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Spray-dried propolis extract, II: Prenylated components of green propolis

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The effect of spray drying conditions on the chemical composition of Brazilian green propolis extract was investigated using a factorial design and high performance liquid chromatography. The raw and dried extract contents of caffeic acid, *p*-coumaric acid, drupanin, isosakuranetin, artepillin C, baccharin and 2,2-dimethyl-6-carboxyethenyl-2*H*-1-benzopyran were quantified using veratraldehyde (3,4-dimethoxybenzaldehyde) as internal standard. The baccharin content in spray-dried propolis was affected by the drying temperature with a 5% significance level, while the coumaric acid and drupanin contents were dependent on drying temperature at a 15% significance level. The other chemical markers, caffeic acid, isosakuranetin, artepillin C and 2,2-dimethyl-6-carboxyethenyl-2*H*-1-benzopyran, showed to be independent of drying conditions. However, all the chemical markers showed some loss on drying, which varied from 30 to 50%. The results showed that prenylated compounds are sensitive to drying, but their losses may be considerably reduced under low temperatures, around 40 °C. The antioxidant activity of the spray dried propolis was determined by the diphenylpicrylhydrazyl (DPPH) method and showed a quadratic dependency on the temperature; extract feed rate and the interaction between them. However, spray dried propolis extracts presented antioxidant activities similar to the original propolis tincturae.

1. Introduction

Propolis is a balsamic or resinous material collected by bees from plants and has the main function of protecting the hive against microbial contaminants. Propolis has been widely used in folk medicine for its therapeutical properties, like antiinflammatory, antimicrobial, antiviral, antitumoral, antioxidant, antiulcer and many others (Reis et al. 2000; Moreno et al. 2000; Sforcin et al. 2000; Bankova and Marcucci 2000; Banskota et al. 2001; Isla et al. 2001; Bazo et al. 2002; Nagai et al. 2003; Santos et al. 2003; Kumazawa et al. 2004).

Until recently, more than 300 compounds, mainly polyphenols, have been identified as constituents of propolis collected in different countries (Marcucci et al. 2001). The composition of propolis depends on the vegetation of the area from where it is collected and on bee biology (Santos 2003). Propolis from tropical zones, like the Brazilian green propolis (Banskota et al. 2000), is rich in prenylated derivatives of *p*-coumaric acid and flavonoids (Simões et al. 2004). It has been suggested that the antitumoral activities of the Brazilian green propolis depend principally on these prenylated compounds (Simões et al. 2004), which in turn are originated from the plant *Baccharis dracunculifolia* L. (Park et al. 2004).

Recently, there has been increasing interest in dry propolis extracts (Bruschi et al. 2003; Marquele et al. 2006). The powdered extract presents well-known advantages in pharmaceutical formulations, like the possibility of easy preparation of tablets and capsules. Furthermore, the spray-dried propolis extract, SDPE, is expected to present greater stability. Bruschi et al. (2003) studied the encapsulation of propolis in gelatin microparticles and found the system to be effective against oral pathogens (Bruschi et al. 2006). A study on the effects of the spray-drying on propolis extracts chemical constituents and antioxidant activities have been recently published (Marquele et al. 2006). The dry extract polyphenol and flavonoid contents and antioxidant activity were studied under different spray drying conditions using response surface methodology. The results indicated that drying temperature and its squared term affected polyphenol content with 0.5% significance. The drying temperature also affected flavonoid content and the antioxidant activity with 5 and 0.1% significance, respectively. However, even under severe drying conditions the spray dried propolis extract presented a very high antioxidant activity, being able to inhibit 50% of lipid peroxidation at concentrations varying from 2.5 to 5.0 μg/ml.

The aim of this work was to investigate the effect of spray drying conditions, e.g. extract feed rate and temperature on the prenylated compounds contents of dry Brazilian green propolis using HPLC and on the antioxidant activity using the radical diphenylpicrylhydrazyl (DPPH). The raw and dried extract contents of caffeic acid, *p*-coumaric acid, drupanin, isosakuranetin, artepillin C, baccharin and 2,2-dimethyl-6-carboxyethenyl-2*H*-1-benzopyran (DCBEN) were quantified by HPLC using veratraldehyde (3,4-dimethoxibenzaldehyde) as the internal standard.

2. Investigations, results and discussion

2.1. Prenylated compounds

The chromatograms of propolis extract, APE, and spray dried propolis extract, SDPE, are shown in Fig. 1a and 1b, respectively. Both chromatograms show that main prenylated compounds could be identified and presented clear, distinct peaks. This demonstrates that the chromatographic conditions used were adequate. In APE chromatogram, it is possible to identify the peaks of caffeic acid (**1**), *p*-coumaric acid (**2**), drupanin (**3**), isosakuranetin (**4**), artepillin C (**5**), baccharin (**6**) and DCBEN (**7**), by comparison with the authentic chromatographic standards. Also, Fig. 1b shows the chromatogram of SDPE obtained in drying experiment nr 3, i.e. 120 °C and 3 mL/min. The chromatograms for both APE and SDPE are similar.

The quantification of the seven compounds was carried out by calibration curves prepared in the range expected of each compound in SDPE and APE, and are presented in Table 1 as percentages of total dry matter. The APE content of caffeic acid (1.56%) was reduced after drying to a content that varied from 0.56 to 1.11%. However, there is the effect of dilution of compounds due to the addition of drying aid (colloidal silicon dioxide). The expected contents of each compound in SDPE can be estimated from the concentration found in APE in dry basis, according to the following mass balance relationship:

$$C^{i}_{C}(\%) = \frac{[M_{APE} \times (1-\phi)]}{[M_{APE} \times (1-\phi)] + M_{DA}} \times C^{i}_{APE}(\%) \qquad (1)$$

where, C^{i}_{C} is the percent content of compound i in SDPE, M_{APE} is the weight of APE in solution for drying, M_{DA} is the weight of drying aid in solution for drying, C^{i}_{APE} is compound i content in APE (dry basis) and ϕ is the solids content in APE.

The SDPE content of caffeic acid, corrected by Eq. (1) was expected to be 1.14%, and as shown in Table 1, it was reduced to contents that varied from 0.56 to 1.11%. Those values represent a loss on drying that varied from 2.6 to 50.9%, depending on drying conditions. All the other constituents also showed a reduction after drying, the loss being 40.8 to 53.5% for *p*-coumaric acid, 31.6 to 43.4% for drupanin, 25.7 to 36.3% for isosakuranetin, 35.1 to 47.3% for artepillin C, 51.8 to 68.3% for baccharin and 36.7 to 45.9% for DCBEN. In general, the chemical markers loss on drying varied around 30–50%, except for baccharin.

Table 1: Spray drying conditions and resulting chemical composition

SPDE	T °C	W_E ml/min	**1** (μg/ml)	**2** (μg/ml)	**3** (μg/ml)	**4** (μg/ml)	**5** (μg/ml)	**6** (μg/ml)	**7** (μg/ml)
1	120	9.0	0.56	0.36	1.78	1;94	2.15	1.37	0.58
2	120	6.0	0.79	0.35	1.78	1.92	2.19	1.27	0.56
3	120	3.0	1.11	0.33	1.70	1.87	1.99	1.12	0.53
4	80	9.0	1.05	0.35	1.63	1.81	1.95	1.50	0.55
5	80	6.0	0.75	0.38	1.66	1.89	2.01	1.57	0.55
6	80	3.0	0.76	0.40	1.74	1.92	2.11	1.60	0.59
7	40	9.0	0.79	0.41	1.80	1.99	2.21	1.72	0.61
8	40	6.0	0.78	0.41	1.77	1.95	2.12	1.70	0.60
9	40	3.0	0.76	0.39	1.76	1.96	2.07	1.69	0.59
3*	120	3.0	0.73	0.42	1.97	2.11	2.40	1.51	0.62
7*	40	9.0	0.56	0.40	1.75	1.88	2.13	1.66	0.58
APE	–	–	1.56	0.98	3.93	3.87	5.05	4.82	1.34

1 – caffeic acid; **2** – coumaric acid; **3** – drupanin; **4** – isosakuranetin; **5** – artepillin C; **6** – baccharin; **7** – 2,2-dimethyl-6-carboxyethenyl-2*H*-1-benzopyran
* Replicates of respective drying experiment

The effect of drying conditions on the loss of propolis constituents can be seen in Fig. 2. In this Figure, the SDPE percent contents of caffeic acid, *p*-coumaric acid, drupanin, isosakuranetin, artepillin C, baccharin and DCBEN are shown for each experimental run. The evaluation of the effect of drying conditions, extract feed rate and temperature on the loss of these compounds is not simple by the visual observation of Fig. 2. For that reason, the technique of response surface analysis (ANOVA) was applied to the data. The resulting significances of drying factors on each compound are shown in Table 2. As can be seen

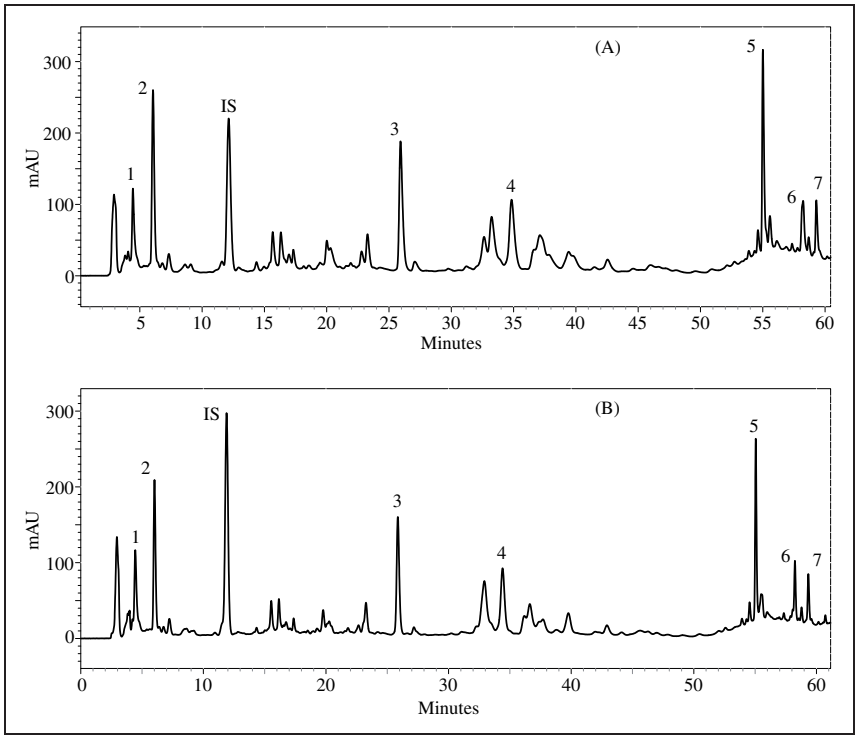

Fig. 1:
Chromatograms of (A) propolis tincturae and (B) spray dried propolis extract, showing the chemical constituents: 1 – caffeic acid; 2 – *p*-coumaric acid; 3 – drupanin; 4 – isosakuranetin; 5 – artepillin C; 6 – baccharin; 7 – 2,2-dimethyl-6-carboxyethenyl-2*H*-1-benzopyran

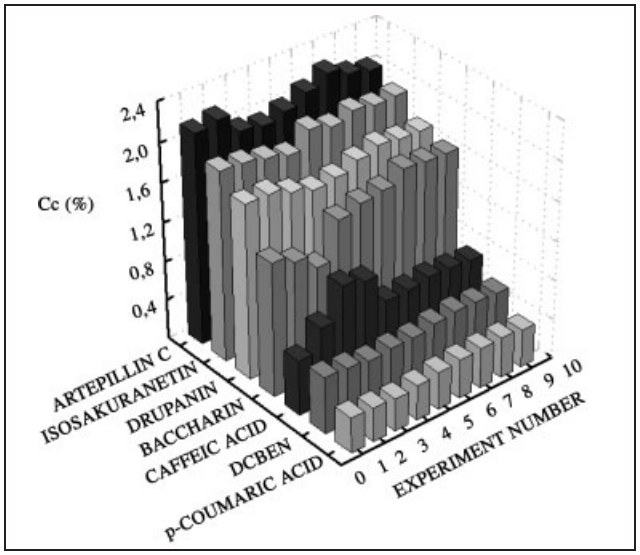

Fig. 2: Chemical composition of spray dried extracts obtained in the drying experiments (Runs 1 to 9)

Table 2: Summary of the analysis of variance on chemical constituent's loss on drying

Factor	1	2	3	4	5	6	7
W_E	NS	NS	NS	NS	NS	NS	NS
T	NS	15%	NS	NS	NS	5%	NS
W_E^2	NS	NS	NS	NS	NS	NS	NS
T^2	NS	NS	15%	NS	NS	NS	NS
$W_E \times T$	NS	NS	NS	NS	NS	NS	NS

NS – non significant; * significance level (%)

in this Table, in most cases the drying conditions did not affect SDPE composition. Exceptions were the *p*-coumaric acid, drupanin and baccharin contents, which depended on drying temperature. The SDPE contents of *p*-coumaric acid and drupanin were affected at 15% significance level by temperature and its squared term, respectively. The criteria adopted in this work was to take into consideration effects with significance levels 15% or higher. The effect of temperature on coumaric acid and drupanin contents in SDPE

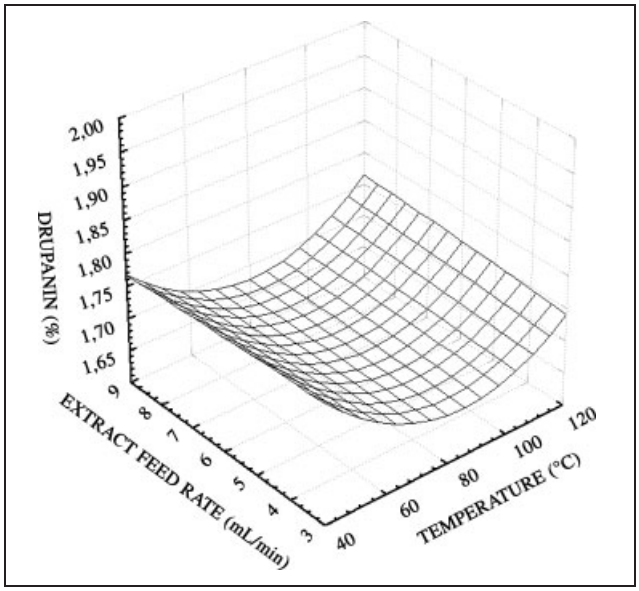

Fig. 4: Surface response of drupanin content in spray dried extract as a function of the drying temperature and extract feed rate

is shown in Figs. 3 and 4. As only the linear term of temperature affected the coumaric acid content, the surface shown in Fig. 3 is flat (linear regression), and confirms that extract feed rate did not affect this compound during drying. On the other hand, the effect of temperature is very pronounced.

The response surface in Fig. 4 is not flat as in Fig. 3 because the squared term of temperature affected drupanin content. The surface shows that drupanin loss on drying is not affected by the APE feed rate, but it depends on temperature and there is a minimum of drupanin content in SDPE when dried at 80 °C.

The baccharin contents in SDPE were very sensitive to the drying operation, as may be proved by its larger losses as compared to other compounds (Table 1), and by its variability in Fig. 2. The influence of drying factors on baccharin content in SDPE is shown in Fig. 5. In this Figure, the effect of temperature is clearly verified and the influence of extract feed rate is less important. The baccharin content decreases as the temperature is increased.

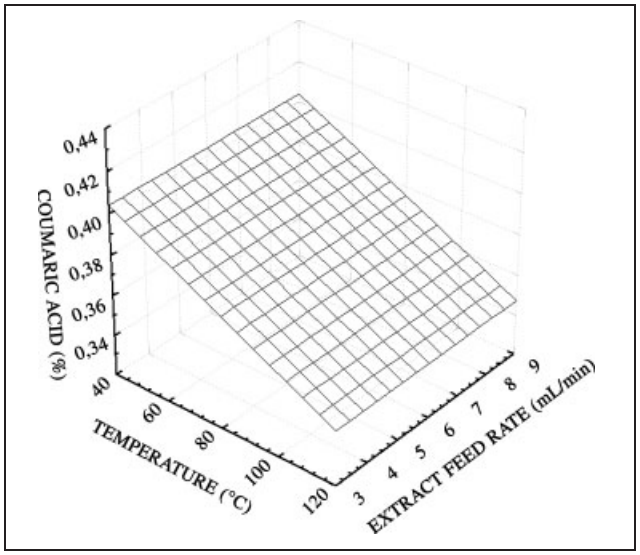

Fig. 3: Surface response of *p*-coumaric acid content in spray dried extract as a function of the drying temperature and extract feed rate

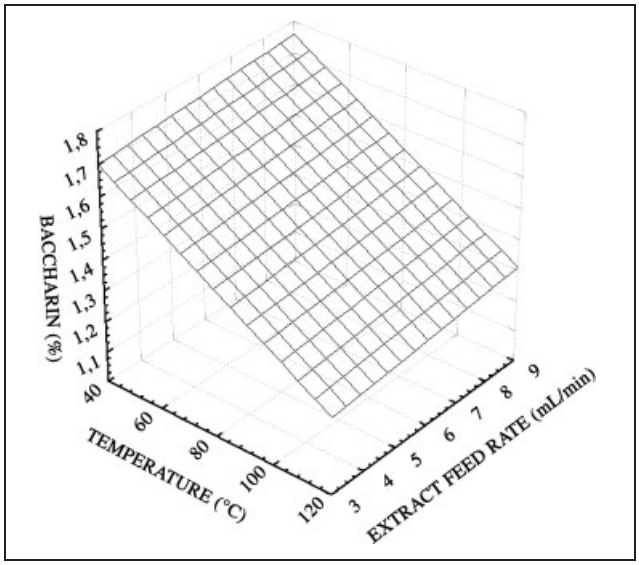

Fig. 5: Surface response of baccharin content in spray dried extract as a function of the drying temperature and extract feed rate

Table 3: Analysis of variance on baccharin data

Factor	Sum of squares	Degree of freedom	Mean square	F_{calc}	p
T	0.2679	1	0.2679	15.4314*	0.0110*
WE	0.0003	1	0.0003	0.3037	0.6052
T^2	0.0053	1	0.0053	0.0156	0.9056
WE^2	0.0005	1	0.0005	0.0280	0.8736
WE × T	0.0008	1	0.0008	0.0468	0.8372
Error	0.0868	5	0.01736		
Total	4.7677				

* Significant at 5%

The response surface analysis (ANOVA) on baccharin data is shown in Table 3. SDPE content showed to depend only on drying temperature at the 5% significance level. Again, the data in Table 1 show that the most sensitive compound is baccharin, since it presented higher losses, which agree well with the ANOVA results.

2.2. Antioxidant activity

The antioxidant activity, determined by the DPPH method, resulted in IC_{50} values ranging from 0.456 to 0.598 mg/mL (Table 4). As the IC_{50} is the concentration of SDPE necessary for inhibition of 50% of radical oxidation, the lower the IC_{50} means higher antioxidant activity. The original propolis tincturae, APE, showed a IC_{50} of 0.480 mg/mL, which is higher than most of IC_{50} found for spray dried propolis. However, the antioxidant activity loss may be considered low in most conditions and neglectable in others. As a means of comparison, the IC_{50} for pure analytical grade ascorbic acid and quercetin were determined using the same DPPH procedure and resulted in 0.099 and 0.077 mg/mL, respectively. The effects of spray drying conditions on the SDPE IC_{50} is shown in Fig. 6. In this Figure, the effect of both drying air temperature and extract feed rate seem to be non linear and the highest antioxidant activities (lowest IC_{50}) are found for intermediary values of T and W_E. Also, the effects of T and W_E are influenced by each other, as noted by the changes in surface curvature. This may be clarified by the response surface analysis using ANOVA. The resulting significances are shown in Table 5. The linear terms of T and W_E did not affect IC_{50} significantly, but the quadratic terms of they both were significant at 15% level. Also, in accordance to surface curvatures in Fig. 6, the interaction between T and W_E is significant at 5% level. This result is in agreement with that of Marquele et al. (2006), who used the mitochondria lipid peroxidation inhibition method for antioxidant activity and determined significant effects of temperature and extract feed rate of spray dried propolis extracts. Furthermore, the DPPH method applied to propolis tincturae from several origins (Banskota et al. 2000), resulted in antioxidant activities in concentrations ranging from 5.9 to 94 μg/mL, which are lower than the IC_{50} observed here in this work.

Table 4: Spray drying conditions and resulting antioxidant activity (DPPH)

SPDE	T °C	W_E ml/min	IC_{50} (mg/mL)
1	120	9.0	0.500
2	120	6.0	0.480
3	120	3.0	0.530
4	80	9.0	0.470
5	80	6.0	0.440
6	80	3.0	0.520
7	40	9.0	0.598
8	40	6.0	0.507
9	40	3.0	0.456
3*	120	3.0	0.559
7*	40	9.0	0.592
APE	–	–	0.480
Vitamin C	–	–	0.099
Quercetin	–	–	0.077

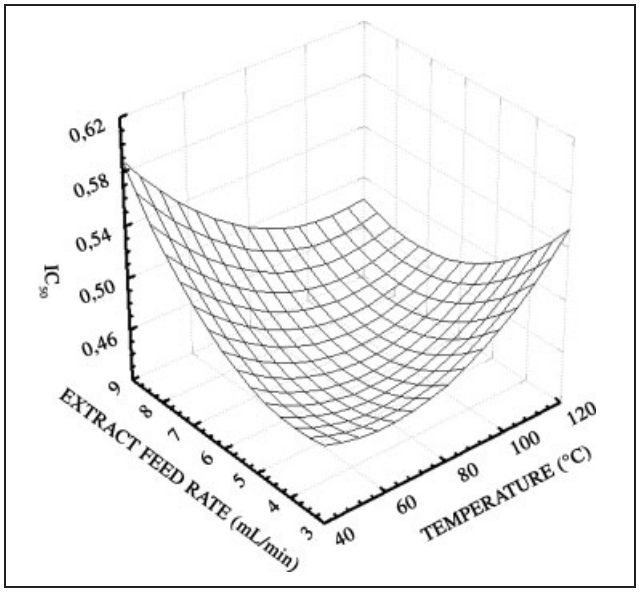

Fig. 6: Surface response of spray dried extract antioxidant activity (IC_{50}) as a function of the drying temperature and extract feed rate

Table 5: Analysis of variance on antioxidant activity (IC_{50}) data

Factor	Sum of squares	Degree of freedom	Mean square	F_{calc}	p
T	0.0006	1	0.0006	0.7292*	0.4321
W_E	0.0030	1	0.0030	3.6489	0.1144**
T^2	0.0006	1	0.0006	0.7292	0.4321
W_E^2	0.0030	1	0.0030	3.6489	0.1144**
W_E × T	0.0106	1	0.0106	12.6754	0.0162*
Error	0.0042	5	0.0008		
Total	0.3974				

Significant at * 5%; ** 15%

3. Experimental

3.1. Propolis extracts

Alcoholic extract of green propolis (tincturae, APE), was purchased in the Brazilian market from Apis Flora Ltda., originating from Oliveira, Minas Gerais. Its total solid content was 9.43% (w/v) and total flavonoid content was 0.46% (w/v).

Spray-dried propolis extract (SDPE): The experimental conditions for drying of APE were similar to those applied by Marquele et al. (2006) in order to facilitate the comparison and discussion. Before the drying step, APE was concentrated in a rotating evaporator to reduce alcohol content and a drying adjuvant was added. The concentrated propolis extract, CPE, was dried in a laboratory scale spray dryer model LM MSD 1.0 (Labmaq Ltda, Brazil) under several different operational conditions. The pneumatic spray nozzle was operated at an airflow rate of 100.0 L/min and a pressure of 2.0 kgf/cm^2. Drying airflow rate was kept constant at 0.60 m^3/min. The SDPE was collected at dryer outlet, weighed and stored in closed flasks protected from light at 4 °C. The factors studied in the drying process were the drying air outlet temperature, T (°C) and the extract feed rate, W_E (mL/min).

Table 6: Factors levels and experimental design

Factors	Levels −1	0	+1
Drying air temperature, T (°C), X1	40	80	120
APE feed rate, W_E (ml/min), X2	3.0	6.0	9.0

Experimental Run	X1	X2
1	+1	+1
2	+1	0
3	+1	−1
4	0	+1
5	0	0
6	0	−1
7	−1	+1
8	−1	0
9	−1	−1

Table 7: Levels of factors for drying experiments

Factors	Levels		
	−1	0	+1
1. Air temperature, T (°C)	40	80	120
2. APE feed rate, W_E (mL/min)	3.0	6.0	9.0

The drying study followed a 3^2 full factorial design, shown in Table 6, which allows for the determination of linear, quadratic and interactive effects (Box et al. 1978). Table 7 shows the levels of the factors applied in this design. In order to follow the levels adopted in this design, the factors studied needed to be decoded. The decoding formula is given by:

$$\text{Coded} \cdot \text{Variable} = \frac{(\text{uncoded} \cdot \text{value} - 0.5 \times (\text{high} \cdot \text{value} + \text{low} \cdot \text{value}))}{0.5 \times (\text{high} \cdot \text{value} - \text{low} \cdot \text{value})} \quad (2)$$

Experimental data were analyzed by response surface regression using the module Visual General Linear Model (VGLM) from the software Statistica '99 (Statsoft, Inc.).

3.2. HPLC methodology

3.2.1. Sample preparation

Samples of spray-dried propolis extract (SDPE, 50 mg) were accurately weighed and dissolved in 10 mL of ethanol 90% containing 80 μg/mL of the internal standard, IS, veratraldehyde (3,4-dimethoxybenzaldehyde). The suspensions were kept under agitation in a shaker for 2 h at 40 °C and 170 rpm. After filtration using a 0.22 mm membrane (Millipore®), the solution was collected and a 1 ml aliquot was transferred to chromatographic vials. 15 μL samples were then automatically injected into the equipment and the analysis were run in triplicate.

3.2.2. Equipment and chromatographic conditions

The analysis was carried out in a Shimadzu HPLC equipped with an automatic injector, connected to a controller SCL-10 Avp, three pumps LC-10AD, diode array detector model SPD-M10Avp and the software Shimadzu Class VP version 5.02. The chromatographic column was a Shimadzu reverse phase Shim-pack CLC-ODS (M), 4.6 mm × 250 mm, with particle and pore sizes of 5 μm and 100 Å, respectively. Also a Pelligard LC-18 pre-column was used. All solvents used were chromatographic grade and the water employed was purified in a Milli-Q-Plus system (Millipore, Bedford, USA). The detector was set to work at 280 nm. Two solvents were used as mobile phase: A) 93.9% water, 0.8% acetic acid, 0.3% ammonium acetate and 5% methanol; B) acetonitrile. The chromatographic was run in gradient mode, with pumps set to 1 mL/min and varying gradually the mixture of solvent from 75% of A and 25% B at the moment of injection to 100% of B after 60 minutes of run.

3.2.3. Chromatographic standards

Veratraldehyde was purchased from Merck (USA). Caffeic and *p*-coumaric acids were supplied by Acros Organics (New Jersey, USA). Drupanin, artepillin C, 2,2-dimethyl-6-carboxyethenyl-2*H*-1-benzopyran, baccharin and isosakuranetin were isolated either from green propolis or *Baccharis dracunculifolia* and are available at the compounds library of the Laboratory of Pharmacognosy from the School of Pharmaceutical Sciences at Ribeirão Preto – University of São Paulo, Brazil.

3.3. Antioxidant activity (DPPH test)

The antioxidant activity was determined using the stable radical 2,2-diphenylpicrylhydrazil (DPPH) (Georgetti et al. 2004). Aqueous solutions of SDPE were prepared in five concentrations and 100 μL aliquots were diluted with 1 mL acetate buffer (pH 5.5), added with 500 μL DPPH (250 mM) and allowed to stand for 10 min at room temperature. The absorbency was read in a spectrophotometer M330 (Camspec, UK) at 510 nm. Negative control was prepared with 1.6 mL ethanol, 1 mL acetate buffer, while positive control, C+, was prepared with 1.1 mL ethanol, 1 mL acetate buffer and 0.5 mL DPPH 250 mM. The percent antioxidant inhibition was estimated by:

$$\%\ \text{inhibition} = 100 - \frac{A^{*}100}{C+} \quad (3)$$

The results were expressed as IC_{50}, e.g. the extract concentration (mg/mL) required to inhibit 50% of the DPPH oxidation. In order to allow comparison, the same procedure was run for pure ascorbic acid (vitamin C) and quercetin. These two compounds were chosen because of their recognized antioxidant activities. DPPH, ascorbic acid and quercetin were all analytical grade (Sigma, USA).

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