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Analysis of propolis from *Baccharis dracunculifolia* DC. (Compositae) and its effects on mouse fibroblasts

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Abstract

This paper confirms *Baccharis dracunculifolia* DC. (Compositae) as the main botanical source of the propolis from southeastern Brazil (state of São Paulo) investigated to ascertain specific biological activity in relation to mouse NIH-3T3 fibroblasts, skin cells directly involved in the cicatrization processes. Flavonoid and total phenolic compounds were determined by spectrophotometry, and chemical composition by HPLC; the chromatographic profile, characterized largely by flavonoids and aromatic acids, was found to be qualitatively similar to that of *Baccharis dracunculifolia* DC. The adsorption of phenolic compounds in the propolis to skin powder was also investigated, and 68% of these compounds adsorbed to the skin powder. At concentrations from 0.12 to 7.81 μg/ml, the propolis revealed no statistical significant differences from its control solutions; however, at concentrations of 31.25 μg/ml or more, the propolis was toxic to NIH-3T3 cells. Thus, the propolis from *Baccharis dracunculifolia* DC. (Compositae) presents an *in vitro* concentration-dependent toxicity on mouse NIH-3T3 fibroblasts.

Keywords: Propolis; Artepillin C; Phenolics; *Baccharis dracunculifolia* DC.; Fibroblasts; Cicatrization

1. Introduction

Propolis is a generic term used to describe a complex mixture of resinous, gummy and balsamic materials from buds, flowers and plant exudates collected by bees; salivary secretions, wax and pollen are added for the obtention of the final product (Brasil, 2001). The uses of this propolis in the life of the colony are related to both mechanical and antibiotic properties (Ghisalberti, 1979).

The first registers of the utilization of propolis were those involving its use in mummification of the ancient Egyptians. It was also used in the treatment of infections and swelling by the Assyrians. Later, the medicinal utility of propolis in both internal and external cicatrization was described by the early Greeks, especially Aristotle, Dioscorides and Hippocrates; the Romans, specifically Pliny and Galen, also described its medicinal uses (Matsuno, 1997; Pereira et al., 2002b). Since then, there have been numerous references to its ethnopharmacological uses. The Incas employed it as an antipyretic (Castaldo and Capasso, 2002), and it was employed in the healing of wounds during the XIX century Anglo-Boer in South-Africa and the XX century Second World War in the ex-Soviet Union (Ioirish, 1981; Matsuno, 1997). It had also been listed in the London pharmacopoeia of the XVII century (Castaldo and Capasso, 2002). Since the 1980s, its use in alternative medicine has been on the increase, and it is used in the prevention of various diseases, such as diabetes, cancer, and cardiovascular dysfunctions, as well as in the treatment of inflammations (Banskota et al., 2001). In addition, it has been used in topical applications as a tissue regenerating agent, which is one of its most popular uses in the world today (Castaldo and Capasso, 2002).

Numerous biological activities involving propolis have been observed experimentally both *in vitro* and *in vivo*, including antitumour, antioxidant, anti-inflammatory, hepatoprotective,

Abbreviations: Artepillin C, 3,5-diprenyl-4-hydroxicinnamic acid; D10, cell culture medium; EEP, ethanolic extract of propolis; HPLC, high-performance liquid chromatography; MEP, methanolic extract of propllis; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt); PMS, phenazine methosulfate; PE, methanolic plant extract; S.D., standard deviation

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immunomodulatory and antibiotic effects (Kujungiev et al., 1999; Menezes et al., 1999; Banskota et al., 2000; Reis et al., 2000; Kimoto et al., 2001; Sforcin et al., 2001; Oršolić and Bašić, 2003; Sá-Nunes et al., 2003; Mishima et al., 2005). Arvouet-Grand et al. (1993) demonstrated enhanced wound healing in animals, while Magro Filho (1991) and Gregory et al. (2002) demonstrated enhanced tissue regeneration in human sulcoplasties and burns, respectively, although the specific mechanism involved in this healing is not yet fully understood.

More than 200 compounds have been identified in the propolis from different geographical origins, including phenolic acids, flavonoids, terpenes, lignans, amino acids, fatty acids, vitamins and minerals (Walker and Crane, 1987; Greenaway et al., 1991; Marcucci et al., 1996; Markham et al., 1996; Tazawa et al., 1998; Marcucci and Bankova, 1999; Bankova et al., 2000; El Hady and Hegazi, 2002; Kartal et al., 2002; Pereira et al., 2002a).

Although there is a significant literature related to the chemical and biological aspects of propolis, its therapeutic use is still incipient. This is currently attributed to the great variability in chemical composition of the propolis from different regions, since honeybees extract raw materials from different plants in different ecosystems for their production of propolis (Greenaway et al., 1990; Tomás-Barberán et al., 1993; Wollenweber and Buchmann, 1997; Bankova et al., 2000; Cuesta-Rubio et al., 2002; Park et al., 2002a,b). However, some investigations suggest common botanical sources and, consequently, similar chemical profiles for large geographical areas. For example, various authors have concluded that *Populus* spp. and its hybrids are the main sources of the propolis produced in temperate zones (Europe, North America and non-tropical regions of Asia), and that this type of propolis is characterized by a predominance of flavonoids (Greenaway et al., 1990; Bankova et al., 2000; Bankova, 2005). For the propolis of southeastern Brazil, *Baccharis dracunculifolia* DC. (Compositae) has been suggested as the main botanical source (Park et al., 2002a, 2004; Kumazawa et al., 2003); this type of propolis seems to be characterized by a predominance of phenolic acids, specially prenylated derivatives of *p*-coumaric acid (Marcucci and Bankova, 1999; Pereira et al., 2002b). Studies of the biological activities of propolis should thus be complemented by information about chemical composition and botanical source of the sample, or at least mention of geographical origin, so that these biological activities can be linked to the specific type of propolis.

The present study thus investigates the influence of a specific type of propolis with known chemical composition and botanical source on mouse NIH-3T3 fibroblasts, which are intimately involved in the cicatrization processes on the dermal level.

2. Materials and methods

2.1. Materials

2.1.1. Propolis and source plant

Three samples of greenish propolis produced by honeybees (*Apis mellifera* L.) in an apiary located in the Environmental Protected Area "Serra do Japi" (in Cabreúva, state of São Paulo, Brazil) were collected at three different times during a year (February, July and August). Buds, unexpanded leaves and parts of what was assumed to be the botanical source (popularly know "alecrim-do-campo" or "vassourinha") were also collected from the area. Since preliminary HPLC analysis of the three samples revealed similar profiles, a single one was selected for chemical quantification and biological testing.

2.1.2. Drugs and reagents

Ferulic, *p*-coumaric and chlorogenic acids were purchased from Fluka (Switzerland); caffeic, gallic and *trans*-cinnamic acids, as well as quercetine dehydrate, were purchased from Sigma–Aldrich (Switzerland); kaempferol, kaempferide and isosakuranetin were provided by Eckhard Wollenweber (Institut für Botanik der Technische Universität Darmstadt, Germany); Artepillin C (3,5 diprenyl-4-hydroxycinnamic acid) was provided by Hayashibara Biochemical Labs, Inc. (Japan). Folin-Denis reagent was prepared by adding 20 g of sodium tungstate dihydrate, 4 g of phosphomolybidic acid and 10 ml of phosphoric acid to 150 ml of distilled water, and the solution refluxed for 2 h, cooled and diluted to 200 ml. The reagents (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS)) and phenazine methosulfate (PMS) were purchased from Promega Co. (USA). Skin powder was purchased from Merck (Germany).

2.2. Preparation of propolis and plant extracts

2.2.1. Ethanolic extract of propolis (EEP)

Ninety grams of crude powered propolis were extracted with 190 ml ethanol for 90 days. The resulting solution was filtered, concentrated under a reduced pressure of 450 mmHg at 75 °C, and dried at 70 °C for 4 h.

2.2.2. Methanolic extract of propolis (MEP) and methanolic plant extract (PE)

Ten grams of crude powered propolis or 10 g of buds and unexpanded leaves of *Baccharis dracunculifolia* DC. (Compositae), were extracted with 150 ml over 8 h in a Soxhlet apparatus. The extracts were concentrated and dried as described for the preparation of EEP.

2.3. Total flavonoid content

A standard curve was built with quercetin reference solutions. Aliquots ranging from 2 to 6 ml of standard quercetin ethanolic solution (50 μg/ml) were pipetted into 25 ml volumetric flasks containing 1.0 ml of aqueous aluminum chloride solution at 2.5% (w/v) and the volume made up with ethanol. The blank was prepared by diluting 1 ml of aluminum chloride solution in a 25 ml volumetric flask with ethanol. After 30 min, the absorbance was measured at 425 nm. For the determination of total flavonoid content in the propolis, 2 ml of propolis solution at a concentration of 2 mg/ml (100 mg of MEP dissolved in 50 ml volumetric flask with ethanol) were used, proceeding in

the same manner described for the reference solutions (Vennat et al., 1992).

2.4. Total phenolic content

A standard curve was built with gallic acid reference solutions. Aliquots ranging from 2 to 9 ml of standard aqueous gallic acid solution (100 μg/ml) were pipetted into 100 ml volumetric flasks containing 70 ml distilled water. Five milliliters of Folin-Denis reagent and 10 ml of saturated sodium carbonate solution was added, and the volume was made up with distilled water. The solution was thoroughly mixed. The blank was prepared in the same way, but without the gallic acid aliquot. After 30 min, absorbance was measured at 760 nm. For determination of the total phenolic content of propolis, 3 ml of aqueous solution at a concentration of 2 mg/ml (200 mg of MEP dissolved in 5 ml of methanol and diluted in water to 100 ml) were used, proceeding in the same manner described for the reference solutions (Waterman and Mole, 1994).

2.5. Adsorption of phenolics on skin powder

The adsorption of phenolics to skin powder was investigated using a modification of the methodology described by Costa (1982). Two hundred milligrams of EEP were dissolved in 5 ml of methanol and the solution diluted in a 100 ml volumetric flask with distilled water to reach a stock solution of 2 mg/ml (S1). An aliquot of 50 ml of the stock solution was mixed with 0.5 g of human skin powder for 1 h. The solution was then filtered, and material (S2) was collected. The percentage of phenolics adsorbed to the skin powder was calculated by the ratio of the concentrations of phenolics in the S1 and S2 solutions, using the standard curve described in Section 2.4.

2.6. High-performance liquid chromatography

One hundred and fifty milligrams of each extract (Section 2.2) were dissolved in 5 ml of methanol and filtered through a teflon mini-sart membrane of 0.45 μm. These solutions (20 μl) were analysed in a HPLC apparatus (10AD VP, Shimadzu, Japan), equipped with two pumps (LCD-M 10AD VP, Shimadzu, Japan), an auto-sample (SIL-10AD VP, Shimadzu, Japan) and a photodiode array detector (SPD-M 10A VP, Shimadzu, Japan). Separation was achieved on a C-18 column (SHIM-PACK CLC-ODS) (250 mm × 4.6 mm; 5 μm particle size) using water–acetic acid (19:1, v/v) (solvent A) and methanol (solvent B). The elution was carried out with a flow rate of 1 ml/min, distributed along the following gradient: 70–60% A (0–15 min), 60–50% A (15–30 min), 50–40% A (30–45 min), 40–25% A (45–65 min), 25% A (65–85 min), 25–10% A (85–95 min), 10–70% A (95–105 min) and 70% A (105–115 min) (Alencar, 2002). Detection was monitored at 280 and 340 nm, and the compounds identified were quantified using standards as references (Marcucci et al., 2001). Quantification was carried out using standard *concentration versus area* curves (calculated using SHIMADZU CLASS VP software) at a minimum of five points.

2.7. Cell culture

Mouse NIH-3T3 fibroblasts (ATCC, USA) were cultivated in cell culture medium (D10; prepared with 90 ml of *Dulbeccos's Modified Eagle Medium*, 10 ml of fetal calf serum, 400 mmol of glutamine, 10,000 UL of penicillin, 10 mg of streptomycin and 2.5 μg of amphotericin B) at 37 °C in 5% CO_2 at 95% humidity.

2.8. Test and control solutions

Stock solution at a concentration of 100 mg/ml was prepared by dissolving EEP in polyethylene glycol 400. An aliquot of the stock solution (or the same volume of solvent for the controls), was diluted in D10, giving two "mother solutions". Serial dilutions (1:2 or 1:1.5) with each solution were carried out in D10, resulting in test solutions ranging from 125 to 0.12 μg/ml each with respective controls.

2.9. Effect of propolis on NIH-3T3 cells

One thousand cells were seeded into 93 wells of a Multi-Well plate and volumes adjusted to 50 μl with D10. The cells were incubated at 37 °C in 5% CO_2 at 95% humidity. After 24 h, 50 μl of test (T) or control (C) solutions were transferred to 90 wells, distributed in triplicates. Three wells were prepared as negative controls (C^-), receiving only 50 μl of D10. Aliquots of 100 μl of D10 were pipetted into the last three wells (without cells) to serve as blanks. After 72 h, the solutions were removed and aliquots of 120 μl of MTS/PMS/D10 solution (prepared with 2 ml of MTS solution diluted in 10.5 ml of D10, followed by the addition of 100 μl of PMS solution; Promega Corporation, 2001) were transferred to the 96 wells, and the cells were incubated at 37 °C in 5% CO_2 at 95% humidity. After 5 h, the absorbance of each well was read at 490 nm with an Elisa-reading spectrophotometer.

2.10. Statistical analysis

To make comparison of results of the effect of propolis on mouse NIH-3T3 cells from different assays feasible, the percentage of cell viability was calculated as the ratio of the absorbance of the test (T) or control (C) to the mean absorbance of its three respective negative controls (100 T/C^- or 100 C/C^-), for each assay. The data are expressed as mean ± S.D. Statistical analyses were carried out for eight assays. Control charts with 99% confidence intervals were established (Box et al., 1978).

3. Results

3.1. Global yields of propolis extracts

The global yields for the MEP and EEP were 53.73 ± 1.55 and 38.34 ± 2.05% (w/w), respectively.

Table 1
Compounds identified in ethanolic extract of propolis (EEP) and methanolic extract of propolis (MEP)

Compounds	Content (%)[a]	
	EEP	MEP
Artepillin C	3.38	5.33
p-Coumaric acid	0.63	1.14
Ferulic acid	+	+
trans-Cinnamic acid	0.05	0.08
Chlorogenic acid	0.21	0.45
Caffeic acid	0.04	0.04
Kaempferol	0.08	0.12
Kaempferide	0.63	0.94
Isosakuranetin	0.12	0.19

+: detected.
[a] Expressed as percentage of crude propolis (w/w), mean of triplicate analyses for each sample.

3.2. Total phenolic and flavonoid contents

The total phenolic and flavonoid contents quantified in the MEP, expressed as gallic acid and quercetin equivalents in crude propolis, were 7.39 ± 0.01 and $2.64 \pm 0.00\%$ (w/w), respectively.

3.3. Adsorption of phenolics to skin powder

The assay showed that $68.14 \pm 2.75\%$ of the phenolic compounds present in the EEP adhered to skin powder.

3.4. High-performance liquid chromatography

Fig. 1 shows the HPLC chromatograms for EEP, MEP and PE, while Table 1 identifies the compounds and their respective quantities in EEP and MEP.

3.5. Botanical source of propolis

The presence of corresponding peaks with the same retention times under the same chromatographic conditions (Fig. 1) on the chromatograms of the MEP and PE indicated that this plant species was indeed a botanical source for the propolis sampled. The species was identified as *Baccharis dracunculifolia* DC. (Compositae) by Mara Magenta of the Institute of Biosciences of the University of São Paulo, in the state of São Paulo in Brazil. The Voucher specimen (*C.S. de Funari 01* – SPF) was deposited in the herbarium of the Institute of Biosciences, University of São Paulo.

3.6. Effect of propolis on NIH-3T3 cells

Fig. 2 shows the general profile for the eight assays (a total of 21 replications of 1.46, 0.73 and 0.37 μg/ml, as well as 24 replications of the other concentrations), with test solutions ranging from 0.12 to 125 μg/ml.

The statistical analysis demonstrated that propolis was toxic to mouse NIH-3T3 cells at 125 and 62.5 μg/ml, whereas the

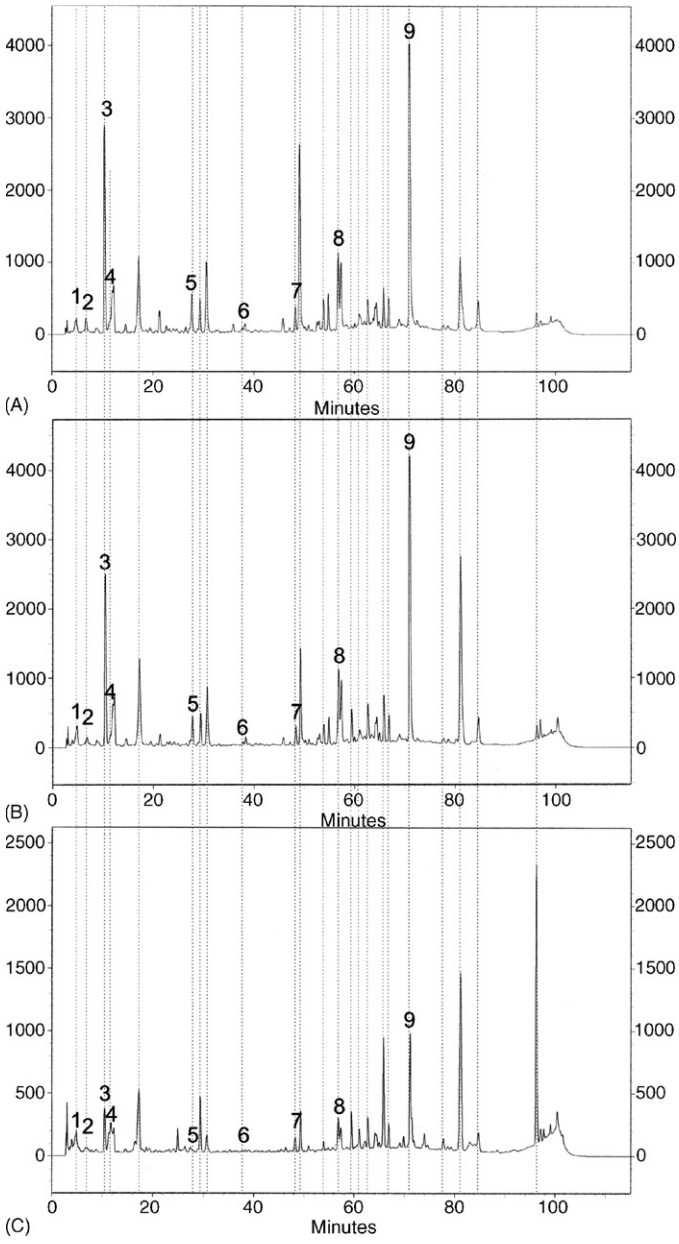

Fig. 1. HPLC chromatograms of the ethanolic extract of propolis (A), methanolic extract of propolis (B) and methanolic plant extract identified as *Baccharis dracunculifolia* DC. (C), at 280 nm. Identified peaks: [1] chlorogenic acid (Rt: 4.851); [2] caffeic acid (Rt: 6.773); [3] *p*-coumaric acid (Rt: 10.506); [4] ferulic acid (Rt: 11.500); [5] *trans*-cinnamic acid (Rt: 27.818); [6] kaempferol (Rt: 37.926); [7] isosakuranetin (Rt: 48.452); [8] kaempferide (Rt: 56.936); [9] Artepillin C (Rt: 71.136). Numbers and parallel lines were inserted across the chromatograms to facilitate comparison.

respective control solutions were not toxic. Even at a concentration of 31.25 μg/ml, half of the replications revealed results outside the lower control limit (data not shown), and should probably be considered toxic. From 15.62 to 0.12 μg/ml of propolis, no significant statistical differences were observed between test solutions (T) and their respective controls (C). Fig. 2 shows that propolis at 125 μg/ml killed about 98% of the cells (2% cell viability) in relation to the viability of the respective negative controls; moreover, the toxicity decreased gradually at

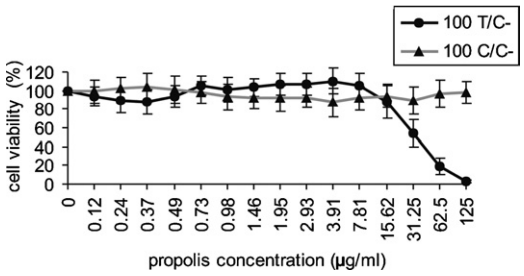

Fig. 2. Concentration-dependent effects of propolis solutions (T) and their controls (C) on mouse NIH-3T3 fibroblasts. Percentage of cell viability was calculated as the ratio of the absorbance of the test (T) or control (C) to the mean absorbance of its three respective negative controls (100 T/C$^-$ or 100 C/C$^-$), for each assay. The data are expressed as mean ± S.D. for eight assays.

concentrations of 62.5 and 31.25 μg/ml, with cell viability of about 20 and 65%, respectively.

4. Discussion and conclusions

Due to the differing nature of the tests to be performed, two different extracts were used. The extract using ethanol (EEP) was designed to reproduce what is done in regional folk medicine; this was used in experiments with both mouse NIH-3T3 fibroblasts and skin powder. The extract using methanol (MEP) was prepared to enable a more rapid preparation and chemical analysis.

The literature attributes the biological activity of propolis largely to the phenolic compounds especially flavonoids and aromatic acids. In Europe, for example, the propolis is cited as having a large flavonoid content often surpassing 20% (Marcucci and Bankova, 1999). The propolis studied here was found to have only 2.64% (w/w) of flavonoids, although this figure is in agreement with the results of other studies of Brazilian propolis using spectrophotometric methods (3, 3.26 and 0.77–2.69% found by Bonvehí and Coll, 1994; Woisky and Salatino, 1998; Chang et al., 2002, respectively). These values may, however, be underestimated, since the use of aluminum chloride is specific for flavones and flavonols (Chang et al., 2002) whereas in the HPLC analysis of the propolis of the present study, the presence of the flavanone isosakuranetin was detected.

In addition to these flavonoid components, the propolis studied here was marked by the presence of other phenolic compounds (a total of 7.39% w/w), although this value may also be underestimated because HPLC analysis of the propolis revealed a value higher than that of the spectrophotometric assay (8.2% w/w; Table 1). Moreover, some of the unidentified peaks (Fig. 1) may also correspond to phenolic compounds, since such aromatic compounds show intense absorption in the ultraviolet region of the spectrum studied here. In fact, Woisky and Salatino (1998) reported the presence of a relatively large percentage of phenolic compounds in different samples of propolis from the state of São Paulo (from 8.78 to 13.72% w/w) with the spectrophotometric method, but using Folin-Ciocalteau reagent instead of the Folin-Denis reagent used here.

The chromatograms of the two extracts of propolis are shown in Fig. 1. The peak profiles are very similar, with the differences in peak intensity observed easily explained by the extractive methods employed (maceration versus soxhlet) and solvent (ethanol versus methanol). The most abundant compound identified (Table 1) was Artepillin C, a prenylated phenolic acid linked to various biological activities: antibiotic (Aga et al., 1994), antioxidant (Nakanishi et al., 2003; Kumazawa et al., 2004; Shimizu et al., 2004) and antitumour (Kimoto et al., 1998; Akao et al., 2003). The abundance of this compound corroborates the findings of Nakanishi et al. (2003), who reported it to be the main compound in propolis from the state of Minas Gerais, also in the southeast of Brazil. These studies thus suggest that Artepillin C may be a phytochemical marker for the propolis from the southeastern region of Brazil.

There are differences between the peak intensities of the methanolic extract of propolis and that of the plant itself (Fig. 1). These differences, however, may have been due to the sampling procedures used, since entire leaves were collected, whereas bees bite off only the margins of young leaves (Kumazawa et al., 2003). The confirmation of *Baccharis dracunculifolia* DC. (Compositae) as a main source of this propolis sample from the Serra do Japi, in the state of São Paulo, corroborates the reports of Park et al. (2002a), Kumazawa et al. (2003) and Park et al. (2004), who also identified this species as the main botanical source of propolis from the southeastern states of São Paulo and Minas Gerais, thus reinforcing the hypothesis of a common botanical source in the southeast of Brazil.

The inclusion of information about the botanical source and chemical composition of the propolis used in a biological study, or even its geographical origin, may help us identify links between specific types of propolis and specific biological activities. Such knowledge may even lead to the development of a classification for propolis which can be used to establish standards of quality for therapeutic intervention.

The strong adherence of phenolics from EEP to human skin powder observed here (68.14%) may have been the result of an ability to form a complex with protein by hydrogen bonding. Propolis, like tannic drugs, may lead to the formation of a thin impermeable film, thus protecting wounds from external aggression and facilitating the onset of cicatrization. This hypothesis will be investigated in future studies.

Despite differences in cell type and concentration, the *in vitro* assays carried out here on mouse NIH-3T3 fibroblasts seem to corroborate the results found by Al-Shaher et al. (2004). Also working with propolis from the state of São Paulo, this author found a decrease in human fibroblast viability from the 75 and 100% observed with 1 mg/ml of propolis to 44 and 50% for 4 mg/ml for periodontal ligaments and dental pulp, respectively. In the present study, concentrations of 31.25 μg/ml or more of propolis lead to cell death of mouse NIH-3T3 fibroblasts while those equal to or less than 15.62 μg/ml revealed no significant effect. In short, the propolis from *Baccharis dracunculifolia* DC. (Compositae) investigated here has been found to exert a concentration-dependent toxic effect on mouse NIH-3T3 fibroblasts.

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