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Evaluation of percutaneous absorption of 4-nerolidylcathecol from four topical formulations

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Abstract

Antioxidants such as vitamins E and C are known to play a significant role in ameliorating or preventing oxidative damage to the skin. However, to provide a satisfactory protection they must first permeate the skin, which serves as a permeation barrier. In this study we evaluated the influence of three different formulations (gel, gel–emulsion and emulsion) on the percutaneous absorption of 4-nerolidylcathecol, an antioxidant compound isolated from *Pothomorphe umbellata* root extracts. Also, the absorption of the isolated 4-nerolidylcathecol was compared with its absorption when dried *P. umbellata* root ethanolic extract was incorporated into a gel formulation. The 'lag time method' was employed for the analysis of the in vitro permeation data. All formulations showed satisfactory percutaneous penetration with the 4-nerolidylcathecol–gel presenting a higher rate of penetration leading to higher dry drug levels in the tissue.

Keywords: 4-Nerolidylcathecol; Antioxidant; Skin permeation

1. Introduction

Chronic oxidative stress, which occurs in modern life as a result of excessive sun exposure and increasing air pollution, may justify supplementation with antioxidants in order to retard the cutaneous aging process (Emerit, 1992). Antioxidants from natural products may provide new possibilities for the treatment and prevention of oxidative stress-mediated diseases. Some enzymes and secondary compounds of higher plants have demonstrated protective effects against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species (Larson, 1988). The Brazilian flora is rich in medicinal plants with a high potential for providing antioxidant substances. Among them, the Piperaceae family, particularly the 'pariparobas', are widely used in Brazilian folk medicine for the treatment of liver

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diseases, among other uses. The root of *Pothomorphe umbellata* (L.) Miq. was included in the first edition of the Brazilian Pharmacopoeia (Silva, 1926).

Crude root ethanolic extracts of *P. umbellata* demonstrated a significant activity in the prevention of in vitro spontaneous brain lipid peroxidation (Barros et al., 1996). This activity was attributed to 4-nerolidylcathecol (Fig. 1), a compound isolated from hexane extracts of roots and leaves of *P. umbellata* (Kijjoa et al., 1980). Experiments comparing the total reactive antioxidant potential and total antioxidant reactivity of 4-nerolidylcathecol and methanolic extracts of *P. umbellata* in a homogenous system indicate a higher antioxidant potential of the extract, suggesting the presence of additional compounds with antioxidant activity in the extracts (Desmarchelier et al., 1997).

In a previous study, we evaluated the antioxidant activity of *P. umbellata* crude root extract incorporated into a hydrophilic ointment to a final concentrations of 0.05, 0.1, 0.2 and 2% w/w of 4-nerolidylcathecol on the skin of hairless mice. This antioxidant activity was evaluated in comparison with different concentrations (0.2, 2, 5 and 10% w/w) of α-tocopherol (Sigma) incorporated into the same vehicle. Lipid peroxidation of skin homogenates incubated at 37 °C for 2 h was evaluated as thiobarbituric acid reactants (TBARS) and chemiluminescence (CL) 18 h after treatment. Animals treated with *P. umbellata* root extract showed a decrease of up to 90% in the lipid peroxidation parameters for concentrations higher than 0.1%. Our model demonstrated a significant decrease in TBARS and CL only for higher concentrations of α-tocopherol (5 and 10% w/w). The results indicated a potent antioxidant activity of *P. umbellata* root extract, suggesting its application in cosmetic formulations (Röpke et al., 1999; Röpke et al., 2000).

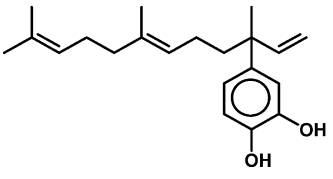

Fig. 1. 4-nerolidylcathecol.

Topical administration of antioxidants provides a manner of enriching the endogenous cutaneous protection system and represents a successful strategy for reducing the ultraviolet radiation-mediated oxidative damage (Saija et al., 1998; Fuchs, 1998; Lopez-Torres et al., 1998).

In order to elicit the antioxidant activity in the case of a topical formulation, the active compound must be capable of first permeating the skin, which serves as a permeation barrier. As the UV radiation penetrates deeply into the skin, topically applied antioxidant drugs may offer a satisfactory photoprotection to the skin only if they are able to reach deeper cutaneous layers (Bonina et al., 1996; Saija et al., 2000). Percutaneous penetration, that is, the passage through the skin, involves the dissolution of a drug in a vehicle, diffusion of the solubilized drug from the vehicle to the surface of the skin, and the penetration of the drug through the layers of the skin, mainly the stratum corneum. This penetration may be improved by selecting the appropriate vehicle (Martin and Bustamante, 1993; Idson, 1983; Pirot et al., 1996).

Thus, in the present study we evaluated the percutaneous absorption of isolated 4-nerolidylcathecol from three different formulations, plus its absorption from *P. umbellata* root ethanolic extract gel. The 'lag time method' was employed for the analysis of in vitro permeation data (Barry, 1983).

2. Material and methods

2.1. Animals

Albino hairless mice of the HRS/J strain were bred in our laboratory. Animals were approximately 10 weeks old at the beginning of the experiment and were housed in cages in a room with controlled temperature and humidity and with a 12-h light:12-h dark cycle. Mice were given food and water ad libitum.

2.2. Test materials

Methanol, ethanol and hexane lichrosolv grade and KCl p.a. were from Merck (Darmstadt, Germany). $LiClO_4$ was from Aldrich. 4-Nerolidylcathecol, used as a standard chemical substance, was isolated as described (Kijjoa et al., 1980) from a specimen growing in our garden.

2.3. Extract preparation

The extract was prepared from dried powdered roots of *P. umbellata* obtained from the medicinal herb garden of the School of Pharmaceutical Science, University of São Paulo. The plant material was identified and a sample was deposited in the Herbarium of the Institute of Biosciences of the University of São Paulo. Powdered roots were percolated according to the Brazilian Pharmacopoeia (Silva, 1926), with ethanol:water (1:1) (100 ml/g of dried powdered roots) and the extract was freeze-dried. The final concentration of 4-nerolidylcathecol in the crude extract was 25.4% (w/w), as determined by HPLC using electrochemical detection.

2.4. Formulations

Three formulations containing 0.1% 4-nerolidylcathecol (hydrophilic gel, gel–emulsion and O/W emulsion) were prepared. A fourth formulation was prepared as a hydrophilic gel containing *P. umbellata* root extract with a final concentration of 0.1% 4-nerolidylcatechol (Table 1). This concentration of 4-nerolidylcathecol was chosen because it was the minimal active concentration in our previous study (Röpke, 1999; Röpke et al., 2000).

2.5. In vitro skin permeation experiments

In vitro studies with intact hairless mouse skin (HRS/J, male) were performed as follows. After ether anesthesia, full thickness skin was obtained from 10-week-old mice. The dorsal skin without adhering subcutaneous fat was mounted on a modified Franz diffusion cell (1.61 cm^2 surface area). Test formulations (600 mg) were applied to the epidermal surface of the skin, completely covering the exposed skin area. The receiving compartment contained 12 ml of ethanol:water (1:1, v/v) to ensure sink conditions (Touitou and Fabin, 1988). Compounds that are essentially insoluble in water may not partition freely from excised skin into an aqueous receptor fluid (Bronaugh and Steward, 1983). The receiving solution was stirred and maintained at 37 °C during the experiment. The receptor fluid was sampled from a side arm at predetermined times (0, 0.5, 1, 2, 4, 6, 8, 11.5 and 24 h) during the absorption process. The sample volume (600 μl for each time) was replaced with fresh solution. Samples (20 μl) were injected directly into the chromatographic system and analyzed for 4-nerolidylcathecol content by

Table 1
Composition (% w/w) of the formulations used to evaluate percutaneous absorption of *P. umbellata* extract and 4-nerolidylcathecol

	Emulsion O/W	Gel–emulsion	Gel	*P. umbellata* gel
Carbopol 940		0.3	0.6	0.6
Propylene glycol	10.0	10.0	10.0	10.0
Triethanolamine		0.25	0.5	0.5
Liquid Vaseline	6.0			
Cetiol V®	3.0	1.5		
Lanette N®	5.0	2.5		
Methyl paraben	0.15	0.15	0.25	0.25
Propyl paraben	0.05	0.05		
4-Nerolidylcathecol	0.1	0.1	0.1	
P. umbellata root extract				4.2
Distilled water qs	100	100	100	100

electrochemical detection. Each experiment was performed in triplicate.

2.6. Quantitative analyses of 4-nerolidylcathecol absorbed into the skin

After the in vitro permeation experiments, the surface of the hairless mouse skin was washed with 140 mM NaCl in 40 mM phosphate buffer, pH 7.4. The skin sheets were cut into small pieces, homogenized (1:10) in an isotonic ice-cold buffer (phosphate buffer, pH 7.4) with an Ultra-Turax blender and subsequently with a Teflon pestle in a tight-fitting glass vessel. 4-Nerolidylcathecol was extracted from the homogenate using a modification of the procedure described by Burton et al. (1985). Briefly, 500 μl of 1 M sodium dodecylsulfate, 1.0 ml of methanol and 6.0 ml of hexane were added to 500 μl of homogenate samples. A fraction of the hexane layer was evaporated under nitrogen, dissolved in ethanol and analyzed by HPLC with electrochemical detection. To determine the recovery from the skin, the intact skin was spiked with known quantities of the compound and homogenized, extracted and analyzed as described above.

2.7. In vivo permeation experiment

Mice were treated topically over the dorsal surface (on a defined site of 4 cm^2) with 80 mg of *P. umbellata* gel. This amount of treatment provides approximately 20 $\mu g/cm^2$ of 4-nerolidylcathecol. Two hours and a half after treatment, mice were killed and the drug-treated skin area was washed with 140 mM NaCl in 40 mM phosphate buffer, pH 7.4. Skin sheets were homogenized, extracted and analyzed as described in Section 2.6. A fraction of the hexane layer was evaporated under nitrogen, dissolved in ethanol and analyzed by HPLC with electrochemical detection.

2.8. Stability of 4-nerolidylcathecol to UV exposure

A group of mice treated exactly as described in Section 2.7 was exposed to a single dose of UVB radiation for 30 min (0.23 mJ/cm^2, ca. 2 MED for these mice) 2 h after treatment. Light was provided by a Philips TL12rs 40 W UVB lamp. The mice were irradiated individually in glass chambers. The distance from the lamps to the dorsal surface of the mice was 26 cm. The output of the light at that distance was monitored with an International Light (Newburyport, MA, USA) model 1700 research radiometer.

After irradiation the mice were killed by cervical dislocation, and the skin was removed immediately. Skin sheets were homogenized, extracted and analyzed as described in Section 2.6. A fraction of the hexane layer was evaporated under nitrogen, dissolved in ethanol and analyzed by HPLC with electrochemical detection.

2.9. HPLC analysis

The HPLC equipment consisted of a Waters Model 510 pump, a 7161 Rheodyne injector equipped with a 20 μl loop and a Hewlett–Packard HP 1049A Electrochemical Detector. The cell contains a solid state, in situ Ag/AgCl reference electrode as a standard with the refillable electrode, and glass carbon is the standard working electrode. The detection conditions were the following: potential = 0.600; polarity, oxidation; mode, amperometry. Integration of the chromatographic peaks was achieved with a SP 4600 Thermoseparation Products integrator. Chromatography was performed on a Supelcosil LC-8, 3 μm, 75 × 4.6 mm column (Supelco, Bellefonte, PA, USA) with a mobile phase of methanol:water (9:1) containing 20 mM LiClO4 and 2 mM KCl. The flow rate was set at 1.0 ml/min. The retention time of 4-nerolidylcathecol was 2.4 min. Each sample was filtered through 0.22 μm cellulose acetate filters (Costar®) and an aliquot of 20 μl was injected into the HPLC apparatus.

2.10. Statistical analysis

Data were analyzed statistically by factorial analysis of variance (ANOVA). The Tukey–Kramer test was then used to determine significant differences between groups.

3. Results

3.1. In vitro skin permeation experiments

The nonlinear portion of a permeation profile, as shown in Figs. 2–5, represents the time period required to establish steady-state diffusion and its duration is referred to as 'lag time'. The steady-state points are the ones considered in the lag-time method. These points are linear and the steady-state flux (J) is determined from the slope of their linear regression, with their x-intercept being the diffusion lag time (T_{lag}) (Barry, 1983; Shah, 1993).

The diffusion coefficient (D), the permeability (P) and the partition coefficient (K) for 4-nerolidylcathecol for the different formulations were determined from the donor phase concentration ($C = 1000$ μg/g), the thickness of the barrier ($e =$ 0.1 cm), the lag time (T_{lag}) and the steady-state flux (J) (Barry, 1983; Shah, 1993). Thus, the steady-state flux J (μg/cm^2 per h) is equal to the slope of the linear permeation profile, the permeability P (cm/h) equals J divided by C, the lag-time T_{lag} (h) is the x-intercept of the linear permeation profile, the diffusion coefficient D (cm^2/h) is expressed by $1/6e^2T_{lag}^{-1}$, and the partition coefficient K equals ePD^{-1}.

The permeation parameters were calculated for each experiment, and the results in Table 2 show the mean values ± standard errors (S.E.) and statistics for three diffusion cell experiments for each formulation.

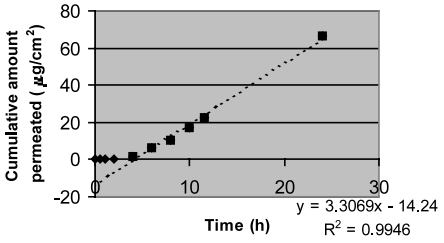

Fig. 2. Permeation profile of the gel formulation containing 0.1% 4-nerolidylcathecol and linear regression of the linear portion of the permeation profile (■ steady state).

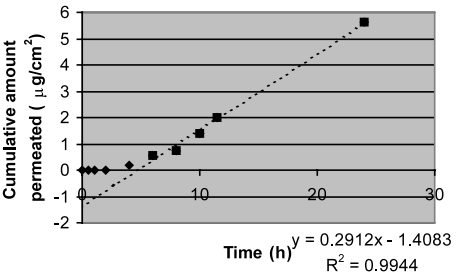

Fig. 3. Permeation profile of the emulsion (O/W) containing 0.1% 4-nerolidylcathecol and linear regression of the linear portion of the permeation profile (■ steady state).

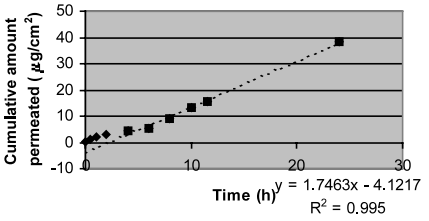

Fig. 4. Permeation profile of the gel–emulsion formulation containing 0.1% 4-nerolidylcathecol and linear regression of the linear portion of the permeation profile (■ steady state).

3.2. Absorption of 4-nerolidylcathecol into the skin after 24 h, using different formulations

Employing the method described in Section 2.6, the 4-nerolidylcathecol recovery rate from skin homogenates was 100%. We observed a significant difference between the 4-nerolidylcathecol–gel formulation and the other formulations (Fig. 6).

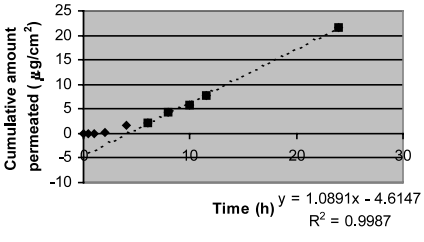

Fig. 5. Permeation profile of the *P. umbellata* gel formulation containing 0.1% 4-nerolidylcathecol and linear regression of the linear portion of the permeation profile (■ steady state).

Table 2
Permeation parameters for 4-nerolidylcathecol in different formulations

	Flux (J, μg/cm^2 per h)	Permeability coefficient (P, 10^{-3} cm/h)	Lag time (T_{lag}, h)	Diffusion coefficient (D, 10^{-3} cm^2/h	Partition coefficient (K)
4-Nerolidylcathecol emulsion	0.3935±0.0963**	0.3935±0.0963**	4.11±1.04	0.42±0.09	0.09±0.02●●
4-Nerolidylcathecol gel-emulsion	1.350±0.515*	1.350±0.515*	4.48±2.57	0.46±0.25	0.31±0.07
4-Nerolidylcathecol gel	3.799±0.984	3.799±0.984	3.52±1.41	0.56±0.030	0.64±0.28
P. umbellata root extract gel	1.516±0.644*	1.516±0.644*	3.26±1.98	0.56±0.39	0.25±0.11●

**, $P < 0.001$; *, $P < 0.01$ compared with 4-nerolidylcathecol gel. ●●, $P < 0.01$; ●, $P < 0.05$ compared with 4-nerolidylcathecol gel.

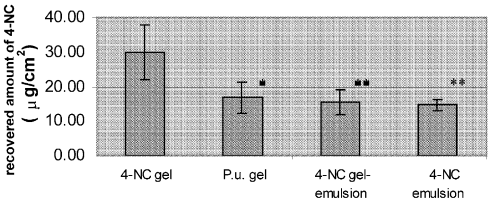

Fig. 6. 4-Nerolidylcathecol absorbed into the skin from different formulations after 24 h. The symbols and bars indicate the mean value±S.E. of three recovery experiments for each formulation. *, $P < 0.05$; **, $P < 0.01$ compared with 4-nerolidylcathecol gel. 4-NC, 4-nerolidylcathecol.

3.3. In vivo permeation experiment

After 2 h 30 min of treatment, approximately 20% of the applied 4-nerolidylcathecol had permeated into the skin. We did not observe any reduction in 4-nerolidylcathecol concentrations between irradiated and non-irradiated mice treated with *P. umbellata* gel (Fig. 7).

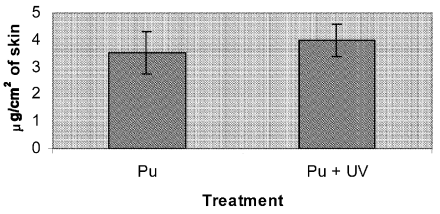

Fig. 7. 4-Nerolidylcathecol concentrations in hairless mouse skin treated with *P. umbellata* gel, before (*P. u.*, 3.53±0.77 μg/g of skin) and after ultraviolet irradiation (*P. u.*+UV, 4.00±0.59 μg/g of skin). Data are the mean±S.D. of six animals. The difference between groups was considered not significant.

3.4. Discussion

Many factors may influence the extent of percutaneous absorption of a drug. Partitioning of the chemical between the vehicle and the stratum corneum results in a concentration gradient developing across the skin, which is influenced by chemical–vehicle–skin interactions (Hilton et al., 1994). There is no doubt that the release of a drug from a topical pharmaceutical preparation can be effectively influenced by the vehicle in which it is applied. An appropriate formulation of the topical agent will ensure that it exerts its maximal activity on the skin (Idson, 1980). The primary requirement for topical therapy is that a drug incorporated into a vehicle reaches the skin surface at an adequate rate and in sufficient amounts.

The relative affinity of the drug for skin and vehicle is represented by the partition coefficient K. There was a significant difference between the K values for the 4-nerolidylcathecol emulsion and the gel (Table 2), a fact possibly explained by the different nature of the two vehicles. A high K value indicates that the vehicle has poor affinity for the drug. A low K value, which indicates a high degree of mutual interaction, reflects the tendency of the drug to remain in the vehicle. Hence, the release of a substance will be favored by selecting vehicles with low affinity for the penetrant (Idson, 1983). In our case, the 4-nerolidylcathecol–gel formulation showed the highest K value. The fact that the *P. umbellata* root extract gel does not present a high K value when compared with

the isolated 4-nerolidylcathecol is probably related to other substances present in the crude extract, which may influence the affinity of 4-nerolidylcathecol for the formulation.

There was also a significant difference in the permeability of 4-nerolidylcathecol in the gel formulation in comparison with the other formulations tested. Permeability is a function of the flux and concentration of the drug in the donor recipient of the diffusion cell. In our case the concentration is constant; thus, the difference is a consequence of flux. The flux J is actually proportional to the gradient of thermodynamic activity, rather than to the concentration. This activity changes according to different formulations (Martin and Bustamante, 1993).

The diffusion coefficient D reflects the facility with which molecules move through the various membrane strata (Idson, 1983; Shah and Maibach, 1993). Diffusivities, which are a function of the molecular structure of the diffusant, did not change in our experiment. The diffusion coefficient is also a function of the barrier material (Shah et al., 1994). In our case the skin barrier seemed not to be affected by the vehicle, since no significant difference in D values was observed among the formulations used in the study.

The steady state is always proportional to the drug concentration in the donor recipient of the diffusion cell, so all formulations had approximately the same 4-nerolidylcathecol 'T_{lag}' values.

The permeation data correlate well with the findings of the 4-nerolidylcathecol quantification experiments. A higher amount of 4-nerolidylcathecol in the skin was found for the gel formulation, which gave the highest rate of percutaneous penetration (Fig. 4). The other formulations (gel–emulsion, emulsion and the crude extract gel) showed intermediate percutaneous absorption rates in relation to the 4-nerolidylcathecol–gel formulation. This does not mean that the possibility of employing the crude extract instead of the isolated active principle should be ruled out. Even with a lower rate of percutaneous absorption, a total reactive antioxidant potential study showed that the crude *P. umbellata* root extract has a higher antioxidant potential than the isolated 4-nerolidylcathecol, suggesting the presence of additional compounds with antioxidant activity (Desmarchelier et al., 1997). Further work is needed to elucidate this difference in antioxidant potential for the skin.

The in vivo permeation experiment confirmed that 4-nerolidylcathecol was also absorbed in biological conditions and that it is stable when exposed to UV irradiation. Considering the high antioxidant activity of the *P. umbellata* root extract, and of its isolated principle 4-nerolidylcathecol (Desmarchelier et al., 1997; Barros et al., 1996; Röpke, 1999), we have demonstrated that both the root extracts and the major antioxidant compound of *P. umbellata* could be effectively administered as protective agents by percutaneous absorption against oxidative stress of the skin.

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