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Original Article

Seasonal Variation, Chemical Composition and Antioxidant activity of Brazilian Propolis Samples

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Total phenolic contents, antioxidant activity and chemical composition of propolis samples from three localities of Minas Gerais state (southeast Brazil) were determined. Total phenolic contents were determined by the Folin–Ciocalteau method, antioxidant activity was evaluated by DPPH, using BHT as reference, and chemical composition was analyzed by GC/MS. Propolis from Itapecerica and Paula Cândido municipalities were found to have high phenolic contents and pronounced antioxidant activity. From these extracts, 40 substances were identified, among them were simple phenylpropanoids, prenylated phenylpropanoids, sesqui- and diterpenoids. Quantitatively, the main constituent of both samples was allyl-3-prenylcinnamic acid. A sample from Virginópolis municipality had no detectable phenolic substances and contained mainly triterpenoids, the main constituents being α- and β-amyrins. Methanolic extracts from Itapecerica and Paula Cândido exhibited pronounced scavenging activity towards DPPH, indistinguishable from BHT activity. However, extracts from Virginópolis sample exhibited no antioxidant activity. Total phenolic substances, GC/MS analyses and antioxidant activity of samples from Itapecerica collected monthly over a period of 1 year revealed considerable variation. No correlation was observed between antioxidant activity and either total phenolic contents or contents of artepillin C and other phenolic substances, as assayed by CG/MS analysis.

Keywords: antioxidant activity – *Apis mellifera* – *Baccharis dracunculifolia* – DPPH – propolis – seasonality

Introduction

Propolis is currently a popular alternative medicine in various parts of the world, including Japan and the European Union. It is a complex mixture of substances collected by honeybees from buds or exudates of plants (resin), beeswax and other substances, such as pollen and sugars. Plant source, physicochemical properties and antibacterial activity are important parameters for propolis quality evaluation (1).

Leaf-buds of *Populus nigra* (black poplar) are sources of propolis resin in temperate regions (2). Propolis resin from Europe and China contain predominantly flavonoids and secondarily phenolic acid esters (3). Iranian propolis has been shown to contain aromatic acids (benzoic and benzenepropanoic), esters of caffeic and

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phenylethyl-*trans*-4-coumaric acids, flavonoids (pinocembrin, chrysin), among other constituents (4). Instead, the resin source of the most prized Brazilian propolis, namely green or alecrim propolis, has been established as buds of *Baccharis dracunculifolia* ('alecrim'), an Asteraceae from southeast and western-central Brazil (5–7). Prenylated derivatives of *p*-coumaric acid predominate in alecrim propolis (5–11). Artepillin C (4-hydroxy-3,5-diprenyl cinnamic acid), drupanin (4-hydroxy-3-prenyl cinnamic acid) and (*E*)-3-prenyl-4-(dihydrocinnamoyloxy)-cinnamic acid were found in both *B. dracunculifolia* and propolis (12). Artepillin C, drupanin, *p*-coumaric and caffeic acids are major constituents of a propolis sample from São Paulo state (southeast Brazil) (11). Recent paper (13) reported a new type of Brazilian propolis. It is red colored and contains compounds not found in alecrim propolis, including isoflavonoids and prenylated benzophenones.

Several biological activities, such as anticancer, antioxidant, anti-inflammatory, antiseptic, antimycotic, bacteriostatic, astringent, anti-ulcer, choleretic, spasmolytic and anaesthetic properties have been reported for propolis and its constituents (12–18). Alecrim propolis with high contents of artepillin C exhibited *in vitro* concentration-dependent toxicity on mouse NIH-3T3 fibroblasts, cells involved in cicatrization processes (13). Both *in vitro* and *in vivo* evidences were raised that alecrim propolis protects against retinal damage (19). Water extract of Brazilian alecrim propolis and some of its constituents, derived from caffeoylquinic acid, protect RGC-5 cells from oxidative stress-induced cell death (13).

Reactive oxygen species (ROS) are implicated in a wide range of human diseases, such as atherosclerosis and certain cancers. When an imbalance between ROS generation and antioxidants occurs, oxidative damage will spread over most cell targets (20) (DNA, lipids, proteins, etc). Hence, the study of antioxidant substances in foods and medicinal natural sources has gained increased interest. Such substances are currently recognized as effective aids for the treatment and prevention of human diseases. Among antioxidants, many stemming from plants have one or more phenolic hydroxyls. Phenolic compounds may exert antioxidant effects as free radical scavengers, as hydrogen donating sources or as singlet oxygen quenchers and metal ion chelators (21). Phenolic compounds are known to counteract oxidative stress in the human body by helping maintaining a balance between oxidant and antioxidant substances (22,23).

Flavonoids and phenolic acids are major classes of phenolic compounds, whose structure-antioxidant activity relationships in aqueous or lipophilic systems have been extensively reported (24). In addition to antioxidant activity, many phenolic compounds have been shown to exert anticancer or anticarcinogenic/antimutagenic activity to a greater or lesser extent (25,26). Their physiological and pharmacological activities may be derived from their antioxidant properties, which are related to their molecular structure (27). Mechanisms of antioxidant action may include suppression of ROS formation, removal or inactivation of oxygen reactive species and up-regulation or protection of antioxidant defenses (28,29).

Development and utilization of more effective antioxidants of natural origin are desired. Naturally occurring polyphenols are expected to help reducing the risk of various life-threatening diseases, including cancer and cardiovascular diseases, due to their antioxidant activity. Propolis possesses antioxidant activity, its constituents being able to scavenge free radicals (30). On the other hand, propolis chemical composition (and hence antioxidant activity) may vary widely according to locality, epoch of collection or simply comparing one hive with another (6). The purpose of the present study is to determine the chemical composition and antioxidant activity of three propolis samples, each from one locality of the state of Minas Gerais (southeast Brazil). It is expected that such analyses may help understanding relationships between composition and antioxidant activity. In addition, it is intended to evaluate the effects of seasonality on chemical composition and antioxidant activity of propolis samples from a same apiary collected over a period of 12 months.

Methods

Material

Propolis samples of Africanized *Apis mellifera* were collected monthly over a period of 1 year in three apiaries from the state of Minas Gerais (southeast Brazil), one of them in the municipality of Itapecerica (It) (20° 32′S, 45° 13′O), another in Paula Cândido (PC) (20° 49′S, 42° 54′O) and the third one in Virginópolis (Vi) (18° 50′S, 42° 43′O). Samples were obtained from five Langstroth-type beehives at each apiary. Colonies were inside wooden boxes with apertures 3 cm wide along both lateral sides, where the produced propolis accumulated. During 12 months, propolis samples produced by five colonies in the three apiaries were monthly collected, powdered and maintained in freezer. Propolis samples were pooled, combining in identical quantities samples from the 12 months of each colony. Samples from Itapecerica apiary were collected at each month, but the identity each month of collection (It/Jan–It/Dec) was preserved, in order to evaluate seasonal influences.

Extraction, Purification and Isolation of Compounds

Samples (5 g) were treated with hexane for 3 h in Soxhlet and the extracts discarded. A second extraction in

Soxhlet was carried out with methanol for 3 h. Waxes still remaining in the extract were eliminated by three consecutive steps of cooling in freezer and filtrating. Wax-free extracts were concentrated under reduced pressure and the residue was dried to constant weight. The obtained residues (dry methanol extracts—DME) were weighed. Bauerenyl acetate, main component of propolis from another sample of Paula Cândido, was isolated according to procedures described in Teixeira *et al.* (31).

Total Phenol Contents

Total phenol contents in crude propolis and DMEs were determined by the Folin–Ciocalteau method according to Woisky and Salatino (32), with minor modifications. Propolis methanol extracts or DME solution (400 p.p.m.) was mixed with 6.0 ml of the Folin-Ciocalteau and 6.0 ml of 20% Na_2CO_3, the absorbance being measured at 760 nm after 2 h. A calibration curve with solutions of gallic acid was used as reference. Total phenol contents were expressed as percentages of total phenolic substances in crude propolis and DMEs and correspond to means of three replicates.

GC/EIMS Analyses of Extracts

Methylation of constituents of part of the wax-free DMEs was carried out with diazomethane. Diazomethane-treated and non-treated DMEs were dissolved in ethyl ether at the concentration of 1000 p.p.m. Ether solutions (1 µl) were injected into a Shimadzu GCMS-QP5050A 17A ChemStation System Mass Spectrometer operating in the EI mode at 70 eV, equipped with auto injector AOC-5000 and mass selective detector. A DBS fused silica capillary column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness), He as carrier gas with flux 1.5 ml min^{-1} and splitless mode were used. Oven temperatures ranged from 100 to 310°C at 10°C min^{-1}, followed by isothermal period of 30 min. The range for mass detection was m/z 40–500. Injector and detector temperature was 300°C. Compounds were identified by computer searches in reference libraries Wiley 229L PMW TOX2 and NIST MS, and comparison of fragmentation patterns with literature data. Solutions of some reference compounds were injected in order to assist in the identification.

Free Radical Scavenging Activity

DMEs were dissolved in ethanol and baurenyl acetate in chloroform. The reaction mixture contained 2 ml ethanol, 0.1 mM free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) and DME. The methanolic extracts from Itapecerica (DME/It), Paula Candido (DME/PC) and Virginópolis (DME/Vi) and 12 DMEs obtained from It (DME/ItJan - DME/ItDec) were dissolved in ethanol at 200 p.p.m. Triplicates were prepared at the proportion 1:6 (v/v), combining DME ethanolic solutions and DPPH solution, respectively. Methanolic extracts were evaluated at the final concentration of 20 µg ml^{-1}. Controls were prepared combining ethanol and DPPH solutions also at the proportion 1:6 (v/v). After 30 min at room temperature, absorbances were measured at 517 nm (33). Intervals of 3 min were maintained between determination of absorbances. Ethanolic solutions of butylhydroxytoluene (BHT) at 200 p.p.m. were used as positive control. All experiments were carried out in triplicates. The antioxidant activity was expressed as percentage inhibition relative control value (BHT), after 30 min reaction, using the formula (33):

$$\%\ \text{Inhibition} = \left[\frac{(\text{Abs DPPH} - \text{Abs test sample})}{\text{Abs DPPH}}\right] \times 100.$$

Comparison of antioxidant activities of DME/It/Jan–DME/It/Dec was statistically evaluated using *F*-test with 5% of significance level. The statistical model included, as fixed effect, month of collection and residual as random effect. Free degree concerning this variation source was decomposed in contrasts and evaluated.

Results

Chemical Analysis

A total of 40 compounds, involving benzoic and cinnamic acid derivatives (phenylpropanoids), triterpenes, sesquiterpenes and diterpenes were found in DME/It, DME/PC and Virginópolis DME/Vi (Table 1), in addition to minor wax constituents (carboxylic acids and linear hydrocarbons). Non-prenylated (compounds **1**–**9**) and prenylated cinnamic acid derivatives (compounds **10**–**17**) were often detected.

Seasonality is an important factor determining propolis composition, since phenologic factors influence biosynthesis of plant secondary metabolites. Chemical composition of the methanolic extracts of propolis samples from Itapecerica, collected monthly along 1 year (DME/ItJan–DME/ItDec), are shown in Table 2.

Total Phenol Contents

Values of total phenol content as determined by the method of Folin–Ciocalteau of samples and extracts (DMEs) from It, PC, Vi and of DMEs of samples from Itapecerica collected monthly (DME/It/Jan–DME/It/Dec) are shown in Table 3.

Table 1. Relative percents of chemical constituents of dry methanolic extracts (DME) of propolis samples from Itapecerica (It), Paula Cândido (PC) and Virginópolis (Vi), municipalities from Minas Gerais state (southeast Brazil)

Constituents	DME/It	DME/PC	DME/Vi
Simple phenylpropanoids			
Dihydrocinnamic acid methyl ester (**1**)	3.8	7.7	–
Dihydrocinnamic acid (**2**)	1.0	1.0	–
p-Hydroxydihydrocinnamic acid (**3**)	0.5	0.4	–
p-Hydroxycinnamic acid (*p*-coumaric acid) (**4**)	1.0	2.0	–
p-Methoxycinnamic acid (**5**)	1.5	2.0	–
cis--3-Methoxy-4-hydroxy-cinnamic acid (**6**)	0.1	–	–
trans-3-Methoxy-4-hydroxy-cinnamic acid (**7**)	1.0	0.1	–
trans-3,4-Dimethoxycinnamic acid (**8**)	2.3	0.7	–
Dihydrocinnamic acid ethyl ester (**9**)	2.5	3.2	–
Prenylated phenylpropanoids			
Allyl-3-prenylcinnamate (**10**)	29.5	23.1	–
4-Hydroxy-3-prenylcinnamic acid (**11**)	1.0	4.4	–
4-Hydroxy-3,5-diprenylcinnamic acid (artepillin C) (**12**)	8.7	14.9	–
4-dihydrocinnamoiloxy-3-prenylcinnamic acid (**13**)	10.2	2.7	–
2,2-Dimethylchromene-6-propenoic acid (**14**)	1.0	2.0	–
2,2-Dimethyl-8-prenylchromene-6-propenoic acid (**15**)	2.0	1.0	–
8-(Methyl-butanechromane)-6-propenoic acid (**16**)	0.1	0.1	–
3-Hydroxy-2,2-dimethyl-8-prenylchromane-6-propenoic acid (**17**)	3.7	2.5	–
Sesqui and diterpenoids			
(-) Caryophyllene oxide (**18**)	–	–	0.4
Farnesol (**19**)	1.0	–	–
Farnesyl acetate (**20**)	–	–	0.8
Spathulenol (**21**)	1.2	1.5	0.3
Viridiflorol (**22**)	2.9	–	–
Dehydrocostus lactone (**23**)	2.2	2.7	–
Isocupressic acid derivative (**24**)	2.0	1.5	–
Triterpenoids and steroids			
Squalene (**25**)	–	–	7.0
Obtusifoliol (**26**)	–	–	1.5
Bauer-7-en-3β-yl acetate (**27**)	–	–	6.5
α-Amyrin (**28**)	–	–	4.1
α-Amyrin acetate (**29**)	–	–	23.5
β-Amyrin acetate (**30**)	–	–	20.5
Lupeyl acetate (**31**)	–	–	7.2
Olean-18-en-3β-yl acetate (**32**)	–	–	4.2
Taraxer-14-en-3β-yl acetate (**33**)	–	–	6.7
Urs-18-en-3β-yl acetate (**34**)	–	–	2.4
Friedooleanan-7,12-dien-3β-yl acetate (**35**)	–	–	1.4
Constituents from other classes			
p-Vinylphenol (**36**)	3.0	4.5	–
p-Vinyl-*o*-prenylphenol (**37**)	8.4	9.8	
Quinic acid (**38**)	0.8	0.6	–
2-Hydroxy-7,12-dimethyl-benzanthracene (**39**)	3.5	3.5	–
Isomaturnin (**40**)	2.0	2.0	–

Table 2. Percents of constituents (Comp.) of methanolic extracts of samples from Itapecerica (Minas Gerais state, southeast Brazil) collected monthly over a period of 1 year

Comp.[a]	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
1	3.8	2.0	3.5	3.9	14.0	2.0	7.5	3.7	4.3	4.8	3.0	2.6
2	3.0	2.5	2.5	1.5	2.0	2.0	2.5	2.5	2.0	1.0	2.5	2.0
3	1.0	0.4	1.0	0.4	1.0	0.5	0.7	0.6	1.5	0.3	0.5	0.3
4	2.0	2.0	2.0	–	2.0	1.5	2.0	1.5	3.8	1.0	1.5	0.5
5	2.0	0.4	3.5	5.5	1.0	3.7	3.0	5.0	1.5	1.5	1.5	1.0
6	0.4	1.0	0.4	–	–	1.0	0.3	1.9	0.4	–	–	–
7	1.1	1.0	1.6	–	–	–	0.4	0.5	0.5	0.7	–	0.4
8	1.5	0.2	4.5	3.2	–	1.0	1.0	3.5	0.8	0.8	–	–
9	2.5	–	0.5	–	–	4.0	3.2	–	–	4.3	–	2.3
10	15.0	25.0	19.6	24.0	23.0	25.0	22.5	27.5	22.7	32.7	24.0	23.1
11	7.0	5.5	6.3	–	1.3	4.9	5.6	3.2	5.2	5.2	4.2	5.9
12	7.0	9.8	7.0	3.2	2.8	5.5	5.6	4.3	8.6	10.2	5.4	12.5
13	3.8	5.5	2.0	12.4	5.0	3.0	4.0	4.0	5.5	4.5	5.5	9.9
14	1.5	1.5	1.0	3.0	3.0	1.5	1.0	1.5	2.0	1.0	1.5	1.5
15	1.5	2.0	1.5	–	2.0	3.5	1.5	2.0	2.0	1.0	3.5	1.5
16	1.5	1.0	1.5	1.0	1.0	1.0	1.5	1.5	3.0	1.0	1.5	1.0
17	1.5	1.5	3.0	2.5	3.0	4.5	3.0	2.0	2.0	2.0	2.0	1.0
19	1.0	1.0	2.0	1.5	1.0	–	–	1.0	–	1.0	1.0	1.0
20	2.5	–	–	–	–	–	–	–	–	–	–	–
21	1.0	1.0	–	–	–	1.0	–	1.0	–	0.5	–	0.5
22	–	–	–	–	1.5	1.4	1.0	–	–	2.5	–	1.0
23	1.5	2.5	1.5	1.0	1.5	1.0	2.0	1.0	1.6	1.0	1.5	1.0
24	1.5	7.5	1.0	–	1.5	2.5	1.0	4.0	2.0	1.5	1.5	1.5
25	1.0	1.5	3.0	3.0	1.5	1.5	1.5	2.0	–	1.0	3.5	1.5
36	5.5	1.0	–	–	5.6	1.5	5.6	–	–	3.0	1.5	–
37	8.2	14.0	11.0	11.0	12.5	11.7	15.6	12.5	16.6	9.6	12.5	9.6
38	–	1	3.5	–	–	–	–	–	1.0	–	4.0	–
39	1.5	3.6	1.5	2.0	1.5	3.5	3.5	2.0	2.5	2.5	2.0	3.0
40	2.0	2.0	3.0	3.0	1.5	3.0	1.5	3.0	3.0	2.0	4.0	2.5

[a]Compounds **26–36** were not detected in Virginópolis samples (Table 1). See Table 1 for correspondence of compound codes.

Table 3. Percents (means ± SE) of total phenolic substances in propolis samples from Minas Gerais (southeast Brazil)

	Total phenols			Total phenols	
Sample origin	Sample	Extract[a]	Sample origin	Sample[b]	Extract[a]
Itapecerica	11.8	20.5 ± 0.0	DME/It/May	16.2	26.4 ± 0.2d
Paula Cândido	12.1	21.9 ± 0.0	DME/It/Jun	12.0	21.5 ± 0.2e
Virginópolis	0.3	1.5 ± 0.0	DME/It/Jul	10.2	18.6 ± 0.2f
			DME/It/Aug	14.4	21.9 ± 0.2e
DME/It/Jan	8.9	15.5 ± 0.2a	DME/It/Sep	12.9	23.0 ± 0.2b
DME/It/Feb	12.4	23.2 ± 0.2b	DME/It/Oct	14.5	23.9 ± 0.2e
DME/It/Mar	12.6	20.9 ± 0.2c	DME/It/Nov	13.7	22.0 ± 0.2e
DME/It/Apr	12.3	22.8 ± 0.2b	DME/It/Dec	13.5	22.8 ± 0.2b

[a]Same letters denote results significantly not different (*F*-test, 5%); [b]DME/It/Jan – DME/It/Dec: extracts obtained from samples collected monthly over a period of 1 year.

Table 4. Antioxidant activity (percents, means ± SE) of dry methanolic extracts (DME) towards the free radical DPPH

Sample origin	Activity[a]	Sample origin[b]	Activity[a]
Itapecerica	47.7	DME/It/May	61.1 ± 2.4b, c
Paula Cândido	50.8	DME/It/Jun	54.6 ± 2.4d, e, f
Virginópolis	0.8	DME/It/Jul	54.4 ± 2.4e, f
Bauer-7-en-3β-yl acetate (a triterpenoid)	0.0	DME/It/Aug	54.2 ± 2.3e, f
DME/It/Jan	50.0 ± 2.4g	DME/It/Sep	58.3 ± 2.3c, d
DME/It/Feb	55.6 ± 2.0d, e	DME/It/Oct	58.3 ± 2.1c, d
DME/It/Mar	66.8 ± 2.7a	DME/It/Nov	63.4 ± 2.1a, b
DME/It/Apr	48.4 ± 2.1g	DME/It/Dec	50.7 ± 2.6f, g

[a]Same letters denote results significantly not different (*F*-test, 5%); [b]DME/It/Jan – DME/It/Dec: extracts obtained from samples collected monthly over a period of 1 year.

Free Radical Scavenging Activity

DPPH scavenging activities of DME are presented in Table 4. A consequence of the monthly compositional instability was a variation of antioxidant activity along the year, which ranged from 48.4% to 66.6%. Results showed significant differences ($P < 0.05$), if the degrees of freedom for the fixed effect (months of the year) were decomposed in contrasts (Table 4). BHT (butylated hydroxytoluene) is a common antioxidant in the food chemistry and was used in this investigation as positive control. The scavenging activity of BTH was 64.6 ± 2.3%.

Discussion

Chemical Composition

Phenolics of DME/It and DME/PC were mainly simple and prenylated cinnamic acid derivatives. Artepillin C (4-hydroxy-3,5-diprenylcinnamic acid) (**12**), usually a major compound in alecrim propolis and so far detected only in Brazilian propolis, was found in the proportion of 8.7% in DME/It and of 14.9% in DME/PC, being second to allyl-3-prenylcinnamate (**10**) in both DME/It (29.5%) and DME/PC (23.1%) (Table 1). This compound was reported in alecrim propolis by Negri *et al.* (8). Other quantitatively important phenolic constituents found in DME/It and DME/PC were dihydrocinnamic acid methyl ester (**1**), 4-dihydrocinnamoiloxy-3-prenylcinnamic acid (**13**), *p*-vinylphenol (**36**) and *p*-vinyl-*o*-prenylphenol (**37**) (Table 1). Although similar chemically, DME/It and DME/PC have some salient differences, comparing the relative amounts of some of the mentioned constituents in one and another sample, such as **1** (higher in DME/PC), **10** (higher in DME/It) and **12** (higher in DME/PC). Other differences correspond to the presence of a compound in one extract and its apparent absence in another; such are the cases of farnesol (**19**) (1.0%) and viridiflorol (**22**) (2.9%) detected in DME/It and apparently absent in DME/PC (Table 1).

GC/MS chromatogram of DME/Vi showed a pattern deprived of phenolics. DME/Vi contained mainly triterpenoids **25-35**, major constituents being α- and β-amyrin acetates, followed by squalene (**25**, an acyclic triterpenoid), bauer-7-en-3β-yl-acetate (**27**), lupeyl acetate (**31**) and taraxer-14-en-3β-yl acetate (**33**) (Table 1). Triterpenoid **27** was reported as the main constituent of another unusual propolis sample (29). Interestingly, Vi sample was also collected in southeast Minas Gerais, a region with predominance of 'green propolis', which is rich in prenylated phenolic compounds. Thus the plant origin of It and PC samples is probably *B. dracunculifolia*. However, the source of Vi sample is probably distinct from that of the two other samples.

Chemical Composition—Seasonal Variation

It seems that propolis samples with composition and physical properties deviating from the usual green pattern are relatively common in southeast Brazil. Seemingly, in samples of alecrim propolis there is a gradient with inversely proportional amounts of triterpenoids and phenolics, most samples characterized by high amounts of phenolics and low contents of triterpenoids. The characteristic alecrim pattern is hard, friable and dark green, with high amounts of phenolic compounds and low amounts of triterpenoids, or none at all. Increasing the amounts of triterpenoids and consequently decreasing those of phenolic compounds, samples progressively turn soft, dark, pitchy, greasy and sticky, or cream and powdery. Patterns such as the one described in this paper and that reported by Teixeira *et al.* (31) are uncommon and represent extremes, characterized by high amounts of triterpenoids and virtual absence of phenolics.

Most compounds of DME/It were detected throughout the year (Table 2). However, farnesyl acetate (**20**) was detected only in January. Other compounds (**4–9**, **11**, **15**, **19–22**, **24**, **25**, **36** and **38)** were detected in some months and not in others. For example, compound **11** appeared at the concentration of 6.3% in March, but was undetected in the following month. Contents of all compounds varied along the year (Table 2). Another study about seasonal chemical composition of Brazilian propolis (34) detected a pattern, according to which diterpenes started appearing in summer and reached a maximum in autumn, being absent along other seasons. No similar regular pattern of chemical variation was observed in the present study.

Resin Plant Sources

Propolis from Paula Cândido and Itapecerica no doubt derive from alecrim plants. On the other hand, a distinct

resin source has to be assigned to Virginópolis sample. Indeed, microscopic observations using methodology published elsewhere (6) detected fragments of *B. dracunculifolia* as predominating plant residues in propolis from Itapecerica and Paula Cândido; on the other hand, in propolis from Virginópolis fragments of *B. dracunculifolia* were rare. Fragments of *B. calvescens* and *Vernonia polyanthes* were also detected, but main resin source of this propolis sample seem to be plant secretions of local species (Teixeira, Message and collaborators, unpublished data).

Total Phenolic Percents

DME/Vi has a very low phenolic content (1.5%), comparing with DME/It and DME/PC (20.5 and 21.9%, respectively). DME/It and DME/PC did not contain triterpenoids (Table 1). Coherent with the analysis of total phenolic substances (Table 3), DME/It and DME/PC exhibited a high diversity of phenolic compounds, while DME/Vi contains mainly triterpenoids (Table 1). DME/It/Jan corresponds to the lowest (15.5%) and DME/It/May to the highest content (26.4%). Such variation of total phenolic contents reflects variation of chemical compositions of the propolis extracts (Table 2).

Antioxidant Activity

DPPH scavenging capacity has been widely used for evaluating antioxidant capacity of natural extracts (35). Radical scavenging activity of phenolic compounds is assigned to the hydrogen-donating ability of compounds (36). Antioxidants intercept the free radical chain oxidation by donating hydrogen from the phenolic hydroxyl groups, thereby forming stable end products, which does not initiate or propagate further oxidation (36,37). Nitrogen based radicals such as DPPH react with phenols by two mechanisms: (i) direct abstraction of phenol H-atom and (ii) electron transfer from ArOH or its phenoxide anion (ArO^-) to DPPH. The contribution of one pathway or another depends on the nature of the solvent and/or the redox potentials of the species involved (38). Radical scavenging activity of phenolic acids and their esters generally depends on numbers of phenolic hydroxyl groups (39–41). The hydro/lipophilicity of a sample does not affect its DPPH scavenging activity (42). Being rapid, simple and independent of sample polarity, the DPPH method is very convenient for the rapid screening of many samples for radical scavenging activity (43). Bioavailability is affected by conjugation of the compounds, and activity is mostly contributed by free forms (36,44).

Artepillin C, a phenol from Brazilian propolis, with a single ring and two prenyl groups, is a bio-available antioxidant, whose activity has been evaluated by several works (36,37). Simple phenols seem to be refractory to conjugation and the two prenyl groups of artepillin C may be an obstacle for conjugation to the hydroxyl (36). This phenolic compound undergoes intestinal absorption and prevents oxidative damage in hepatocytes and is assumed to prevent degenerative diseases by acting on cellular DNA (36). Other compounds with phenolic hydroxyls observed in the present work are **11**, **37** and **39** (Table 1). The phenylpropanoid with highest relative content in DME/It and DME/PC, compound **10**, is devoid of hydroxyl groups and hence probably has low antioxidant activity.

High antioxidant activities were obtained with DME/PC (50.8%) and DME/It (47.7%), a result coherent with the relatively high contents of phenolic compounds in boths DMEs. On the other hand, antioxidant activity in DME/Vi was hardly noticed (0.8%, Table 4). This sample contains mainly triterpenoids and virtually no phenolic substances (Table 1). One of the triterpenoids detected is bauer-7-en-3β-yl acetate (**27**, Table 1), which was obtained as a major constituent of a propolis sample also from Paula Cândido, Minas Gerais (31). This compound was shown to have no antioxidant activity (Table 4). A puzzling circumstance of propolis production, which is a serious barrier towards standardization of the product, is the occurrence of samples with such distinct chemical compositions in the same geographic region.

Antioxidant Activity—Seasonal Variation

DME/It and DME/PC showed 74.6% and 79.5% of the BHT activity, respectively. Months with higher DPPH free radical scavenging activity of DME/It were March and November, followed by May, September and October (Table 4). Activities of DME/It/Mar (66.8%) and DME/It/Nov (63.4%) were statistically not distinct from BHT activity. Less effective extracts were DME/It/Apr, DME/It/Jan and DME/It/Dec. DME/It/May, in spite of bearing the highest total phenolic content value (26.4%, Table 3), exhibited only the third highest DPPH scavenging activity (61.1%, Table 4). The extracts that exhibited lower total phenolic contents were DME/It/Jan, DME/It/Jul and DME/Itj/Mar, with 15.5, 18.6 and 20.9%, respectively (Table 3). While DME/It/Jan is among the extracts with weakest DPPH free radical scavenging activity (50.0%), DME/It/Mar showed an antioxidant activity similar to BHT (66.8%, Table 4).

Factors Influencing Antioxidant Activity

Comparing antioxidant activities with total phenolic contents, no clear correlation is apparent regarding these DME/It parameters along the year. Antioxidant activities are dependent on structures of phenolic compounds. For example, assuming identical patterns of hydroxyl and methoxyl substitution, hydroxycinnamic acids are

more effective than hydroxybenzoic acids (39,45). In addition, antioxidant activity of phenolic acid derivatives depends on characteristics of both propane side chain and phenolic hydroxyls (22). Thus, structural aspects are important in determining antioxidant activity, which makes the subject of antioxidant activity too complex to be explained just in terms of quantity of phenolic compounds.

There is still the possibility of correlations between antioxidant activity and contents of total hydroxylated substances. Regarding propolis composition, likely compounds with high antioxidant activity are those bearing phenolic hydroxyls, such as compounds **3**, **4**, **6**, **7**, **11**, **12**, **36**, **37** and **39** (Table 1). Months with higher antioxidant activities were March, May and November (periods with scavenging activity above 60%, Table 4). However, months when the sum of the percents of those compounds reached higher values (above 31.0%) were July (39.3%), September (39.1%), February (38.3%), January (33.7%), October (32.5%) and December (32.2%) (Table 2). So, no correlation is apparent between DPPH scavenging activity and concentration of assumed active antioxidant compounds, revealing again how difficult it is to assign antioxidant efficacy to a limited set of components in complex mixtures, such as the case of propolis extracts. Constituents other than the most obvious powerful antioxidants probably play important roles in the final and observable effect. Even synergisms cannot be overruled in such cases.

Antioxidant Activity—Concluding Remarks

Lipid peroxidation is a probable cause of many health problems. Brazilian propolis has been shown to exert neuroprotective effect by inhibiting neurotoxicity in neuronally differentiated PC12 cell cultures. A protection against oxidative stress by propolis has been suggested to be responsible, at least partly, for the observed neuroprotection (46). Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ), are widely used in the food industry, although BHA and BHT have been under suspicion of being responsible for liver damage and carcinogenesis in laboratory animals (47,48). Suppression of lipid antioxidant reactions in food is a major cause of quality deterioration and off-flavor development. Antioxidants may be used to preserve food quality from oxidative deterioration of lipids. Therefore, antioxidants may be used to avoid lipid deterioration and play an important role in food industry.

In addition to being a source of natural products capable of promoting radical scavenging beneficial effects in human and animal healthcare, propolis might end up as a source of model compounds for antioxidants useful in the food industry.

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