Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 342 (2007) 40–48
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Development of topical functionalized formulations added with propolis extract: Stability, cutaneous absorption and *in vivo* studies

F. Marquele-Oliveira, Y.M. Fonseca, O. de Freitas, M.J.V. Fonseca *

Department of Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences of Ribeirão Preto-USP, Av. do Café s/n, 14049-903 Ribeirão Preto, SP, Brazil

Received 7 March 2007; received in revised form 20 April 2007; accepted 24 April 2007
Available online 5 May 2007

Abstract

Propolis, which is a natural product widely consumed in the folk medicine, is a serious candidate to be applied topically due to its outstanding antioxidant properties. So, the purpose of this study was to develop stable topical formulations added with propolis extract in an attempt to prevent and/or treat the diseases occurring in skin caused by UV radiation. The antioxidant activity using a chemiluminescent method was used to evaluate the functional stability and the permeation/retention in skin of these formulations. In the long-term stability study, the formulations were stored at 25 ± 2 °C/AH and at 40 ± 2 °C/70% RH for 360 days. It was found in this study, that the formulations prepared with Polawax® showed functional and physical stability in the period of study. In addition, this formulation presented good results in the percutaneous study, allowing the antioxidant compounds present in the propolis extract to reach lower layers in pig ear skin and in the whole hairless mice skin (retention = 0.12 and 0.13 μL of propolis/g of skin, respectively). In the *in vivo* study, it was also suggested that this formulation may be effective in protecting skin from UVB photodamage, nevertheless other assays need to be done in order to have a complete understanding of the protective effect of formulations added with propolis extract.

Keywords: Propolis; Topical formulation; Antioxidant activity; Cutaneous absorption

1. Introduction

Skin is a biological interface with the environment and, due to its barrier function, is a potential target organ of oxidative stress from external insults, such as UV-irradiation, ozone, ionizing radiation, various toxic chemicals, etc. This oxidative stress, which could be an initiator in the pathogenesis of skin cancer and photoaging, is referred as a condition of imbalanced prooxidant/antioxidant equilibrium, in favor of the former (Saija et al., 1998).

Antioxidants from natural sources may provide new possibilities for the treatment and prevention of oxidative stress-mediated diseases, such as the ones developed in skin (Afaq and Mukhtar, 2006). Therefore, in recent years, a lot of researches trying to establish and characterize natural antioxidants to be applied topically have been performed. Many studies have been carried out not only with isolated compounds, such as quercetin (Casagrande et al., 2006a) and other flavonoids (Georgetti et al., 2003), but also with natural extracts, such as *Glycyrrhiza glaba*, *Ginkgo biloba* (Di Mambro and Fonseca, 2005), green tea (Hsu, 2005), *Pothomorphe umbellata* (Ropke et al., 2002), etc.

Propolis, which is a natural product widely consumed in the folk medicine since ancient times, is a serious candidate to be added to topical formulations due to its antioxidant properties (Kumazawa et al., 2004). Besides antioxidant activity, epidemiological studies have detected that propolis has many pharmacological properties, such as antibacterial, antifungal, antiviral, antitumor, anti-inflammatory (Dobrowolski et al., 1991; Kujumgiev et al., 1999; Castaldo and Capasso, 2002; Russo et al., 2004) among others. In addition, besides its properties, this material is still used as a remedy in modern medicine due to a general "back to nature trend" (Castaldo and Capasso, 2002).

Our group has previously published a study assessing the antioxidant activity of propolis extracts (Marquele et al., 2005) and we have found that this material presented important

* Corresponding author. Tel.: +55 16 602 4726; fax: +55 16 633 1941.
E-mail address: magika@fcfrp.usp.br (M.J.V. Fonseca).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.04.026

antioxidant activity, scavenging several free radicals using several antioxidant systems. So, it may be suggested that the assessment of the antioxidant activity could be a useful tool to evaluate the functional activity of propolis extracts and it could be applied as a quality parameter. It is noteworthy that the antioxidant method is also able to evaluate the activity of the whole extract, since the compounds present in the extract might be acting in a synergic form. In addition, with the evaluation of the antioxidant activity there is no need for markers, which could be a difficult task, since propolis extracts are constituted of several compounds (propolis from Brazil and other tropical zones are mainly prenylated derivatives of *p*-coumaric acid, various diterpenes and flavonoids) depending on the collecting area (Park et al., 2002).

We have also reported that the antioxidant activity could be used to evaluate the functional activity of topical functionalized formulations (Marquele et al., 2005) and even to evaluate the release of such formulations (Marquele et al., 2006). However, we have to be concerned that not all the antioxidant methods are able to assess the antioxidant activity of such formulations, due to lack of sensibility or interferences (Marquele et al., 2005).

During the development step of formulations added with propolis extract, it is necessary to perform release, skin retention and stability studies of these formulations in order to acquire efficacy and functional and physical stability. Stability testing represents a crucial part of the testing program because the instability of the product modifies the three essential requisites, i.e. quality, efficacy and safety (Bilia et al., 2001). And finally, *in vivo* studies are desirable, since it will show if the expected action of the formulation developed was reached. So, in this paper, our purpose was to develop physically stable formulations added with propolis extract and to establish the antioxidant activity (using a chemiluminescent method) as a suitable method to assess their functional stability and their penetration into skin. In addition, it was intended to assess *in vivo* photoprotection effect of a formulation added with propolis extract.

2. Materials and methods

2.1. Chemicals

Marketed Brazilian extracts of propolis were purchased from APIS FLORA (Ribeirão Preto, SP, Brazil. The extracts were standardized using propolis from several sites of Brazil. Patent number PI 0405483-0, published in Revista de Propriedade Industrial no. 1778 from 01/02/2005). Two extracts were evaluated: ethanolic extract of propolis (EEP) and glycolic extract of propolis (GEP), however both of them presented the same chemical composition (11% dry weight). Luminol, xanthine and xanthine-oxidase (XOD) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of reagent grade and were used without further purification.

2.2. Test formulations

The types of formulations developed in this study were cream (F2 and F4), gel-cream (F1, F3 and F5) and gel (F6), as it can be seen in Table 1. All the raw-materials for the formulations were purchased from Galena (Campinas, SP, Brazil) or were a gift from Clariant (São Paulo, SP, Brazil). The concentration used of each raw-material was in accordance with the manufacturer's recommendations. Creams were developed varying the self-emulsifying wax (Polawax® or Crodabase® CR2). Gel-cream formulations were obtained using a self-emulsifying agent (Hostacerin® SAF) or using a self-emulsifying wax (Polawax®) with an anionic hydrophilic colloid (Carbopol® 940). In formulation 6, the anionic hydrophilic colloid (carboxypolymethylene, Carbopol® 940) was added as a stabilizing agent in order to form a gel formulation. The pH of the formulations added with Carbopol® 940 (F5 and F6) were adjusted to 6.5 by adding triethanolamine. Macadamia nut oil, isodecyl oleate and isopropyl palmitate were added as emollient, and glycerol as a moisturizer. 2.5% of extracts of propolis (alcoholic or glycolic) and preservatives (mixture of phenoxyethanol and parabens) were firstly solubilized in propylene glycol and next incorporated to

Table 1
Percent composition (w/w) of the emulsion media of the formulations

Component	F1 (%)	F2 (%)	F3 (%)	F4 (%)	F5 (%)	F6 (%)
Hostacerin® SAF[a]	6	–	6	–	–	–
Crodabase® CR2[b]	–	10	–	–	–	–
Polawax® [c]	–	–	–	8	4	–
Isodecyl oleate	0.5	1	–	1.5	0.5	–
Isopropyl palmitate	0.5	1	–	1.5	1	–
Macadamia nut oil	1.5	2	–	–	–	–
Propylene glycol	5	5	5	5	5	5
Glycerol	–	3	–	3	2	2
Carbopol® 940 (dispersion 2%)	–	–	–	–	20	30
Phenoxyethanol and parabens	0.5	0.5	0.5	0.5	0.5	0.5
Deionized water	86.6	77.5	88.5	80.5	67	63

[a] Self-emulsifying agent prepared without heating (ammonium acryloyldimethyl-taurate/VP copolymer + rapeseed oil sorbitol esters + trilaureth-4 phosphate + mineral oil + isopropyl palmitate).
[b] Self-emulsifying wax (mineral oil + petrolatum + lanolin alcohol + fatty alcohol + ethoxilated fatty alcohol).
[c] Self-emulsifying wax (cetostearyl alcohol and polyoxyethylene derived of a fatty acid ester of sorbitan 2OE).

the formulations at room temperature. The stability studies were performed with formulations containing both EEP and GEP. The permeation/retention and *in vivo* studies were performed with formulations containing only EEP.

2.3. Stability studies

2.3.1. Short-term physical stability

The formulations were packaged in impermeable polypropylene containers and stored for 30 days at 25 ± 2 °C/AH (ambient humidity) and at 40 ± 2 °C/70 ± 5% RH (relative humidity). Samples from each formulation container were evaluated every 7 days at room temperature in the following methods:

- Centrifugation assay. This test was carried out weighting 2 g of each sample and centrifuging them at 3000 rpm for 30 min.
- Organoleptic test. The organoleptic features of the samples were examined at the same temperature, lighting and packaging conditions to assess variations in appearance, phase separation, color and smell.
- pH measurements. One gram of each formulation was weighted and diluted with distilled water till 10 mL. After homogenized, the pH measurement of the samples was carried out with pHmeter.

2.3.2. Long-term physical and functional stability

After the previous physical stability study, the most stable formulations (F3 and F4, containing 2.5% of EEP or GEP and propolis-free formulations) were chosen to start the long-term physical and functional stability study as further discussed. The formulations were prepared again and stored in the same conditions mentioned above for a period of 1 year. The extracts as raw-materials (EEP and GEP) were also stored in the same aforementioned conditions. Samples were evaluated after 1, 7, 14, 30, 60, 90, 180, 270 and 360 days of preparation.

2.3.2.1. Physical stability. Besides carrying out the physical tests mentioned above (Centrifugation assay, Organoleptic test, pH Measurements), a Microscopy study was also performed to evaluate the formulations.

- Microscopy study. For this evaluation, a small amount of each sample was gently spread on a plate and covered by a tiny plate. The area of the emulsions oil droplets were measured by Leica DMLB Microscopy (1000×) coupled to LEICA Q Win DD 300 software. The mean area (μm^2) was calculated on the basis of at least 15 droplets.

2.3.2.2. Functional stability. The antioxidant activity was evaluated by the superoxide anion scavenging activity, measured by the inhibition of chemiluminescence intensity of the xanthine/luminol/xanthine-oxidase system. Chemiluminescent mixture was prepared immediately before analysis by mixing: 400 μL glycine buffer (0.1 M pH 9.4, 1 mM EDTA), 150 μL xanthine (6 mM in glycine buffer), 10 μL sample, 10 μL of luminol (0.6 mM). Adding 100 μL xanthine-oxidase (XOD) solution (20 IU/mL) started the reaction. Chemiluminescence was measured for 5 min at 25 °C with an Autolumat LB 953 apparatus (Girotti et al., 2000) and the percentage of inhibition of chemiluminescence of each sample was calculated as we previously reported (Marquele et al., 2005).

Firstly, in order to verify the antioxidant activity of the extracts, dose–response curves were built. So, the extracts of propolis were solubilized with propylene glycol (1:10) and then, diluted using the medium of the reaction to final concentrations ranging from 0.002 to 0.075 μL/mL. During the stability studies, formulations containing 2.5% EEP or GEP were diluted 1:5 and 1:20 with the medium of the reaction, rending final concentrations of 0.075 and 0.019 μL/mL. The extracts of propolis as raw-material were evaluated at the same final concentrations. The propolis free-formulations served as blank for the analytical measurements, when formulations were evaluated.

2.3.2.3. Statistical analyses. Stability data were statistically analyzed by one-way analysis of variance (ANOVA) followed by Tukey test for the evaluation of the pH value and the droplet area to evaluate the influence of the time and the condition of storage. These evaluations were determined using GraphPad Prism® software (3.02 version, 2000). The results were considered significantly different at P-values < 0.05. Regarding the functional stability, it was established the limit of 10% loss of the initial activity for the concentration of 0.019 μL/mL, which caused about 70% of inhibition of chemiluminescence (Casagrande et al., 2006b).

2.4. In vitro permeation/retention study

Preparation of the skin. After institutional approval had been obtained from animal care committee, male hairless mice (HRS/J strain) were sacrificed and full thickness dorsal skin was obtained. Pig ear was obtained from local slaughter house (Pontal, São Paulo, Brazil) and full thickness dorsal skin was obtained too. In both cases, the adhering subcutaneous fat was carefully removed. The cleaned skin was then mounted in a modified Franz diffusion cell (Bentley et al., 1999; Ropke et al., 2002) with 1.77 cm^2 diffusion area. Samples of 1 g of the formulations (F3 and F4) containing or not EEP were placed on the skin (facing the stratum corneum) and the receptor compartment was filled with 12 mL of receptor solution: isotonic phosphate buffer 20 mM (pH 7.4) added of 0.5% (v/v) of polyoxyethylene (20) sorbitan monolaurate in order to ensure "sink conditions" (data not shown). Receptor solution was stirred by a rotating Teflon-coated magnet and were maintained at 37 °C by means of a water bath, circulator and a jacket surround the cells for 12 h. Six diffusion cells were used in this experiment.

After the end of the permeation studies, the formulation was completely removed and the circular area of diffusion was extracted. This tissue was homogenized in methanol (3 mL) using Turratec TE-102-homogeneitor (Tecnal, São Paulo, Brazil) at 18,000 rpm for 1 min, then it was led to ultrasound for 15 min, vortex-mixed for 1 min, and centrifuged for 15 min at 9000 rpm. After filtering the supernatant (0.45 μm filter, Millipore, Brazil) and placing it in a second test-tube, it

was evaporated to dryness under air-flow and then reconstituted in methanol. Nevertheless, before homogenizing the whole pig skin, this tissue was firstly submitted to the retreat of stratum corneum by the tape stripping technique. For this procedure, 15 tapes were successive stripped (Invisible tape) on the area of diffusion and extracted in methanol (3 mL), using ultrasound for 15 min and vortex-mix for 1 min. After filtering the supernatant (0.45 μm filter, Millipore, Brazil), it was placed in a second test-tube and evaporated to dryness under air-flow and then reconstituted in methanol.

The antioxidant activity of the receptor solution and of the skin retention samples was evaluated by the inhibition (%) of chemiluminescence, as described above. However in these studies, the sample volume changed to 50 μL, but the final volume in the test tube was maintained. This method was validated as we previously published (Marquele et al., 2006). The inhibition (%) of light luminescence obtained was transformed in antioxidant activity as propolis extract equivalent (AAPEE) μL/mL, using the regression equation obtained from the calibration curve built by plotting the concentrations of propolis extract (range 0.005–0.014 μL/mL) against the inhibition (%) of each concentration. The blank vehicles without active agents served as references in the analytical measurements.

The recovery of the extraction method was also performed. For this purpose, blank samples of skin homogenate were spiked with known amounts of propolis extract and extracted as described above (Larrucea et al., 2001). The chemiluminescent inhibition found for these samples were compared to freshly prepared samples.

2.5. *In vivo* study to evaluate the photoprotective effect

In vivo experiments were performed on male hairless mouse of the HRS/J strain (30 g) at 3 months of age. Skin erythema was induced by UVB irradiation using an ultraviolet lamp model TL/12RS 40 W Philips. This source emits in the range of 270–400 nm with an output peak at 313 nm resulting in an irradiation of 2.6075×10^{-4} W/cm^2 at a distance of 20 cm as measured by an IL 1700 radiometer (Newburyport, MA, USA) equipped with UVB and UV detector. The minimal erythemal dose (MED) was preliminarily determined (188 mJ/cm^2 for these mice) and irradiation dose corresponding to 2 MED was used.

The protocol consisted of two series of experiments. In the first series (protocol of treatment 1), the test formulations containing or not EEP were applied (200 mg) three times a day, for 3 days on the upper side of the animals ($n = 3$) and on the third day, 1 h after the last treatment, the animals were irradiated. In the second series of experiments (protocol of treatment 2), the animals received the same treatment as group 1, and in addition, they were treated for more 2 days (three times a day) after the irradiation. During irradiation, the back of each animal was divided in two sides using a black tape in order to protect the lower side from the irradiation. The animals were irradiated within their cages. Visual inspection of the erythema, swelling and desquamation was done 24 and 48 h after the exposure. The animals were fed with a standard diet and allowed to drink water *ad libitum*. They were housed within cages with a 12-h light and 12-h dark cycle.

3. Results and discussion

3.1. Stability studies

3.1.1. Short-term physical stability

The formulations developed in this study presented different characteristics, mainly in their lipid content, so several physical instabilities could occur when the complex compounds present in the propolis extracts were added. This is the reason, for a short-term physical stability study.

The centrifugation test is of major interest since it provides fast information about comparable stability properties of different emulsions. In this test, F1 and F2 (formulations containing the propolis extracts and propolis-free formulations) showed phase separation in both storage conditions and in all the period of study. While the other formulations showed stability during the 30 days of study. So, F1 and F2 were withdrawn from the study.

Regarding the organoleptic characteristics, it was observed that the addition of the propolis extracts to the formulations lead to formulations with characteristic appearance, color and smell. The formulations containing the dispersion of Carbopol® 940 (F5 and F6) added with the extracts, presented darkness some days after their manufacturing. The other formulations presented stability in their characteristics during the time and conditions of storage. So, F5 and F6 were withdrawn from the study. The darkness observed in these formulations may be related to their pH value, since they presented pH values (around 6.5) higher than the other stable formulations (around 4.5–5.5).

The measurement of pH of the formulations is necessary to detect pH alterations during time storage, ensuring that the pH value is compatible with the components of the formulation and with the application place, avoiding irritation. It was observed that the addition of the propolis extracts in the formulations caused the decrease in their pH value. The pH values showed minimal changes within the 5.5–5.11 and 4.55–4.4 range for F3 and F4, respectively, which were the most stable formulations in the other physical studies, during the time and conditions of storage.

3.1.2. Long-term physical and functional stability

In this study, F3 and F4 formulations, containing or not the extracts, were evaluated not only in relation to their physical stability, but also in relation to their functional stability. It is noteworthy to point these formulations present different lipid content. F3 can be established as a gel-cream formulation (lower lipid content) and F4 as a cream formulation (higher lipid content). The propolis extracts were also evaluated regarding their functional stability as raw-material.

3.1.2.1. Physical stability. In the centrifugation study, both formulations were stable. In the organoleptic study, both formulations presented characteristic performance when stored at 25 ± 2 °C/AH, however F3 started to present slight darkness after 60 days of preparation, when stored at 40 ± 2 °C/70 ± 5%

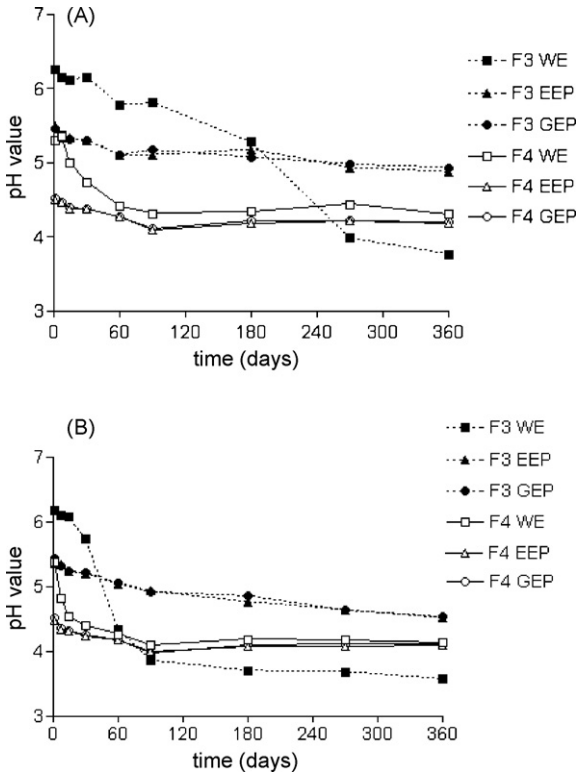

Fig. 1. pH measurement of F3 and F4 stored at 25 ± 2 °C/AH (A) and at 40 ± 2 °C/70 ± 5% RH (B). Results are represented by mean ± S.E.M. of 2 experiments run in parallel (WE = formulation without extract; EEP = formulation added with ethanolic extract of propolis; GEP = formulation added with glycolic extract of propolis).

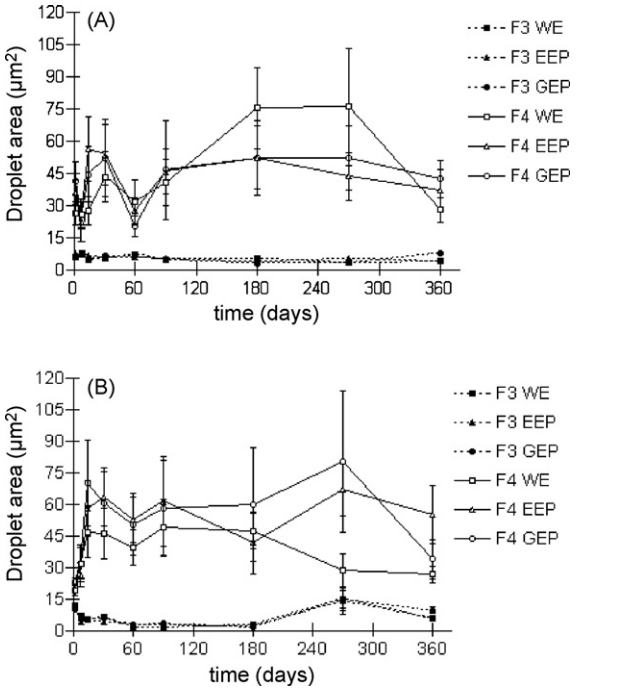

Fig. 2. Droplet area of F3 and F4 stored at 25 ± 2 °C/AH (A) and at 40 ± 2 °C/70 ± 5% RH (B). Results are represented by mean ± S.E.M. of 15 droplets (WE = formulation without extract; EEP = formulation added with ethanolic extract of propolis; GEP = formulation added with glycolic extract of propolis).

RH. In Fig. 1 it can be observed the pH variation of both formulations stored at 25 ± 2 °C/AH and at 40 ± 2 °C/70 ± 5% RH. The data obtained from the pH measurements suggest that both formulations requested a period to stabilize themselves, while the pH value decreases slightly. But after this period, their pH value stabilize and they do not change, which are compatible with topical use. This characteristic was observed in both conditions of study, however at 40 ± 2 °C/70 ± 5% RH, the period to stabilize is longer. For F3 it was observed statistical difference in the period of study and in both conditions of storage. On the other hand, the pH value of F4 became stable after 60 days of manufacturing, when stored at 25 ± 2 °C/AH, and 180 days, when stored at 40 ± 2 °C/70 ± 5% RH.

In Fig. 2, it can be observed the droplet area of the formulations. It can be clearly observed that the droplet size of F3 was much smaller than F4. This characteristic is probably due to the emulsifiers present in the formulations. The droplets from F4, besides being bigger, they did not show homogeneous area. However, these characteristics do not seem to mean instability. When the formulations were stored at 25 ± 2 °C/AH, it can be observed that both formulations maintained their droplet size distribution. This is also true for F4, when stored at 40 ± 2 °C/70 ± 5% RH. But when F3 was maintained at this condition, the droplet area firstly decreased, and then they increased, meaning instability of this formulation. Regarding the statistical analysis, F3 showed statistical difference in both storage conditions in the period of study. However, F4 did not show difference in the period of study ($p < 0.05$, ANOVA, followed by Tukey test).

The physical stability observed for F4 may be related to the compounds of the Polawax® self-emulsifying wax, which leads to the formation of liquid crystal structures. Liquid crystals exhibit optical properties typical of the crystalline state (anisotropy to light, birefringence) and mechanical properties typical of the liquid state (fluidity and surface tension) (Cioca and Calvo, 1990). The liquid crystal structures were observed using microscopy with polarized light (figure not shown). It is proposed that in such emulsions, multilayers of liquid crystals concentrate at the oil droplet–water interface. These multilayers stabilize the emulsion by their ability to cause a reduction in London-van der Vaals forces of attraction, which combined with the high viscosity of the liquid crystalline layers, dramatically delays the processes of coalescence (Eccleston, 1997).

3.1.2.2. Functional stability. As it is well known that propolis has more than 150 identified compounds (Russo et al., 2004), it is a very hard task to establish just a marker compound to evaluate the extract as a raw-material and the formulations developed. In addition, it is suggested that the antioxidant activity desired when propolis extract is added to topical formulations is the result of synergic action of several compounds present in the extract. So, for these reasons, the functional stability of the formulations and of the raw-material (propolis extract) was performed assessing the antioxidant activity by measuring the inhibition of chemiluminescence using the xanthine/luminol/XOD system.

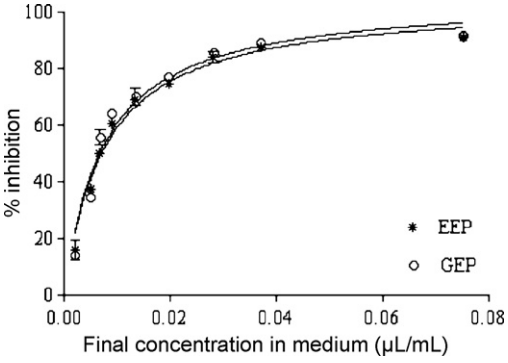

Fig. 3. Inhibition of light emission from xanthine/luminol/XOD luminescent reactions found for different concentrations (0.002–0.037 μL/mL) of ethanolic and glycolic extract of propolis. Results are means ± standard error of 3 experiments run in parallel. The tendency line was built using nonlinear regression (one site binding—hyperbola).

In Fig. 3, it can be observed the dose–response curve of the extracts evaluated in the inhibition of chemiluminescence. The IC_{50} value (the concentration which caused 50% inhibition) found was about 0.007 μL/mL for both EEP and GEP. This IC_{50} value is lower than the values we previously found with other antioxidant methods, such as inhibition of lipid peroxidation (0.016 μL/mL), inhibition of deoxirribose degradation (0.024 μL/mL), inhibition of chemiluminescence using H_2O_2/luminol/HRP system (0.22 μL/mL) (Marquele et al., 2005) and scavenging of the DPPH free radical (0.087 μL/mL) (data not published). Moreover, besides its high sensitivity, this chemiluminescent method showed rapidity and low interferences from formulation compounds (Marquele et al., 2005).

The inhibitory capacity of chemiluminescence was also reported by Pascual et al. (1994) evaluating extracts of propolis from Cuba. These extracts had strong antioxidant activity against superoxide radicals, alkoxyl radicals and in the oxidation reaction of luminol on hydrogen peroxide. In addition, propolis has also shown to inhibit chemiluminescence using neutrophils (Krol et al., 1994; Simões et al., 2004).

In Figs. 4–6, we can find the antioxidant activity of the samples from the stability study. Both extracts as raw-materials maintained their activity in the storage conditions (Fig. 4). The same was found for F4 (Fig. 6), however F3 (Fig. 5) lost some antioxidant activity at the concentration of 0.019 μL/mL during the period of study, when stored at 40 ± 2 °C/70 ± 5% RH. The antioxidant activity of F3 with both extracts, started to decrease significantly after 90 days of storage at this condition, presenting loss of more than 10% of the initial antioxidant activity.

Thus, relating the functional instability with some physical instabilities, such as the droplet area and the color of F3, it can be suggested that this formulation added with both propolis extracts present tendency to instability. On the other hand, F4 added with both propolis extracts presented suitable functional and physical stability. As the formulations presented different lipid content, F3 is a gel-cream formulation and F4 is a cream formulation, it could also be suggested that the different lipid content may be related to the stability of formulations added with propolis extract.

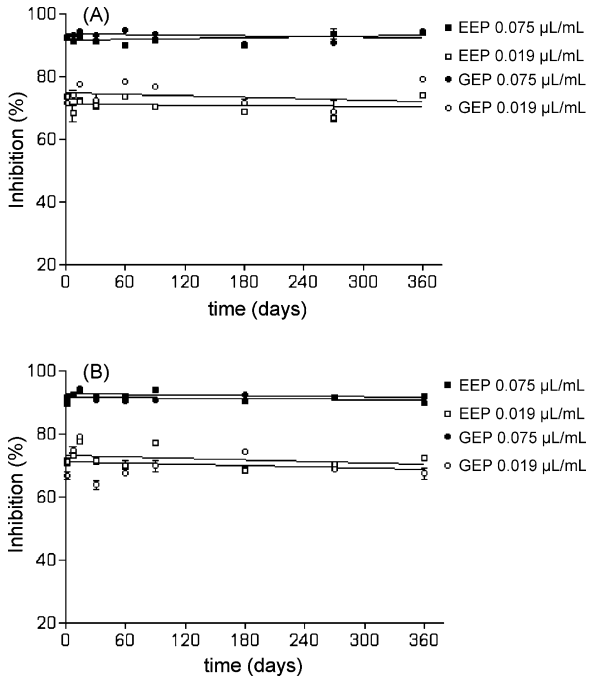

Fig. 4. Functional stability of the propolis extracts as raw-material stored at 25 ± 2 °C/AH (A) and at 40 ± 2 °C/70 ± 5% RH (B). Results are represented by mean ± S.E.M. of 3 experiments run in parallel (—) linear fit.

After the conclusion of the stability study, it was observed that the propolis extracts evaluated (EEP and GEP) presented the same functional stability, not only alone, but also when added to formulations, so only EEP was chosen to continue the following studies, since this extract is most available in market.

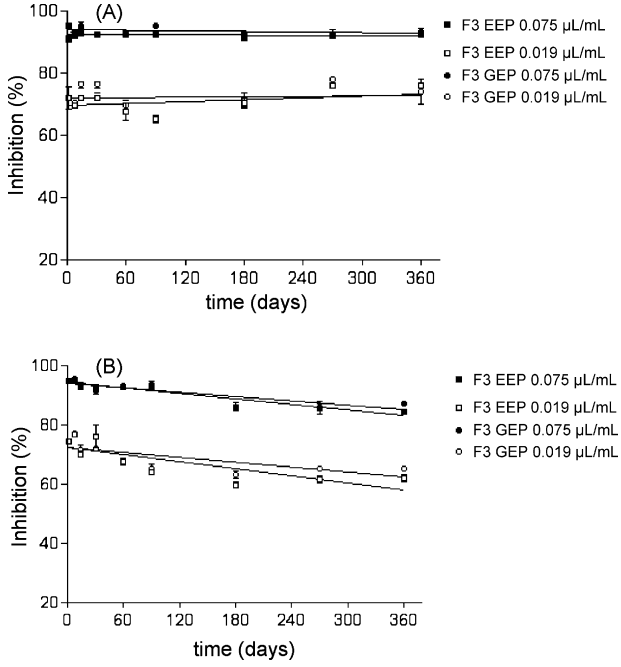

Fig. 5. Functional stability of F3 added with EEP and GEP stored at 25 ± 2 °C/AH (A) and at 40 ± 2 °C/70 ± 5% RH (B). Results are represented by mean ± S.E.M. of 3 experiments run in parallel (—) linear fit.

Table 2
Percentage of inhibition and correspondent AAPEE concentration estimated to the permeation studies after 12 h of experiment

	Permeation					
	Hairless mouse skin			Pig ear skin		
	Inhibition (%)[a] ± S.D.	C_{12}[b] (μL)	Q_{12}[c] (μL/cm^2)	Inhibition (%)[a] ± S.D.	C_{12}[b] (μL)	Q_{12}[c] (μL/cm^2)
F3	42.05 ± 7.50	0.86	0.49	37.51 ± 17.4	0.70 μL	0.40
F4	38.99 ± 14.99	0.76	0.43	22.8 ± 23.49	–[d]	–[d]

[a] Percentage of inhibition found for 50 μL of sample in the reaction medium, $n = 5$.
[b] Total AAPEE content found in the receptor solution ($v = 12$ mL) in 12 h of experiment.
[c] Total AAPEE content found in the receptor solution ($v = 12$ mL) in 12 h of experiment in relation to the permeation area.
[d] The mean value of the inhibition is under the quantification limit of the method, so the correspondent AAPEE content was not estimated.

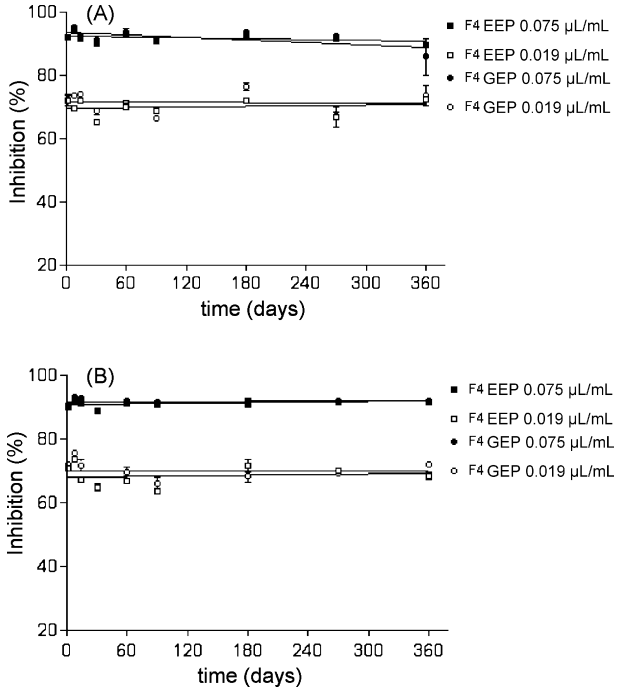

Fig. 6. Functional stability of F4 added with EEP and GEP stored at 25 ± 2 °C/AH (A) and at 40 ± 2 °C/70 ± 5% RH (B). Results are represented by mean ± S.E.M. of 3 experiments run in parallel (—) linear fit.

3.2. In vitro permeation/retention study

Topical administration of antioxidants provides an efficient way to enrich the endogenous cutaneous protection system, and thus may be a successful strategy for diminishing ultraviolet radiation-mediated oxidative damage in skin (Saija et al., 1998). When drugs, be it modern or traditional, are applied topically on the skin, an active agent must be released from the carrier (vehicle) and be able to penetrate into the stratum corneum and lower layers of the skin. But as we have already pointed out, in the case of propolis, and other phytopharmaceuticals, there are many constituents that are able to carry out pharmacological activities, and even the expected activity may be the result of synergic action of several compounds acting together. So, once again, the inhibition of chemiluminescence using the xanthine/luminol/XOD system was the antioxidant method chosen to evaluate the permeation and the skin retention.

In Tables 2 and 3 it can be observed the results of the permeation and the retention of the formulations 3 and 4 added with EEP. In the stratum corneum samples from pig skin, provided by the tape stripping technique, it was observed that all the percentages of inhibition were lower than 10% and they could not be estimated as AAPEE, since they were lower than the quantification limit of the method (0.005 μL/mL, corresponding to 30% of chemiluminescence inhibition) (Marquele et al., 2006). This barrier was separated from the viable epidermis of pig skin, since the stratum corneum is considered the main rate-determining step in skin penetration (Pagliara et al., 1999).

Table 3
Percentage of inhibition and correspondent AAPEE concentration estimated to the retention studies after 12 h of experiment

	Retention					
	Hairless mouse skin[a]			Pig ear skin[b]		
	Inhibition (%)[c] ± S.D.	C_{12}[d] (μL/cm^2)	Q_{12}[e] (μL/g^1)	Inhibition (%)[c] ± S.D.	C_{12}[d] (μL/cm^2)	Q_{12}[e] (μL/g^1)
F3	38.51 ± 15.04	0.014	0.14	14.70 ± 13.03	–[f]	–[f]
F4	33.58 ± 23.08	0.013	0.13	51.28 ± 9.41	0.03	0.12

[a] Full skin.
[b] Skin without stratum corneum.
[c] Percentage of inhibition found for 50 μL of sample in the reaction medium, $n = 5$.
[d] Total AAPEE content retained in the skin after 12 h of experiment and estimated in relation to the permeation/retention area.
[e] Total AAPEE content retained in the skin after 12 h of experiment and estimated in relation to the weight of the skin in the permeation/retention area.
[f] The mean value of the inhibition is under the quantification limit of the method, so the correspondent AAPEE content was not estimated.

The results obtained in this entire study suggest that the antioxidant compounds present in the propolis extract were able to reach lower layers of both skin samples as the antioxidant activity was observed in the viable epidermis of pig skin and in the whole hairless mouse skin. There was not significant difference between the retention values caused by the formulations in the hairless mouse skin (33.5–38.5% of inhibition, corresponding to 0.013–0.014 μL AAPEE/cm^2 for F3 and F4, respectively). However, F4 showed best retention in pig skin (Table 3). It was found around 51.2% of inhibition of the chemiluminescence for F4 (corresponding to 0.03 μL AAPEE/cm^2), against 14.7% for F3 (correspondence to AAEEP not determined). In addition, in the permeation study, some antioxidant activity (from 22.8 to 42.05% of inhibition) was found in the receptor solution for both skin models (Table 2), implying that some antioxidant compounds are able to cross the skin barrier and reach the systemic circulation.

During the evaluation of the extraction method employed, we observed that the recovery was 100 and 93% for the final concentrations in medium of 0.014 and 0.007 μL/mL (corresponding to about 55 and 45% of chemiluminescence inhibition), respectively. So these results are in accordance with the literature recommendations (Bronaugh et al., 1999).

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 (Ropke et al., 2002). Therefore, after evaluating the data obtained, it can be inferred that the objective of the functionalized formulation was attained mainly for F4, which presented the best retention in pig ear skin. Pig ear skin is considered the best skin model, when human skin is not available, since it has been shown to have histological and physiological properties and epidermal lipid biochemistry similar to those of skin from man (Bronaugh et al., 1999). In our studies, we also used skin from hairless mouse as barrier model, because this tissue is also used in such studies (Bronaugh et al., 1999).

3.3. *In vivo study to evaluate the photoprotective effect*

The *in vivo* study to evaluate the photoprotective effect of propolis extract was performed using F4 added with EEP (2.5%), since this was the most stable formulation and in the permeation and retention (hairless) studies, it showed to be similar to F3. The animals from the control groups (no treatment) and the ones with the EEP free-formulations for both protocols of treatment showed evident erythema and swelling after 24 h of irradiation, and evident erythema and slight desquamation after 48 h of irradiation. The animals treated with the formulations added with EEP from both protocols of treatment showed slight decrease in erythema and no swelling after 24 h of irradiation. After 48 h of irradiation, the animals treated with the formulations added with EEP from protocol of treatment 1 showed erythema and desquamation, suggesting that this treatment was only effective in decreasing swelling. On the other hand, in the same period, the animals treated with the formulations added with EEP from protocol of treatment 2 showed decrease in erythema and they mainly presented a great desquamation, which suggests that this treatment must be accelerating the cellular renewing (cicatrisation) and inhibiting the inflammatory process. In spite of the lack of studies evaluating topical formulations added with propolis extract in literature, our findings are in accordance with the studies of Gregory et al. (2002), who reported that a topical formulation added with propolis has improved the cicatrisation and decreased the inflammation in human minor burns.

The evaluation of the erythema was also performed with a chromameter, which is able to measure the erythema (redness) on skin. With this equipment, it was also observed a decrease in the redness of the treated animals; however it was only found significant difference in the animals treated with the formulations added with EEP from protocol of treatment 2 after 48 h of irradiation (data not shown).

Therefore, it can be suggested that the treatments proposed are not able to avoid all the visible alterations caused on skin by UV radiation, however the protocol of treatment 2 seems effective in diminishing photodamages on skin, suggesting that more studies in this area are still necessary.

4. Conclusions

It can be inferred from our results, that the analytical method employed to evaluate the antioxidant activity brought successful results regarding the functional stability and skin retention of topical functionalized formulations.

Regarding to the development of formulations added with propolis extract, it can be concluded that the most stable formulation was prepared with the self-emulsifying wax Polawax®. This formulation retained its physical and functional stability when stored for 360 days at 25 ± 2 °C/AH and at 40 ± 2 °C/70 ± 5% RH. In addition, this formulation also presented good results in the retention study, allowing the antioxidant compounds present in the propolis extract to reach lower layers in pig ear skin and in the whole hairless mice skin. With the visual inspection, it is also possible to suggest that this formulation may be effective in protecting skin from UVB photodamage. Nevertheless, the results here have to be interpreted carefully and other assays need to be done in order to have a complete understanding of the protective effect of formulations added with EEP.

Acknowledgements

The authors are grateful to CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and CNPq, Brazil (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for financial support and Dr. Yara M.L. Valim and Dr. Vitória L.B. Bentley for the lending of Autolumat LB953 and IL 1700 radiometer, respectively.

References

Afaq, F., Mukhtar, H., 2006. Botanical antioxidants in the prevention of photocarcinogenesis and photoaging. Exp. Dermatol. 15, 678–684.

Bentley, M.V.L.B., Marchetti, J.M., Ricardo, N., Ali-Abi, Z., Collett, J.H., 1999. Influence of lecithin on some physical chemical properties of poloxamer gels: rheological, microscopic and in vitro permeation studies. Int. J. Pharm. 193, 49–55.

Bilia, A.R., Bergonzi, M.C., Morgenni, F., Mazzi, G., Vincieri, F.F., 2001. Evaluation of chemical stability of St. John's wort commercial extract and some preparations. Int. J. Pharm. 213, 199–208.

Bronaugh, R.L., Hood, H.L., Kraeling, M.E.K., Yourick, J.J., 1999. Determination of percutaneous absorption by in vitro techniques. In: Bronaugh, R.L., Malbach, H.I. (Eds.), Percutaneous Absorption—Drugs, Cosmetics, Mechanisms and Methodology, 3rd ed. Marcel Dekker, New York, pp. 229–234.

Casagrande, R., Georgetti, S.R., Verri Jr., W.A., Dorta, D.J., dos Santos, A.C., Fonseca, M.J.V., 2006a. Protective effect of topical formulations containing quercetin against UVB-induced oxidative stress in hairless mice. J. Photochem. Photobiol. B 84, 21–27.

Casagrande, R., Georgetti, S.R., Verri Jr., W.A., Jabor, J.R., Santos, A.C., Fonseca, M.J.V., 2006b. Evaluation of functional stability of quercetin as raw material and in topical formulations by its antilipoperoxidative activity. AAPS PharmSci. 7, E1–E8.

Castaldo, S., Capasso, F., 2002. Própolis, an old remedy used in modern medicine. Fitoterapia 73, S1–S6.

Cioca, G., Calvo, L., 1990. Liquid crystals and cosmetics applications. Cosmet. Toiletries 105, 57–62.

Di Mambro, V.M., Fonseca, M.J.V., 2005. Assays of physical stability and antioxidant activity of topical formulations added with different plant extract. J. Pharmaceut. Biomed. 37, 287–295.

Dobrowolski, J.W., Vohora, S.B., Sharma, K., Shah, S., Naqvi, S.A.H., Dandiya, P.C., 1991. Antibacterial, antifungal, antiamoebic, antiinflammatory and antipyretic studies on própolis bee products. J. Ethnopharmacol. 35, 77–82.

Eccleston, G.M., 1997. Functions of mixed emulsifiers and emulsifying waxes in dermatological lotions and creams. Colloid Surf. A 123/124, 169–182.

Georgetti, S.R., Casagrande, R., Di Mambro, V.M., Azzolini, A.E.C.S., Fonseca, M.J.V., 2003. Evaluation of the antioxidant activity of different flavonoids by the chemiluminescent method. AAPS PharmSci. 5, 210–214.

Girotti, S., Fini, F., Ferri, E., Budini, R., Piazzi, S., Cantagalli, D., 2000. Determination of superoxide dismutase in erytrocytes by a chemiluminescent assay. Talanta 51, 685–692.

Gregory, S.R., Piccolo, N., Piccolo, M.T., Piccolo, M.S., Heggers, J.P., 2002. Comparison of propolis skin cream to silver sulfadiazine: a naturopatic alternative to antibiotics in treatment of minor burns. J. Altern. Complement. Med. 8, 77–83.

Hsu, S., 2005. Green tea and the skin. J. Am. Acad. Dermatol. 52, 1049–1059.

Krol, W., Czuba, Z., Sheller, S., Paradowski, Z., Shani, J., 1994. Structure–activity relationship in the ability of flavonols to inhibit chemiluminescence. J. Ethnopharmacol. 41, 121–126.

Kujumgiev, A., Tsvetkova, I., Serkedjieva, Y., Bankova, V., Chistov, R., Popov, S., 1999. Antibacterial, antifungal and antiviral activity of própolis of different geographic origin. J. Ethnopharmacol. 64, 235–240.

Kumazawa, S., Hamasaka, T., Nakayama, T., 2004. Antioxidant activity of propolis of various geographic origins. Food Chem. 84, 329–339.

Larrucea, E., Arellano, A., Santoyo, S., Ygartua, P., 2001. Combined effect of oleic acid and propylene glycol on the percutaneous penetration of tenoxicam and its retention in the skin. Eur. J. Pharm. Biopharm. 52, 113–119.

Marquele, F.D., Di Mambro, V.M., Georgetti, S.R., Casagrande, R., Valim, Y.M.L., Fonseca, M.J.V., 2005. Assessment of the antioxidant activities of Brazilian extracts of propolis alone and in topical pharmaceutical formulations. J. Pharmaceut. Biomed. 39, 455–462.

Marquele, F.D., de Oliveira, A.R.M., Bonato, P.S., Lara, M.G., Fonseca, M.J.V., 2006. Própolis extract release evaluation from topical formulations by chemiluminescence and HPLC. J. Pharmaceut. Biomed. 41, 461–468.

Pagliara, A., Reist, M., Geinoz, S., Carrupt, P.A., Testa, B., 1999. Evaluation and prediction of drug permeation. J. Pharm. Pharmacol. 51, 1339–1357.

Park, Y.K., Alencar, S.M., Aguiar, C.L., 2002. Botanical origin and chemical composition of Brazilian propolis. J. Agric. Food Chem. 50, 2502–2506.

Pascual, C., Gonzalez, R., Torricella, R.G., 1994. Scavenging action of propolis extract against oxygen radicals. J. Ethnopharmacol. 41, 9–13.

Ropke, C.D., Kaneko, T.M., Rodrigues, R.M., da Silva, V.V., Barros, S., Sawada, T.C.H., Kato, M.J., Barros, S.B.M., 2002. Evaluation of percutaneous absorption of 4-nerolidylcathecol from four topical formulations. Int. J. Pharm. 249, 109–116.

Russo, A., Cardile, V., Sanchez, F., Troncoso, N., Vanella, A., Garbarion, J.A., 2004. Chilean propolis: antioxidant activity and antiproliferative action in human tumor cell lines. Life Sci. 76, 545–558.

Saija, A., Tomaino, A., Trombetta, D., Giacchi, M., De Pasquale, A., Bonina, F., 1998. Influence of different penetration on *in vitro* skin permeation and *in vivo* photoprotective effect of flavonoids. Int. J. Pharm. 175, 85–94.

Simões, L.M.C., Gregório, L.E., Da Silva Filho, A.A., de Souza, M.L., Azzolini, A.E.C.S., Bastos, J.K., Lucisano-Valim, Y.M., 2004. Effect of Brazilian green propolis on the production of reactive oxygen species by stimulated neutrophils. J. Ethnopharmacol. 94, 59–65.