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Propolis extract release evaluation from topical formulations by chemiluminescence and HPLC

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Abstract

Propolis is a resinous bee hive product that has many biological activities. Among these activities, the antioxidant activity deserves special interest since it suggests propolis could be successfully applied topically to prevent and treat skin damages. The skin is continuously exposed to free radicals generated in the aging process and by external stimuli such as sunlight. Thus, the development of topical formulations added with propolis extract is justified. However, it raises the necessity of being concerned about the methodologies that could be used to evaluate the propolis extract release from these formulations. So, *p*-coumaric acid content using HPLC and the antioxidant activity using chemiluminescence were used to assess the release of propolis extract from topical formulations. A low fat content formulation (F1) and a high fat content formulation (F2) were evaluated and they showed that after 6 h, 4.6 μg/cm^2 (F1) and 2.75 μg/cm^2 (F2) of the *p*-coumaric acid was released, while it was found that both formulations released about 0.85 μL/cm^2 of the antioxidant activity as propolis extract equivalent (AAPEE). Thus, once the antioxidant activity of propolis extract may be the result of the synergic action of several compounds, the obtained results indicate that a release study would be more conclusive if the antioxidant activity was evaluated, besides the measurement of a marker compound content.

Keywords: Propolis; Release studies; Topical formulation; Chemiluminescence; Antioxidant activity; HPLC

1. Introduction

Propolis is a resinous bee hive product that has been used by man since ancient times for its pharmaceutical properties and it is still used as a constituent of "bio-cosmetics", "health food" and for numerous other purposes [1]. Numerous studies have reported that propolis has a broad spectrum of biological activities such as antioxidant [2–5], cytotoxic, hepatoprotective [6], antiinflamatory [7,8], immunomodulatory [9], antibacterial, antifungal and antiviral [1].

From the biological activities found for propolis, the antioxidant activity deserves special interest since it suggests propolis could be successfully applied topically to prevent and treat skin damages. The skin is continuously exposed to free radicals generated in the aging process and by external stimuli such as sunlight. It is well known that sunlight coupled with living in an oxygen-rich atmosphere causes unwanted and deleterious stresses on skin, since sunlight can overwhelm the antioxidant system, making natural protective controls inadequate, resulting in oxidative damage [10]. Thus, the development of topical formulations added with propolis extract is justified.

When drugs, be it modern or traditional, are applied topically on the skin, an active agent must be released from the carrier (vehicle) before it contacts the epidermal surface and be available for penetration into the stratum corneum and lower layers of the skin, but in the case of propolis, and other phytopharmaceuticals, there are many constituents that are able to be released from the formulation. For example, more than 150 compounds have been identified as constituents of propolis [11], thus, it is difficult to establish just a marker compound to evaluate a release study since the activity desired when propolis extract is added to topical formulations is the result of synergic action of several compounds present in the extract. The objectives of this study were, therefore, to elaborate and validate a HPLC analysis of

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p-coumaric acid (a component present in large amounts in propolis), to evaluate and validate an antioxidant activity of propolis extract by chemiluminescence, and to study the release profile of propolis extract from topical formulations using both validated methods.

2. Experimental

2.1. Chemicals and reagents

p-Coumaric acid was supplied by Sigma–Aldrich (St. Louis, MO, USA), ethanolic propolis extract was purchased from Apis Flora (Ribeirão Preto, Brazil—the extract was 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). Methanol was purchased from Merck (Darmstadt, Germany) and was of chromatographic grade. Acetic acid was supplied by Zilquímica (Ribeirão Preto, Brazil). Sodium chloride and sodium dihydrogen phosphate were obtained from Merck (Darmstadt, Germany) all of analytical grade. The water used to prepare the solutions or mobile phase was purified in a Milli-Q-plus System (Millipore, Bedforte, MA, USA). 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. All the raw materials for the formulations were purchased from Galena (Campinas, SP, Brazil) or were a gift from Clariant (São Paulo, SP, Brazil).

2.2. Determination of p-coumaric acid by HPLC

2.2.1. Apparatus and chromatographic conditions

Analyses were conducted using a Shimadzu (Kyoto, Japan) liquid chromatograph, equipped with an LC-AT VP solvent pump unit and an SPD-10A UV–visible detector operating at 268 nm. Injections were performed manually through a 50 μL loop with a Rheodyne model 7125 injector (Rheodyne, Cotati, USA). Data were collected using a Chromatopak CR6A integrator (Shimadzu, Kyoto, Japan). The separation of *p*-coumaric acid was carried out at 22 ± 2 °C on Lichrospher 100 RP-8 column (Merck, Darmstadt, Germany) using methanol–acetic acid solution 1% (25:75, v/v), at flow rate of 1.2 mL/min. A CN column (4 mm × 4 mm, 5 μm particle size, Merck, Darmstadt, Germany) was used as guard column.

2.2.2. Standard solutions

Stock standard solutions of *p*-coumaric acid were prepared in methanol in the concentration range of 1–200 μg/mL. They were stored frozen at −20 °C, remaining stable for at least 3 months. Working solutions of *p*-coumaric acid were prepared daily in receptor solution in the concentration range of 25–5000 ng/mL. Both standard and working solutions were protected from direct light, since we previously observed *p*-coumaric acid was degraded by light [12]. The reception solution content is further described.

2.2.3. Validation of the method

Calibration curves were obtained by spiking aliquots of 1 mL drug-free receptor solutions with standard solutions of *p*-coumaric acid in the concentration range of 25–5000 ng/mL. No internal standard was used in this method.

The sensitivity of the method was evaluated by determining the quantification limit (LOQ). The LOQ was defined as the lowest concentration that could be determined with accuracy and precision below 20% [13] over five analytical runs and it was obtained using receptor solution (1 mL) spiked with 25 ng/mL of *p*-coumaric acid. Precision was expressed as relative standard deviation (R.S.D.%) and accuracy as percent of deviation between the true and the measured value. To assess within-day precision and accuracy, replicate analyses ($n = 10$) of 1 mL of receptor solution spiked at concentrations of 75 and 4000 ng/mL of *p*-coumaric acid were performed. For between-day assays, quintuplicate receptor solution of *p*-coumaric acid were analyzed for 5 consecutive days ($n = 5$). The selectivity of the method was assured in the release studies, which are further described. For this determination, blank receptor solutions were analyzed before and after release studies.

Freeze–thaw cycle stability and short-term room temperature stability were determined. To perform the freeze–thaw cycle stability test, three aliquots ($n = 3$) at the low (25 ng/mL) and high concentration (4000 μg/mL) of the quality control samples were stored at −20 °C for 24 h and thawed at room temperature protected from direct light. When completely thawed, the samples were refrozen for 12 h under the same conditions. The freeze–thaw cycle was repeated twice more, and then the samples were analyzed on the third cycle. For the determination of short-term room temperature stability, three aliquots of each quality control sample (at the same concentrations as described above) were prepared and kept at room temperature (22 ± 2 °C) for 12 h protected from direct light. After this period, the samples were analyzed. The peak areas obtained from both stability tests were compared to the peak areas obtained with freshly prepared samples. Student's *t*-test was applied, with the level of significance set at $p \leq 0.05$.

2.2.4. Determination of p-coumaric acid in propolis extract

p-Coumaric acid was chosen as marker compound in propolis extract and it was determined after diluting propolis extract 1:1000 in methanol and injecting into the HPLC instrument.

2.3. Determination of the antioxidant activity by chemiluminescence

2.3.1. Apparatus of chemiluminescence

This method was slightly modified from Girotti et al. [14] and Marquele et al. [15]. Chemiluminescent mixture was prepared immediately before analysis by mixing 360 μL glycine buffer (0.1 M pH 9.4, 1 mM EDTA), 150 μL xanthine (6 mM in glycine buffer), 50 μL sample and 10 μL of luminol (0.6 mM). Adding 100 μL xanthine-oxidase solution (20 IU/mL) started the reaction. The buffer and the xanthine solution were stable for 4 and 2 weeks, respectively, when kept at 4 °C, while the XOD and luminol solutions were freshly prepared each time.

Chemiluminescence was measured for 5 min at 25 °C with an Autolumat LB 953 apparatus. Light emission was recorded in counted photons per minute (cpm). The area under the time-course curves (AUC) was calculated. Percentage of inhibition of chemiluminescence of each sample was calculated by the formula:

$$\text{Inhibition}\,(\%) = 100 - \frac{100 \times \text{AUC}_1}{\text{AUC}_0}$$

where AUC_0 represents the area under the curve observed for the control (extract absence) and AUC_1 (experimental samples).

2.3.2. Standard solutions

Stock standard solutions of propolis extract were prepared daily in propylene glycol in the concentration of 100 μL/mL. Working solutions of propolis extract were prepared diluting the stock solution in the drug-free receptor solution in the final medium concentrations range of 0.005–0.014 μL/mL.

2.3.3. Validation of the method

Calibration curve was obtained in the concentration range of 0.005–0.014 μL/mL of propolis extract in medium. The LOQ was defined as the lowest concentration that could be determined with accuracy and precision below 20% [13]. Precision was expressed as relative standard deviation and to assess within-day precision, replicate analyses ($n = 10$) of the concentrations 0.005, 0.007 and 0.0014 μL/mL were performed. For between-day assays, quintuplicate of the same concentrations were analyzed for 5 consecutive days ($n = 5$).

An antioxidant activity stability test was also performed. To perform the freeze–thaw cycle stability test, three aliquots ($n = 3$) at the low (0.005 μL/mL), medium (0.007 μL/mL) and high concentration (0.014 μL/mL) of the quality control samples were stored at −20 °C for 24 h and thawed at room temperature. When completely thawed, the samples were refrozen for 12 h under the same conditions. The freeze–thaw cycle was repeated twice more, and then the samples were analyzed on the third cycle. The antioxidant activities obtained were compared to the activity obtained with freshly prepared samples. The antioxidant activity stability of propolis extract was also evaluated in the receptor solution maintained at 37 °C for 24 h. For this determination the content of propolis extract present in 1 g of formulation (25 μL) was firstly solubilized in propylene glycol and then added to the receptor solution (12 mL) in the same conditions of the release study. An aliquot (600 μL) was withdrawn at specific times during 24 h. The antioxidant activity obtained from the different times were then compared to the first aliquot (time = 0). Student's *t*-test was applied, with the level of significance set at $p \leq 0.05$.

2.3.4. Determination of the antioxidant activity of propolis extract and of p-coumaric acid

The propolis extract was solubilized 1:10 in propylene glycol and then diluted in receptor solution rending the final concentrations in the medium: 0.002, 0.005, 0.007, 0.009, 0.014, 0.019, 0.028, 0.037 and 0.075 μL/mL. *p*-Coumaric acid was diluted in methanol rending the final concentrations in the medium: 0.375, 0.75, 1.5, and 3.0 μg/mL.

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

Component	F1 (%)[a]	F2 (%)[b]
Hostacerin® SAF[c]	6	–
Polawax®[d]	–	8
Isodecyl oleate	–	2
Isopropyl palmitate	–	1.5
Propylene glycol	5	5
Glycerol	–	3
Phenoxyethanol and parabens	0.5	0.5
Water	88.5	80

[a] Lower fat content.
[b] Higher fat content.
[c] Self-emulsifying agent prepared without heating (ammonium acryloyldimethyl-taurate/VP copolymer + rapeseed oil sorbitol esters + trilaureth-4-phosphate + mineral oil + isopropyl palmitate).
[d] Self-emulsifying wax (mineral oil + petrolatum + lanolin alcohol + fatty alcohol + ethoxilated fatty alcohol).

2.4. Formulations

Emulsions were developed using two different self-emulsifying agents, as it can be seen in Table 1. The developed formulations presented different fat material contents next (F1) which had the presence of hydrophilic colloid as emulsion stabilizing agent, presented lower fat content than F2. The preservative was a mixture of phenoxyethanol and parabens. Extract of propolis (2.5%, v/w) and preservatives were firstly solubilized in the propylene glycol amount and next incorporated to the formulations at room temperature. The formulations were allowed to equilibrate for 24 h at room temperature before carrying out the studies.

2.5. In vitro release studies from topical formulations containing propolis extract

The release experiments were performed using modified diffusion cells (Franz type) with 1.77 cm^2 diffusion area and a cellulose ester membrane HA (nitrate 75–80% and acetate—Millipore, Brazil) with pore size of 0.45 μm. Samples of 1 g of the formulations were placed on the membrane 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". Receptor solution was stirred by a rotating Teflon-coated magnet and were maintained at 37 °C by means of a circulating water bath with a jacket surrounding the cells.

Samples from the receptor solution (600 μL) were collected from the Franz cells in the following intervals: 2, 4, 6, 8, 10, 12 and 24 h and the same volume of fresh receptor solution was replaced. The samples were stored until use at −20 °C. The amount of *p*-coumaric acid released in the receptor solution was assayed by HPLC using a calibration curve of standard *p*-coumaric acid. The antioxidant method was used to determine the inhibition (%) of light luminescence caused by the receptor solution. 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 against the inhibition (%) of each concentration. The blank vehicles without active agents served as references in the analytical measurements. Six modified diffusion cells were used in the experiments.

3. Results and discussion

Propolis extract has presented a very important antioxidant activity in several assays that we have performed in our laboratory. Its activity may be compared with other plant extracts and even with known isolated antioxidant compounds. As an example, in the inhibition of lipid peroxidation assay, it showed IC_{50} (concentration which caused 50% of inhibition of the system) of 0.016 μL/mL, while *Glycyrrhiza glabra* showed 0.070 μL/mL, *Ginkgo biloba* 0.05 μL/mL [16], Isoflavin Beta® 0.033 μg/mL, Red clover 0.032 μg/mL (data not published), quercetin 0.34 μg/mL [17] and α-tocopherol 1 μg/mL [16]. Among these methods that we have performed, such as inhibition of lipid peroxidation and degradation of deoxyribose, scavenging of DPPH radical, inhibition of chemiluminescence using H_2O_2/luminol/HRP, the inhibition of chemiluminescence using xanthine/luminol/XOD showed the best sensitivity and reproducibility [15]. So this method was chosen to evaluate the release study, since this study could request a method which is able to quantify a low quantity of antioxidant compounds.

3.1. Determination of p-coumaric acid by HPLC

p-Coumaric acid was chosen as a marker compound to be evaluated in propolis extract because it is a compound found in large amounts in Brazilian propolis collected in southeastern region [18].

The HPLC method developed here to perform the separation of *p*-coumaric acid, proved to be linear over the concentration range of 25–4000 ng/mL, with correlation coefficient, $r \geq 0.9995$. The precision and accuracy of the method were assessed for both within-day (10 spiked receptor solutions for each concentration on the same day) and between-day (5 spiked receptor solution for each concentration for 5 consecutive days) determinations. Table 2 shows the results achieved with two concentrations in the evaluation of the precision and accuracy of the method; neither R.S.D.s nor relative errors exceeded a value of 4.9%, in agreement with literature recommendations [13]. The lowest concentration quantified by the validated method (LOQs) was 25 ng/mL (Table 2). The method developed here proved to be selective since the receptor solutions of the blank formulations analyzed under the established chromatographic conditions did not show any interferences in the retention time of *p*-coumaric acid (Fig. 1C and E). The stability test showed no statistically significant difference between freeze–thaw cycles and short-term room temperature stability studies with *p*-value ≥0.11.

Fig. 1A and B shows the chromatograms of the *p*-coumaric separation alone in receptor solution and in propolis extract.

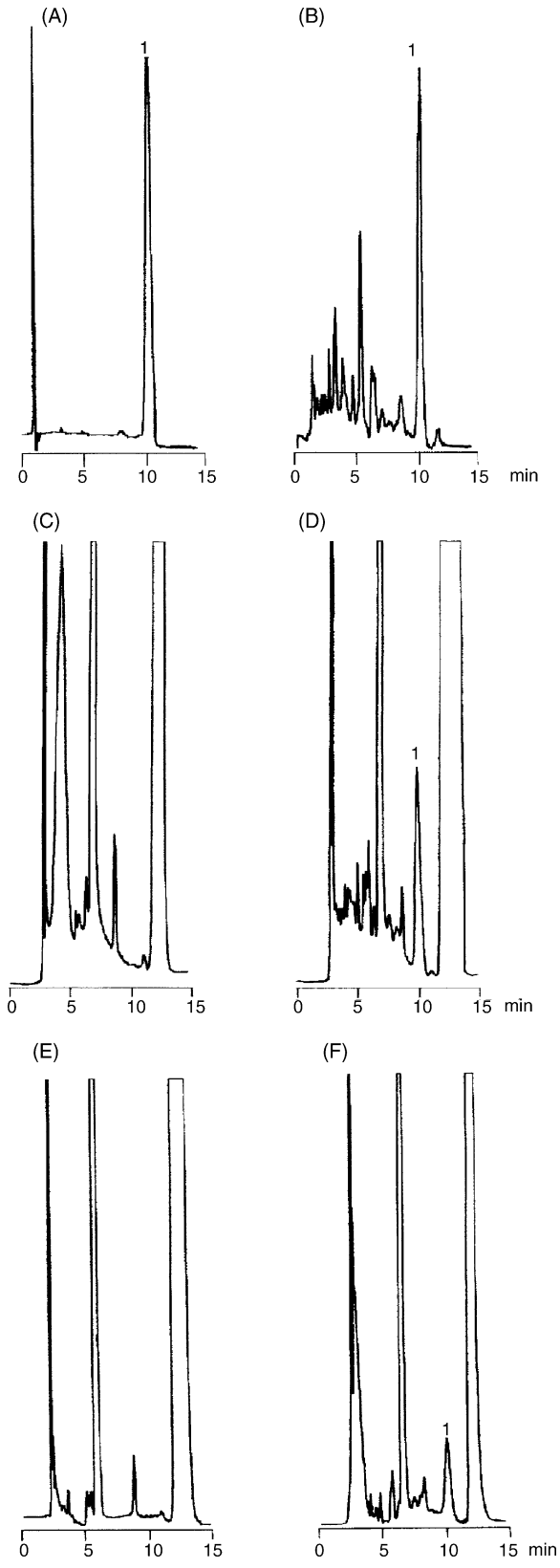

Fig. 1. Chromatograms referring to the separation of *p*-coumaric acid (1). (A) Receptor solution spiked with *p*-coumaric acid, (B) propolis extract diluted 1:1000 in methanol, (C) receptor solution from the blank of formulation 1, (D) receptor solution of formulation 1 after 6 h of release, (E) receptor solution from the blank of formulation 2, (F) receptor solution of formulation 2 after 6 h of release.

Table 2
Precision and accuracy of *p*-coumaric acid evaluated in receptor solution

Concentration added (ng/mL)	Concentration found (ng/mL)	Accuracy[a]	Precision[b]
Within-day ($n = 10$)[c]			
25[d]	26.1	4.73	4.90
75	75.1	0.14	3.38
4000	3900	−2.60	2.19
Between-day ($n = 5$)[e]			
75	73.8	4.25	−1.50
4000	3806	4.77	−4.83

[a] Expressed as deviation from theoretical values.
[b] Expressed as relative standard deviation.
[c] Number of samples.
[d] Quantification limit, $n = 5$.
[e] Number of days.

Table 3
Precision of antioxidant activity of propolis extract evaluated in receptor solution

Concentration added in medium (μL/mL)	Inhibition found (%)	Precision[a]
Within-day ($n = 10$)[b]		
0.005[c]	41.0	8.32
0.007	55.5	2.95
0.014	69.0	2.01
Between-day ($n = 5$)[d]		
0.005	43.4	7.22
0.007	54.5	5.4
0.014	70.8	3.47

[a] Expressed as relative standard deviation.
[b] Number of samples.
[c] Quantification limit, $n = 5$.
[d] Number of days.

It was found 0.82 mg of *p*-coumaric acid in 1 mL of propolis extract.

3.2. Determination of the antioxidant activity by chemiluminescence

As we have already pointed, the chemiluminescent method, due to its high sensitivity, rapidity and reproducibility was chosen for the evaluations of the antioxidant activity of propolis extract and of *p*-coumaric acid. The xanthine/luminol/XOD system emits luminescent light by oxidation of luminol, used as detector, by superoxide anions ($O_2^{\bullet -}$) generated in the following reaction:

$$\text{Xanthine} + O_2 \xrightarrow{\text{XOD}} \text{Uric acid} + O_2^{\bullet -}$$

The ability of a substance in scavenging superoxide anions lead to the inhibition of the luminescent light emission. The inhibition (%) of chemiluminescence versus the amount of propolis extract (μL) in the reaction medium is shown in Fig. 2A.

The method proved to be linear over the final medium concentration range of 0.005–0.014 μL/mL, with correlation coefficient, $r \geq 0.9675$. This correlation coefficient could be properly acceptable, since this method presents multi-stages which involve formation and scavenging of free radicals, besides the presence of an enzyme. The precision was assessed for both within-day (10 replics of the receptor solutions for each concentration on the same day) and between-day (5 replics of each receptor solution for each concentration for 5 consecutive days) determinations. Table 3 shows the results achieved with three concentrations in the evaluation of the precision of the method; the R.S.D.s of the concentrations analyzed did not exceed a value of 8.3%, in agreement with literature recommendations [13]. The lowest concentration in the linear range quantified with R.S.D. lower than 20% (LOQs) was 0.005 μL/mL. The stability tests showed no statistically significant difference in the freeze–thaw cycles and in the antioxidant activity present in the receptor solution during 24 h with *p*-value ≥0.097.

The ability of *p*-coumaric acid in scavenging superoxide anions was also evaluated. The inhibition (%) of chemiluminescence versus the amount of *p*-coumaric acid (μg) in the reaction medium is shown in Fig. 2B.

3.3. In vitro release studies from topical formulations containing propolis extract

The antioxidant defense in skin may be overwhelmed by external stimuli such as prolonged sun exposition [19], pollution and also by internal stimuli such as in the aging process. So free radicals are formed in excess and may lead to several

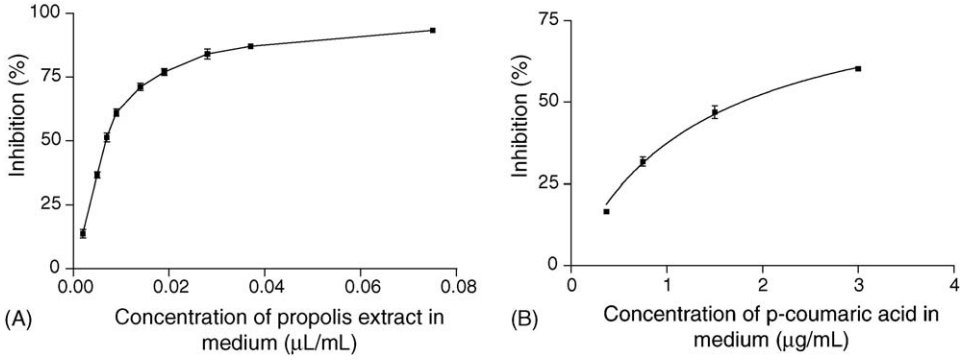

Fig. 2. (A) Inhibition of light emission from xanthine/luminol/XOD luminescent reaction by propolis extract. Results are mean ± S.E. of eight experiments run in parallel. (B) Inhibition of light emission from xanthine/luminol/XOD luminescent reaction by *p*-coumaric acid. Results are mean ± S.E. of three experiments run in parallel.

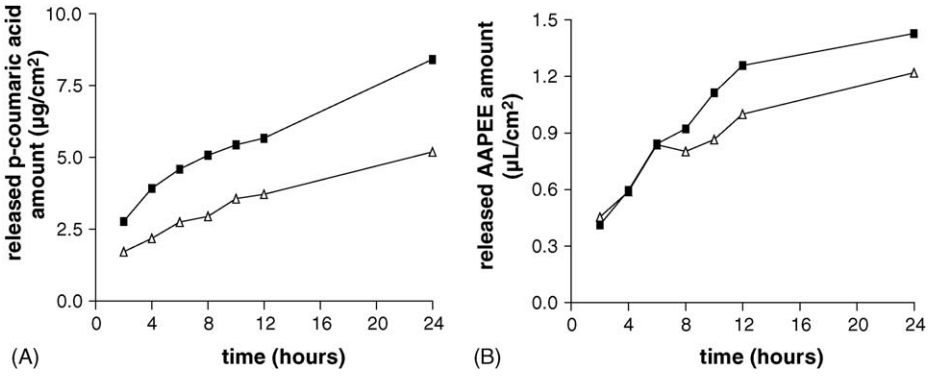

Fig. 3. Released *p*-coumaric acid (A) and AAPEE (B) amount from formulations 1 (■) and 2 (△), added with propolis extract.

damages. To prevent and treat skin damages, propolis extract may be used as a topical antioxidant in formulations. However, when a formulation containing propolis extract is developed, raises the necessity of being concerned with the release of this extract. In release studies the measure of drug release as a determination of bioavailability has been a subject of debate for many years. A consensus has been reached that an in vitro release test can serve as a valuable tool for initial screening of experimental formulations in the product development area and can serve to evaluate bioavailability [20]. Thus, a compound which is in large amount in the extract is usually used as marker. But as the antioxidant activity is the desired action, besides assaying a marker, it would be of very great interest, evaluating the antioxidant activity found in the receptor solution. So, the HPLC and the antioxidant validated methods were used to evaluate the release of propolis extract from formulations by assessing a marker (*p*-coumaric acid) and the antioxidant activity, respectively. The antioxidant activity as propolis extract equivalent was used to estimate the antioxidant activity found in the receptor solution.

Fig. 1D and F shows the chromatograms of the receptor solution of formulations 1 and 2, respectively. The release profiles of *p*-coumaric acid from both formulations are presented in Fig. 3A. It was found that after 6 h, 4.6 μg/cm^2 of the *p*-coumaric acid was released from the formulation 1 (lower fat content), while 2.75 μg/cm^2 was released from formulation 2 (higher fat content), and this difference in the release was maintained in the following hours.

Fig. 3B shows the release profile of the AAPEE. It was found that both formulations released about 0.85 μL/cm^2 of AAPEE in 6 h, but formulation 1 released a higher amount of AAPEE in the following hours. This difference must be due to the fat content of the formulations. The results of the first 6 h suggest that in this period there was mainly a release of the less liposoluble compounds for both formulations. However, in the following hours, the most liposoluble compounds were probably released and the fat content of the formulations influenced the release profile. So F1, which has lower fat content, was able to release these compounds more easily. While for F2, which has higher fat content, presented more likeness to these compounds and prevented them to be released in the same way that F1.

The release data were plotted against square root of time for the determinations of the release rate from the profiles (Fig. 4). Table 4 summarizes the slopes representing the diffusion rates and also the released *p*-coumaric acid and AAPEE amounts after 12 h. The plots were linear as function of square root of time, indicating that the release follows Higuchi model.

From the release studies, it is apparent that *p*-coumaric acid and the AAPEE are better released from formulation 1 (lower fat content) than from formulation 2 (higher fat content). This behavior may be due to several influences, among them, the rheological parameters of the vehicle [21], the pH of the formulations and also due to the solubility of propolis extract in the formulations. Furthermore, knowing that *p*-coumaric acid as well as the antioxidant compounds of propolis extract must be phenolic compounds, these results are in accordance with Gete et al. [20], who concluded that a hydrophilic cream may function best in delivering flavonoids, which are phenolic compounds.

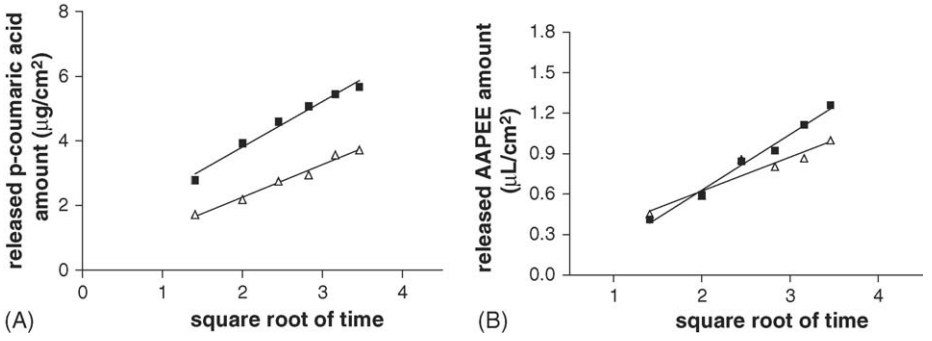

Fig. 4. Cumulative released *p*-coumaric acid (A) and AAPEE (B) through synthetic membrane from formulations 1 (■) and 2 (△), added with propolis extract.

Table 4
Comparison of the diffusion rates, correlation coefficients and the cumulative released *p*-coumaric and AAPEE amounts after 12 h

	p-Coumaric acid			AAPEE		
	Diffusion rate[a]	r^2[b]	Q_{12} (μg/cm^2)[c]	Diffusion rate[a]	r^2[b]	Q_{12} (μL/cm^2)[d]
F1	1.408	0.9895	5.68	0.413	0.9941	1.26
F2	1.012	0.9916	3.72	0.252	0.9502	1.00

[a] Slope of the plots of amount of drug released against square root of time.
[b] Linear correlation coefficients.
[c] Amount of *p*-coumaric acid released in 12 h, assayed by HPLC.
[d] Amount of AAPEE released in 12 h, assayed by chemiluminescence.

Comparing the release profiles in both formulations, it can be concluded that *p*-coumaric acid was released more easily than the AAPEE. This is probably due to the difference in the solubility of *p*-coumaric acid and the propolis extract antioxidant compounds. The high release of *p*-coumaric acid, but low AAPEE release can be explained by the low antioxidant activity of *p*-coumaric acid compared to the propolis extract. Propolis extract showed IC_{50} of 0.007 μL/mL while *p*-coumaric acid showed 1.78 μg/mL. It is also important to note that *p*-coumaric acid may suffer photoisomerization when exposed to light [12], which was observed by the HPLC determination (data not shown), however, the antioxidant activity was not lost. This finding suggests that the antioxidant activity method evaluates the activity which is present, even if a compound had suffered decomposition.

In addition, in order to prove that it was not the *p*-coumaric acid alone, the compound responsible for the antioxidant activity found in the receptor solution, we used the regression equation obtained by plotting the concentrations of *p*-coumaric acid against the inhibition (%) of each concentration, and we estimated the *p*-coumaric acid content that would be corresponding to the inhibitions found in the receptor solution during the release study. After 24 of study, the receptor solution showed 73.1 and 64.9% of inhibition of chemiluminescence for F1 and F2, respectively. To these data, we compared the *p*-coumaric acid content determined by HPLC (Tables 5 and 6). Next, it was observed after 24 h of release, that 1.24 and 0.77 μg/mL of *p*-coumaric acid were released and determined by HPLC for F1 and 2, respectively, while if it was the *p*-coumaric acid, the responsible for the antioxidant activity, the release would be 52.9 and 44.0 μg/mL for F1 and 2, respectively. With this comparison it became easy to observe that many other compounds must have been released and that the antioxidant method is able to determine the whole activity that is released from the formulation.

It is also important to note that the chemical composition of propolis is diversified from region to region even in the same country, as observed by Park et al. [18], the propolis from the southeastern region of Brazil has as majority compounds coumaric acid, pinobanksin and kaempferide, while the propolis from the southern has as majority compounds pinobanksin 3-acetate, chrysin, etc., and in the northeastern, none of these compounds are present. These data also suggest that an antioxidant method would be the most appropriate to evaluate propolis from different regions in order to establish quality parameters and profile of activity.

The results obtained with the antioxidant method show the real antioxidant activity released and which is able to penetrate into the skin. So, the evaluation of the antioxidant activity showed to be an important method to perform release studies of isolated compounds and mainly of extracts constituted for a mixture of antioxidant compounds.

Table 5
Comparison of the *p*-coumaric acid content released from F1 determined by HPLC and determined by its antioxidant activity

Release time, *T* (h)	Inhibition found for the receptor solution in the chemiluminescent assay (%)	Corresponding *p*-coumaric acid content (μg/mL)[a]	*p*-Coumaric content determined by HPLC (μg/mL)
2	38.2	14.4	0.41
4	45.4	22.2	0.58
6	55.1	33.1	0.68
8	56.8	34.9	0.75
10	63.2	42.1	0.80
12	67.7	47.0	0.84
24	73.1	52.9	1.24

[a] The inhibition found in the receptor solution was estimated as of *p*-coumaric acid content by using the regression equation obtained by plotting the concentration of *p*-coumaric acid against the inhibition (%) of each concentration.

Table 6
Comparison of the *p*-coumaric acid content released from F2 determined by HPLC and determined by its antioxidant activity

Release time, *T* (h)	Inhibition found in the chemiluminescent assay (%)	Corresponding *p*-coumaric acid content (μg/mL)[a]	*p*-Coumaric content determined by HPLC (μg/mL)
2	40.0	16.4	0.25
4	45.0	21.9	0.32
6	55.7	33.8	0.40
8	51.5	29.2	0.43
10	52.6	30.4	0.53
12	57.0	35.3	0.55
24	64.9	44.0	0.77

[a] The inhibition found in the receptor solution was estimated as of *p*-coumaric acid content by using the regression equation obtained by plotting the concentration of *p*-coumaric acid against the inhibition (%) of each concentration.

4. Conclusion

As propolis extract is known for its great antioxidant activity, the use of this extract added to topical formulations to prevent and treat skin diseases is justified. During formulation development and the release studies, besides evaluating a marker compound such as *p*-coumaric acid, we showed that the antioxidant activity may also be suitably evaluated. The results obtained with both analyses are more conclusive because the antioxidant activity desired is not only based in one marker compound, but it is the result of the synergic action of several compounds present in the extract.

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