Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 461–468
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Propolis extract release evaluation from topical formulations by chemiluminescence and HPLC

Franciane D. Marquele [a], Anderson R.M. Oliveira [b], Pierina S. Bonato [b], Marilisa G. Lara [a], Maria José V. Fonseca [a,*]

[a] *Department of Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences of Ribeirão Preto, USP Av. do Café s/n, 14049- 903 Ribeirão Preto, SP, Brazil*
[b] *Department of Physics and Chemistry, Faculty of Pharmaceutical Sciences of Ribeirão Preto, USP Av. do Café s/n, 14049- 903 Ribeirão Preto, SP, Brazil*

Received 11 October 2005; received in revised form 8 December 2005; accepted 15 December 2005
Available online 9 March 2006

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

* Corresponding author. Tel.: +55 16 602 4315; fax: +55 16 633 1941.
E-mail address: magika@fcfrp.usp.br (M.J.V. Fonseca).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.12.022

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.