 9 h ($P < 0.05$).

affect the assay (data not shown). Then, the maximal antioxidant activity observed for formulations 1 and 2 after 12 h of experiment was 54.0% ± 5.5 and 42.0% ± 5.9, respectively, without significant statistical difference between the formulations. Furthermore, a gradual increase in the antilipoperoxidative activity over time (3, 6, 9 and 12 h) was detected with formulation 1 containing quercetin. Statistical significant differences were detected after 6 and 9 h compared to 3 h, and after 12 h compared to 3, 6 and 9 h. For formulation 2 there was no statistical difference over time for quercetin activity.

4. Discussion

The present study is divided in two parts: (i) the stability study of two different lipid content quercetin formulations, and (ii) the evaluation of quercetin release from these formulations and percutaneous absorption. The present results explain the recent *in vivo* data demonstrating that the same formulations used herein diminish the UVB-induced oxidative/inflammatory skin damages (Casagrande et al., 2006).

The stability evaluation of active principles of formulations stored at different climatic conditions for given time constitutes an important step for the development of new products. It provides information about the shelf lives of pharmaceutical products, as well as the conditions for their storage (Wessels et al., 1997; Singh, 1999). There are several inherent problems with the estimation of shelf life since it is applied to linearly degradable drugs, it does not consider change in degradation mechanisms above the critical temperatures, and does not include the errors associated with the determination of the drug content (Singh, 1999). On the other hand, The ICH Harmonized Tripartite Guidelines on Stability Testing of New Drug Substances and Products summarize the fundamental recommendations for stability studies. According to the ICH guidelines, accelerated stability studies have to be carried out for 180 days (Simon et al., 2004).

Therefore, this stability study evaluated two different lipid content quercetin formulation for 180 days (6 months) at different temperature conditions, and to ensure the stability of quercetin, focused on it's antioxidant activity. This is a manner to ensure and guarantee the efficacy of a product with a specific function (D'León, 2001). Additionally, functional stability of the raw material and formulations containing quercetin was evaluated using an easy, inexpensive, fast and precise methodology to measure the antioxidant activity, the DPPH• assay (Nuutila et al., 2003).

DPPH• is a stable free radical that can accept an electron or hydrogen radical converting into stable, diamagnetic molecule. Because of its odd electron, DPPH• has a strong absorption band at 517 nm. As this electron becomes paired off, the absorp-

tion decreases stoichiometrically with respect to the number of electron taken up. Such a change in absorbance produced in this reaction has been widely used to test the ability of several molecules to act as free radical scavengers (Dinis et al., 1994). The adequacy of the methodology was determined by a dose-dependent curve and the absence of formulations' interference in the detection of quercetin antioxidant activity using DPPH• assay.

Focusing in the stability study, after 180 days (6 months), stored at different temperatures (4 °C, RT, and 40 °C ±70% RH) quercetin maintained its hydrogen-donating ability in all of the systems and temperatures tested as in the first day of the experiments, which suggests that quercetin remained stable. Despite of it, there was an apparent loss of activity in some days and temperatures, especially for the raw material. However, this loss could be attributed to errors associated with the DPPH• assay (coefficient of variation = 5%). Corroborating, it has been previously suggested the chemical stability of quercetin (Smith et al., 2000). Therefore, it was concluded that the formulations developed in this work remained functionally stable under the evaluated conditions (data not shown in figures).

There is no doubt that the vehicle in which a drug is applied can effectively influence its release from a topical pharmaceutical preparation. An appropriate formulation of the topical agent will ensure that it exerts maximal activity in the skin. The primary requirement for topical therapy is that a drug incorporated into a vehicle reach the skin surface at an adequate rate and in sufficient amounts (Röpke et al., 2002). Therefore, as the formulations remained stable, the evaluation of quercetin release rates and/or percutaneous absorption became a crucial step to further substantiate the possible usefulness of quercetin in those formulations. Thus, quercetin diffusion through the different formulations developed was examined *in vitro* using a nitrocellulose membrane mounted in a vertical diffusion cell.

First of all, the optimal receptor medium for these studies was selected. The selection of the receptor solution is an important decision to create *in vitro* conditions, which can adequately replicate the *in vivo* conditions. The receptor solution has an important influence on the *in vitro* absorption of compounds with low aqueous solubility such as quercetin. Solubilizers are commonly used to increase the solubility of the drugs in the receptor solution to guarantee sink conditions without apparent damage to the skin. As a general rule, the concentration of the permeant should not be allowed to exceed 10% of saturation solubility (Surber et al., 1991; Bronaugh and Collier, 1993; Valenta and Janisch, 2003). Therefore, for the present study, phosphate buffer (pH 7.6; 0.1 M) containing polyoxyethylene (20) sorbitan monolaurate (Tween 20) (0.5%) was used as receptor solution.

The diffusion coefficient reflects the facility by which molecules move through the membrane (Shah and Maibach, 1993) and also through the formulation (Larrucea et al., 2001; Merclin et al., 2004). Due to the lipophilic characteristics of quercetin (log *P* octanol–water = 1.48) (Meylan and Howard, 1995), it was important to test formulations of different lipid content. Lipophilic substances can be solubilized in the lipophilic phase of emulsions, and thus, solubility will increase proportionally to lipid content (Carstensen, 1995). Both formulations tested are oil-in-water emulsions, but the aqueous external phase of formulation 2 represents about 90% of the total volume, besides the presence of hydrophilic colloid, which compared to formulation 1, probably decreases the affinity of the lipophilic quercetin for this formulation, facilitating its diffusion.

Despite the confirmation that quercetin can be released from the formulations, the *in vitro* skin permeation studies demonstrated that quercetin provably did not permeate the skin. Furthermore, although the HPLC is the most used analytic methodology in percutaneous absorption studies, it did not detect quercetin in the receptor medium after 12 h in the permeation experiments using pig ear skin (data not shown in figures). Corroborating, quercetin antilipoperoxidative activity was also not detected in the receptor medium (data not shown in figures). Some raised possibilities were that the drug did not permeate or the permeated amount was below the methodology limit detection of HPLC (18 ng/mL). Another important issue to mention is that the recovery of a given drug is improved by changing the receptor medium at regular time points or using constant flow receptor medium (drip method). Although Crutcher and Maibach (1969), did not compare those conditions with fixed volume (present study), it is conceivable that changing the receptor or using constant flow the recovery would be improved (Crutcher and Maibach, 1969). Therefore, it is possible that using a different approach quercetin could be detected in the receptor medium. Nevertheless, even considering those possibilities, the absence of antioxidant activity in the receptor medium compared to the high activity in the skin samples suggests that the quercetin main effect is topical.

These results are in accordance with Saija et al. (1998), demonstrating that although the liposolubility of quercetin is theoretically adequate to penetrate the skin, it did not permeate well compared to other flavonoids tested (hesperetin e naringenin) when the acetone solution was used as vehicle and the skin was pretreated with absorption promoters such as D-limonene and lecithin (Saija et al., 1998). As drug skin permeation is not only determined by the partition coefficient, but also by other physico-chemical properties as water solubility, molecular size and diffusivity (Zatz, 1985), the nearly absent solubility of quercetin in water might justify its poor capability to permeate the skin (Bonina et al., 1996).

Moreover, the HPLC analyses were inadequate to detect retained quercetin in the pig ear skin. Probably, the tissue interferences were not eliminated by the extraction procedure, which was also the case of DPPH• assay (data not shown). Therefore, the lipid peroxidation assay was used as an alternative approach to evaluate the possibly retained quercetin in the skin.

Lipid peroxidation is a free radical mediated event, and its primary products are a complex mixture of peroxides, which then break down to produce carbonyl compounds such as MDA (Gutteridge, 1981). Quercetin is a chain-breaking inhibitor of the peroxidation process by scavenging intermediary peroxyl and alkoxyl radicals, and chelating iron ions (Morel et al., 1993; Saija et al., 1995; van Acker et al., 1998).

Thus, it was recently demonstrated that both formulations containing quercetin tested in the present study inhibit the UVB-induced GSH depletion, myeloperoxidase activity increase

and proteinase secretion/activity. These data demonstrated that quercetin effectively inhibit the oxidative stress and inflammation induced by UVB irradiation (Casagrande et al., 2006). There are multiple mechanisms involved in the UVB irradiation-induced skin lesions including ROS generation and the activation of transcription factors such as NFκB, which are certainly related to the production of cytokines (e.g. TNF-α and IFN-γ) and induction of cyclooxygenase resulting in prostaglandin E_2 (Fuchs et al., 2001b; Morikawa et al., 2003; Casagrande et al., 2006). It has been described that quercetin inhibits TNF-α and prostaglandin E_2 production (Morikawa et al., 2003). Additionally, there are at least three ways that UV irradiation affects antioxidant concentrations in the skin: (i) direct absorbance of light, (ii) interaction with ROS generated by interaction of photosensitizers with UV light, and (iii) antioxidant recycling mechanisms, whereby one antioxidant can be spared at an expense of another (Shindo et al., 1994). There is no evidence until now that quercetin works either as chemical or physical sunscreen. Thus, the mechanisms of protection by quercetin may be the alteration of GSH metabolism and the scavenging of ROS, protecting the cells from the deleterious consequences of GSH deficiency (Skaper et al., 1997; Ishige et al., 2001). Moreover, quercetin might reach viable epidermis since it presents anti-inflammatory/antioxidative activities *in vivo* (Casagrande et al., 2006), and these effects are local since it did not permeate the skin.

Reinforcing and explaining absorption percutaneous results, the present data demonstrated that sufficient active quercetin is retained in the skin. In addition, this faster effect of formulation 2 in the skin is probably correlated with a faster quercetin release from this formulation when compared to formulation 1 as detected by the diffusion coefficients. Nevertheless, the retention profile of formulation 1 was time-dependent, being better than the profile of formulation 2. Corroborating, in a previous study (Casagrande et al., 2006), formulation 1 presented higher *in vivo* antioxidant/anti-inflammatory activity compared to formulation 2, which might be explained by their different lipid content. These data reinforce the importance of appropriate formulations. Furthermore, the absence of quercetin in the receptor media demonstrates that it is only retained in the skin, really functioning as a topical drug.

In addition to the successful preclinical study described above (Casagrande et al., 2006), the potential use of antioxidants to protect the skin from UV-induced lesions was demonstrated in other preclinical studies (Wheeler et al., 1986; Fuchs et al., 1989; Shindo et al., 1994; Evelson et al., 1997; Weber et al., 1997) as well as by small phase I clinical studies (Saija et al., 1998, 2000; Dreher et al., 1999). Nevertheless, this field surely merits more and intensive studies.

Concluding, the present study suggests an efficient approach to evaluate quercetin skin retention with minimal interference of the tissue products. Furthermore, the present data on skin retention and the previous study on quercetin *in vivo* activities (Casagrande et al., 2006) suggest that these functionally stable formulations containing quercetin may be used as topical active product to control UVB-induced oxidative skin damages.

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