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Spray-dried propolis extract. I: Physicochemical and antioxidant properties

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The effect of spray drying on the chemical and biological properties of alcoholic extract of green propolis was investigated. The total polyphenol and flavonoid contents in spray-dried propolis extract were determined by the Folin-Ciocalteu and aluminum chloride colorimetric methods, respectively. The antioxidant activity of the dry extract was assessed by the membrane lipid peroxidation inhibition method, using quercetin as reference. The polyphenol content was shown to depend on the drying air outlet temperature and its square at the 0.5% significance level, while the flavonoid content depended only on the square of the outlet temperature at the 5% significance level. Polyphenol and flavonoid recovery after spray-drying ranged from 45.1 to 54.9% and 30.6 to 40.8%, respectively. The antioxidant activity of the spray-dried propolis was shown to be affected by the extract feed rate and air outlet temperature at a significance level of 0.1%. The spray-dried propolis extract showed significant antioxidant activity, with 50% lipid peroxidation inhibition at concentrations ranging from 2.5 to 5.0 μg/ml.

1. Introduction

Propolis (bee glue) is a resin collected by bees for use in and around the hive. It has been used by man since ancient times for its pharmaceutical properties and is still widely used as a remedy in folk medicine, as a constituent of "bio-cosmetics" and for numerous other purposes. Commercial interest in propolis has shown a steady increase in recent years (Bankova and Marcucci 2000).

Up to now, more than 150 compounds, mainly polyphenols, have been identified as constituents of propolis collected in different countries (Pascual et al. 1994). The composition of propolis depends on the vegetation of the area from where it was collected and on the bee species. Propolis from tropical zones, like Brazilian green propolis, consists mainly of prenylated derivatives of *p*-coumaric acid and flavonoids (Simões et al. 2004).

It has been suggested that the biological activities of propolis depend mainly on the presence of this large number of flavonoids. These are reported to have antioxidant, anti-inflammatory, anticancer and antiviral activities, as well as antimicrobial effects (Pascual et al. 1994; Moreno et al. 2000; Sforcin et al. 2000; Banskota et al. 2001; Nagai et al. 2003; Kumazawa et al. 2004).

Antioxidant activities of alcoholic and aqueous extracts of propolis from different origins have been studied extensively worldwide. Scavenging of free radicals generated in inflammatory processes was reported by Krol et al. (1996). Recently, Cuban red propolis has shown scavenging action against oxygen radicals (Pascual et al. 1994). Argentine propolis has been reported to inhibit lipid oxidation (Isla et al. 2001). Almost all propolis extracts evaluated by Banskota et al. (2000) showed scavenging activity towards DPPH free radicals to a similar extent.

Recently, there has been an increasing interest in dry extracts of natural products, including propolis. The powdered extract presents obvious advantages in pharmaceutical formulations, such as the possibility of easy preparation of tablets and capsules. Furthermore, the spray-dried propolis extract, SDPE, is expected to present better stability. Available literature indicates that no previous studies on the effect of spray-drying of propolis extract on its chemical constituents and antioxidant activity have been performed.

In this work the effect of the drying process on total polyphenol, TPC, and flavonoid content, TFC, was studied. Also, the antioxidant activity, AOA, of spray-dried propolis extract (SDPE) was assessed using the peroxidation of cellular membrane lipids method, using mitochondria as the lipid source. In this system generation of reactive oxygen species (ROS) occurs, which is increased in the presence of Fe^{2+}, leading to oxidative damage to lipids. Response surface methodology, RSM, was used to evaluate the effect of drying parameters on SDPE composition and antioxidant activity.

2. Investigations, results and discussion

The full experimental design used in this study can be seen in Table 1. The results shown in this Table are averaged values of TPC, TFC and AOA, for illustrative purposes only. The response surface analysis was carried out with values of TPC and TFC in duplicate and AOA in quintuplicate. In general the powders obtained by APE spray-drying showed excellent physical aspect, uniform color, low apparent density and good flow properties. This is due to the use of colloidal silica as a drying aid.

Table 1: Experimental design and resulting TPC, TFC and AOA

EXPER. RUN	X1 W_E	X2 T	TPC (μg/mg)	TFC (μg/mg)	AOA (U/μg)
1	+1	+1	42.29	12.54	0.248
2	+1	0	40.8	11.90	0.15
3	+1	−1	43.53	13.50	0.223
4	0	+1	42.32	13.43	0.166
5	0	0	37.83	10.58	0.162
6	0	−1	46.02	14.03	0.257
7	−1	+1	43.47	12.56	0.558
8	−1	0	40.17	12.72	0.695
9	−1	−1	45.57	12.15	0.895

2.1. SDPE polyphenol and flavonoid content

The Folin-Ciocalteu method is currently used to determine total polyphenol, and in the present study, this method was used to determine the total polyphenol content of the propolis extract samples. This method is based on the oxidizability of phenols at basic pH, the Folin-Ciocalteu reagent working as an oxidant agent. Phenols are responsible for most of the antioxidant capacity in most plant-derived products, so that their determination can be very informative (Singleton et al. 1999).

The total flavonoid content was determined by the aluminum chloride colorimetric method. The principle of this method is that aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones (chrysin, apigenin, luteolin) and flavonols (rutin, morin, quercetin, myricetin, kaempferol, quercitrin and galangin). In addition, aluminum chloride forms acid labile complexes with the ortho-dihydroxyl groups in the A or B ring of flavonoids. Quercetin was shown to be a reliable reference compound as compared to 15 other flavonoid standards investigated by Chang et al. (2002). The acid complex of quercetin, similarly to other flavonoids, has an absorption maximum at 415 nm.

For the response surface analysis of TPC and TFC the samples were analyzed in duplicate. The effect of spray-drying temperature, T (°C) and extract feed rate, W_E (ml/min), on SDPE polyphenol content is shown in Fig. 1. The response surface plot shows that extract feed rate did not affect the TPC, while the drying air outlet temperature exerted a strong non-linear effect on TPC. However, the effects of those factors on TPC can be more reliably evaluated by analysis of variance on the experimental data. The resulting analysis can be seen in Table 2.

Table 2: Analysis of variance on TPC data

Factor	Sum of squares	Degree of freedom	Mean square	F_{calc}
WE	2.240	1	2.240	1.636
T	16.509	1	16.509	0.984*
WE^2	1.347	1	1.347	12.057
T^2	72.800	1	72.800	53.170*
WE × T	0.342	1	0.342	0.264
Error	16.430	12	1.369	
Total	109.687			

* Significant at 0.5%

The ANOVA confirmed the tendencies observed in Fig. 1, showing that temperature affected the TPC at a 0.5% significance level. The square of temperature affected the TPC at a 0.5% significance level, confirming the quadratic effect observed in Fig. 1. On the other hand, the extract feed rate and its square did not affect TPC to a significant level. Also, there was no interaction between the two factors studied. In this work, only factors with significance levels higher than 10% were considered. The response surface analysis permits the fitting of a polynomial equation of the dependent variable, TPC as a function of the significant factors. The fitted equation, with correlation coefficient $R^2 = 0.9221$, is given by:

$$\mathrm{TPC} = 39.2 - 1.17\left(\frac{\mathrm{T}-80}{40}\right) + 4.27\left(\frac{\mathrm{T}-80}{40}\right)^2 \quad (1)$$

The effect of the spray-drying factors on the SDPE total flavonoid content, TFC, can be seen in Fig. 2. The surface plot in this Figure shows a profile similar to the one observed for the TPC (Fig. 1), e.g. the non-linear effect of temperature and the undefined effect of extract feed rate. To evaluate those effects better, an analysis of variance was carried out and the results are shown in Table 3.

The ANOVA showed that neither extract feed rate, its square, the temperature nor the interactive terms were significant. Only the square of temperature was shown to be significant at the 5% level. The response surface analysis allows for the fitting of TFC as a function of the signifi-

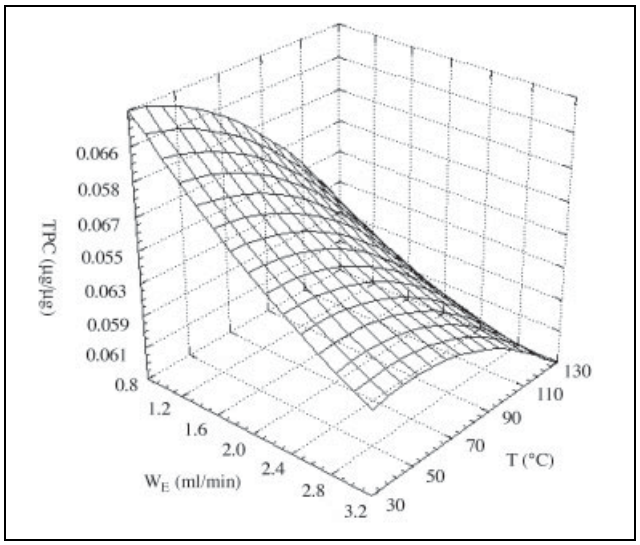

Fig. 1: Response surface of TPC after APE spray-drying

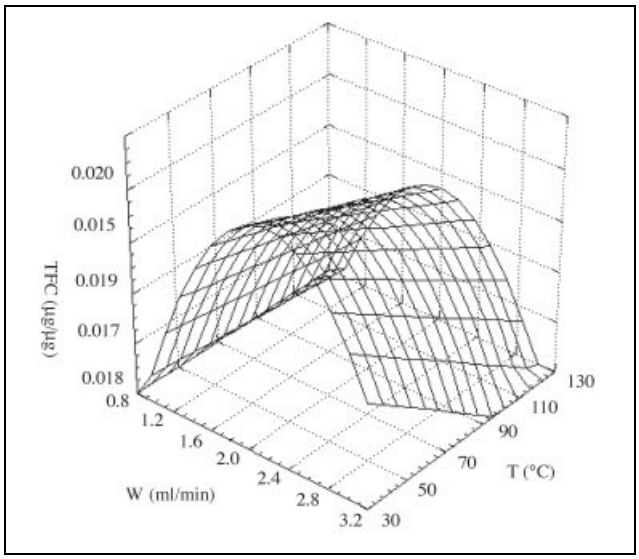

Fig. 2: Response surface of TFC after APE spray-drying

Table 3: Analysis of variance on TFC data

Factor	Sum of squares	Degree of freedom	Mean square	F_{calc}
WE	0.0884	1	0.0884	0.1121
T	0.4349	1	0.4349	0.5517
WE^2	0.0554	1	0.0554	0.0703
T^2	6.7316	1	6.7316	8.5388*
WE × T	0.9428	1	0.9428	1.1959
Error	9.4602	12	0.7884	
Total	17.7133	17		

* Significant at 5%

cant factor. The fitted equation, with correlation coefficient $R^2 = 0.6826$, is given by:

$$TFC = 11.82 + 1.30\left(\frac{T - 80}{40}\right)^2 \quad (2)$$

The value of TPC obtained for the APE on a dry basis was 0.122 μg per 1 μg of total solids, while for the SDPE the values of TPC ranged from 0.055 to 0.067 μg/μg. Given that SDPE dry matter corresponds to the sum of solids in APE plus the amount of drying adjuvant, and the values of TPC shown above were calculated on a dry basis of solids in APE, TPC recovery varied from 45.1 to 54.9%. Regarding the TFC, the APE showed a flavonoid content of 0.049 μg/μg on a dry basis, and the SDPE varied from 0.015 to 0.020 μg/μg, showing a recovery ranging from 30.6 to 40.8%. These results suggest that extreme drying conditions may lead to significant loss of components with AOA and consequently an overall loss of SDPE activity. Next, the *in vitro* AOA of APE and SDPE are assessed and discussed.

2.2. SDPE anti-oxidant activity

As regards the evaluation of the lipid peroxidation induced by Fe^{2+}/ascorbate, the initiation of the peroxidation sequence of a membrane in a free lipid peroxide system refers to the attack of any species that has sufficient reactivity to abstract a hydrogen atom from a polyunsaturated fatty acid.

As the membrane fractions (mitochondria) used in this experiment were isolated from disrupted cells, they may contain some lipid peroxides, which are formed enzymatically in tissues by cyclooxygenase and lypoxygenase enzymes. When iron salts are added, these lipid peroxides are decomposed to generate peroxyl ($LOO^{\bullet}$) and alkoxyl ($LO^{\bullet}$) radicals that can abstract hydrogen from polyunsaturated acyl chains and propagate lipid peroxidation (Halliwel and Gutteridge 1990; Dinis et al. 1994).

The break down of peroxides will produce carbonyl compounds. The 3-carbon compound malondialdehyde (MDA) is one such carbonyl, which forms a characteristic chromogenic adduct with 2 molecules of thiobarbituric acid (TBA), which is determined at 535 nm. On the basis of the inhibition of the MDA-TBA complex formed, the relative inhibitory activity of the samples was estimated at different concentrations. The percent inhibition caused by each sample was calculated as:

$$I\% = 100 - 100\,\frac{I_A}{I_0} \quad (3)$$

where I_0 and I_A represent the absorbancies observed for the control (antioxidant absent) and experimental samples, respectively.

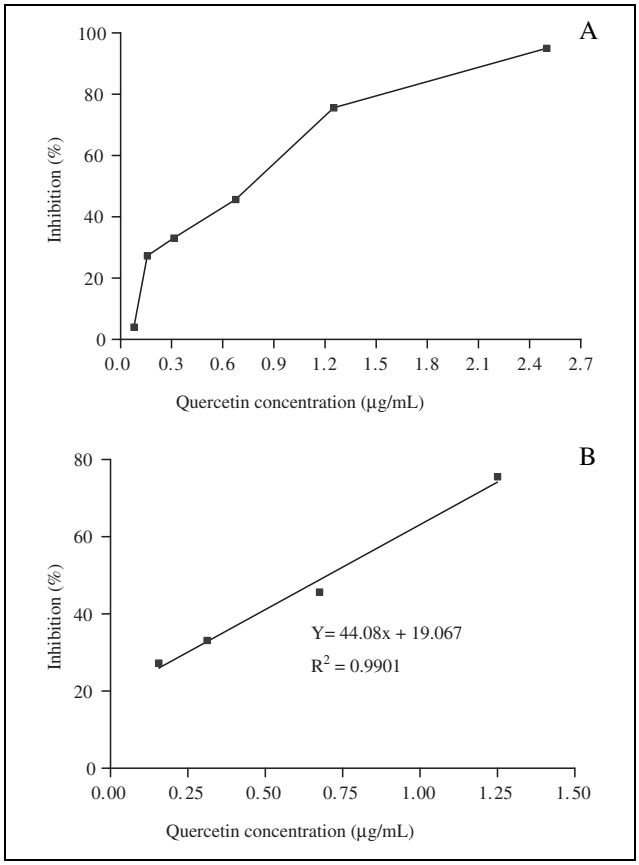

Fig. 3: Quercetin calibration curve – Percentage inhibition by quercetin of lipid peroxidation using mitochondria. Results are means ± S.D. of three measurements run in parallel. (A) Inhibition caused by quercetin at final medium concentrations: 2.5 μg/mL, 1.25 μg/mL, 0.625 μg/mL, 0.312 μg/mL, 0.156 μg/mL, 0.078 μg/mL. (B) Inhibition linearity curve caused by quercetin at final medium concentrations: 1.25 μg/mL, 0.625 μg/mL, 0.312 μg/mL, 0.156 μg/mL

Fig. 3 shows the concentration-response curves for the lipid peroxidation inhibitory effects of spray-dried propolis extracts obtained under varyious drying conditions, e.g., extract feed rates and outlet temperatures. SDPE and APE were evaluated at the same final concentration (2.5, 5 and 10 μg/ml). To evaluate this loss of antioxidant activity, the units of AOA were calculated for both APE and SDPE on a basis of total solids from the original propolis extract.

Percent peroxidation inhibition was measured as a function of quercetin solutions with different concentrations. The dose-response curve obtained is shown in Fig. 4A. The inhibition index, I%, increased with increasing amount of the reference flavonoid (quercetin) added to the assay mixture. The linear bands in the dose-response curves were fitted to the calibration equations by linear regression, as shown in Fig. 4B.

Samples of the SDPE and the APE were evaluated for lipid peroxidation at concentrations at which inhibition rates gave a linear response in the quercetin curve (Fig. 4B). With the aid of the calibration equations obtained from quercetin lipid peroxidation inhibition, the equivalent antioxidant (AOA) units were calculated for each propolis extract sample when its inhibition of lipid peroxidation was about 50%. The AOA units for APE and SDPE were also calculated on a dry basis of the original APE solids content.

Fig. 5 presents the effect of drying factors on the SDPE antioxidant activity, AOA. The surface plot in Fig. 5 indicates that the extract feed rate affects the AOA while it

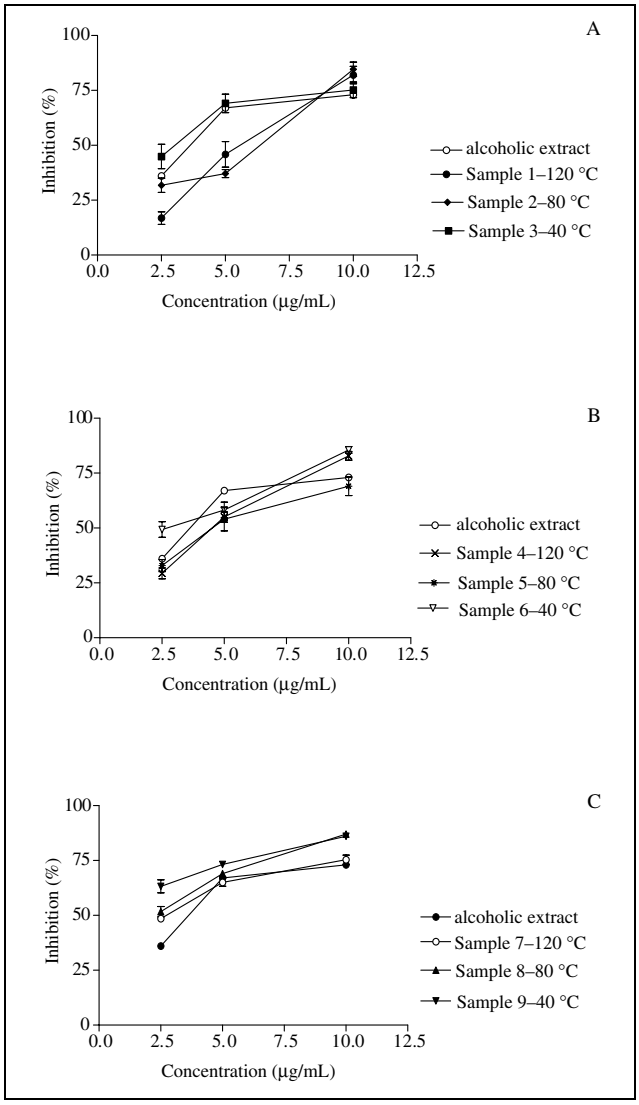

Fig. 4: Percentage inhibition by spray-dried propolis extracts of lipid peroxidation using mitochondria. Results are means ± S.D. of nine measurements run on different days. (A) Inhibition caused by spray-dried propolis extract produced with a feed rate of 3 mL/min at final medium concentrations: 2.5 μg/mL, 5 μg/mL and 10 μg/mL, (B) Inhibition caused by spray-dried propolis extract produced with a feed rate of 2 mL/min at final medium concentrations: 2.5 μg/mL, 5 μg/mL and 10 μg/mL (C) Inhibition caused by spray-dried propolis extract produced with a feed rate of 1mL/min at final medium concentrations: 2.5 μg/mL, 5 μg/mL and 10 μg/mL

seems that temperature is not important for SDPE activity. To support those observations, the data were submitted to variance analysis. The resulting analysis is shown in Table 4 and shows that both temperature and extract feed rate affect AOA at the 0.1% significance level. The effects of the square of both factors and their interaction are not significant. The AOA data were put into an equation, taking into account only the significant terms. The resulting equation, with correlation coefficient $R^2 = 0.9240$, is given by:

$$AOA = 119.84 - 10.17(W_E - 2) - 3.30\left(\frac{T - 80}{40}\right) \quad (4)$$

The APE showed lipid peroxidation inhibition of 47.5% at a final concentration of 2.5 μl/ml, while some SDPE varied from about 43 to 58% at 5 μg/ml and others from about 48 to 63% at 2.5 μg/ml. This comparison of activity

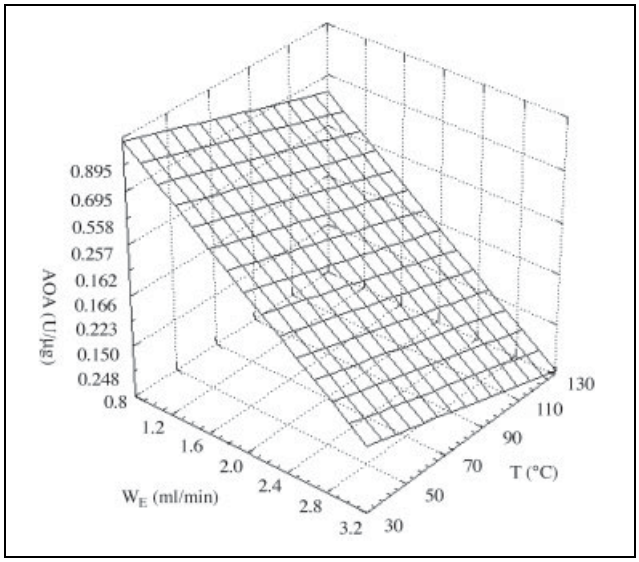

Fig. 5: Response surface of AOA after APE spray-drying

Table 4: Analysis of variance on AOA data

Factor	Sum of squares	Degree of freedom	Mean square	F_{calc}
WE	3100.8	1	3100.8	203.638*
T	307.2	1	307.2	20.173*
WE^2	5.9	1	5.9	0.386
T^2	27.8	1	27.8	1.824
WE × T	24.2	1	24.2	1.589
Error	593.9	39	15.2	
Total	4059.8	44		

* Significant at 0.1%

becomes clearer when the percentages of lipid peroxidation inhibition are estimated as antioxidant units, and these antioxidant units are calculated per μg of total solids in the original APE. The final concentration of 2.5 μg/ml of APE in the medium of lipid peroxidation corresponds to 0.2 μg/ml in total solids. However for SDPE this same final concentration (2.5 μg/ml) corresponds to 1.7 μg/ml of total solids in the lipid peroxidation medium. The AOA recovery ranged from 9 to 28%, showing that some activity is lost during the spray-drying process. Better recovery is found for SDPE submitted to spray-drying at APE feed rates of 1 ml/min.

Despite SDPE having lost some of their TPC, TFC and AOA, the dry extracts could be successfully utilized in antioxidant therapy. The SDPE, when evaluated at the final concentrations of 2.5 and 5.0 μg/ml in the medium were able to inhibit about 50% of lipid peroxidation. These AOA values are plausible, since 50 μg/ml of alcoholic and water propolis extracts from other areas have been evaluated by copper mediated lipid peroxidation in unfractioned serum and the inhibition observed varied from 20 to 80% (Isla et al. 2001).

Also, Banskota et al. (2000) demonstrated that alcoholic and water propolis extracts from several origins were able to scavenge the stable free radical DPPH at levels ranging from 5.9 to 94 μg/ml. These results, together with the fact that the process can be modified to allow higher chemical and AOA recovery, suggests that despite SDPE having lost some of their activity they still present excellent antioxidant activity and this can be a valid process to obtain new pharmaceutical forms of propolis.

The chemical and antioxidant activities of spray-dried propolis extract were shown to be affected by drying conditions. The air outlet temperature was shown to affect significantly the overall polyphenol and flavonoid content and the antioxidant activity of the dry extract, with a linear or quadratic relationship. The extract feed rate significantly affected only the antioxidant activity. The recovery of polyphenols and flavonoids was significantly higher than the recovery of antioxidant activity. This may be an indication that the antioxidant activity is only partially related to the two classes of compounds observed here, and there may be other chemicals involved in its activity. It is also possible that some structural changes in flavonoids and polyphenols occurred during the drying process that caused the loss of antioxidant activity but did not interfere significantly with the determination of flavonoid and polyphenol content. Finally, the spray-dried propolis extract obtained under the conditions studied here presented a high level of lipid peroxidation inhibition and can be successfully used for biopharmaceutical purposes. The data also show the complex phenomena involved in the drying of biological materials and the resulting changes in their properties. With the increasing interest in natural products for therapeutic use, there is a need to direct efforts for better understanding of such drying/biopharmaceutical relationships.

3. Experimental

3.1. Alcoholic propolis extract (APE)

Alcoholic extract of green propolis, was purchased on the Brazilian market from Apis Flora Ltda., and originated from Oliveira, Minas Gerais. Its total solids content was 9.43% (w/v).

3.2. Spray-dried propolis extract (SDPE)

Before the drying step, APE was concentrated in a rotating evaporator to reduce its 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 Ltd., Brazil) under various operational conditions. The factors studied in the drying process were the drying air outlet temperature, T (°C) and the extract feed rate, W_E (ml/min). The pneumatic spray nozzle was operated at an airflow rate of 40.0 l/min and a pressure of 2.0 kgf/cm^2. Drying airflow rate was kept constant at 0.60 m^3/min. The range of drying conditions was chosen to operate outside the limits of flammability for the ethanol-air mixture (Berthold and Loffler 1993). The SDPE was collected at the dryer outlet, weighted and stored in closed flasks protected from light at 4 °C.

The drying study followed a 3^2 full factorial design, which allows for the determination of linear, quadratic and interactive effects (Box et al. 1978). Table 5 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:

$$\text{Coded} \cdot \text{Variable} = \frac{(\text{uncoded} \cdot \text{value} - 0.5 \times (\text{high} \cdot \text{value} + \text{low} \cdot \text{value}))}{0.5 \times (\text{high} \cdot \text{value} - \text{low} \cdot \text{value})} \quad (5)$$

The response function applied was a quadratic equation, as given below:

$$Y_i = A_o + A_1X_1 + A_2X_2 + A_3X_1^2 + A_4X_2^2 + A_5X_1X_2 \quad (6)$$

where, Y_i = dependent variable = TPC, TFC or AOA; X_1 = coded CPE feed rate; X_2 = coded air inlet temperature and A_i = polynomial coefficients.

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

Table 5: Levels of factors for drying experiments

FACTORS	LEVELS		
	−1	0	+1
1. CPE feed rate, W_E (ml/min)	1.0	2.0	3.0
2. Air temperature, T (°C)	40	80	120

3.3. Polyphenol and flavonoid contents

3.3.1. Extraction of flavonoid and polyphenol contents from APE and SDPE

Spray-dried propolis extract (SDPE, 0.1 g) (accurately weighed) was first dissolved in 10 mL of 80% ethanol. After centrifugation at 1000 × g for 10 min, the supernatant was collected and the precipitate was then twice extracted with 5 mL of 80% ethanol. Finally, the supernatant was combined with the previous supernatant and adjusted to 25 mL with 80% ethanol. Alcoholic propolis extract (APE) was directly diluted 50 and 100 times with 80% ethanol.

3.3.2. Polyphenol content

Total polyphenol contents, TPC, in APE and SDPE were determined by the Folin-Ciocalteu colorimetric method. 0.5 ml of APE and SDPE solution were mixed with 0.5 ml of the Folin-Ciocalteu reagent and 0.5 ml of 10% Na_2CO_3, and the absorbance was measured at 760 nm with a Hitachi U2001 spectrophotometer, after 1 h incubation at room temperature. Gallic acid was used to make the calibration curve. 25 mg of gallic acid was dissolved in 50% ethanol and then diluted to 1, 5, 10, 15 and 20 μg/ml (Kumazawa et al. 2004).

3.3.3. Flavonoid content

Total flavonoid contents, TFC, were determined using the aluminum chloride colorimetric method. To 0.5 ml of APE or SDPE solutions, 0.5 ml of 2% $AlCl_3$ ethanol solution was added. After 1 h at room temperature, the absorbance was measured at 420 nm with a Hitachi U2001 spectrophotometer. Quercetin was used to make the calibration curve. 10 mg of quercetin was dissolved in 80% ethanol and then diluted to 1, 5, 10, 20 and 25 μg/ml. The amount of 2% aluminum chloride was substituted by the same amount of distilled water for the blank (Kumazawa et al. 2004).

3.4. Lipid peroxidation

In addition to the lipid peroxidation inhibition tests performed for APE and SDPE, the same test was also performed for quercetin. Quercetin was used as an antioxidant reference with the purpose of comparing activities of APE and SDPE. The choice was due to the potent antioxidant activity of this flavonoid, which shows dose-dependent lipid peroxidation inhibition.

3.4.1. Sample preparation

Quercetin was diluted to yield media concentrations of 5 μg/mL, 2.5 μg/mL, 1.25 μg/mL, 0.625 μg/mL, 0.312 μg/mL, 0.156 μg/mL and 0.078 μg/mL. 25 mg of SDPE sample was diluted in ethanol to obtain solutions of 1 mg/mL, 500 μg/mL and 250 μg/mL, yielding final concentrations in the reaction medium of 10 μg/mL, 5 μg/mL and 2.5 μg/mL. The APE was also weighted and diluted, yielding final medium concentrations of 10 μg/mL, 5 μg/mL and 2.5 μg/mL.

3.4.2. Inhibition of lipid peroxidation

10 μL of each sample were added to 1.0 mL of a reaction mixture with sucrose 125 mM, KCl 65 mM and Tris-HCl 10 mM, pH 7.4, and mitochondria were added to yield a final concentration of 1 mg of protein plus 50 μM ferrous ammonium sulfate and 2 mM sodium citrate for 30 min, at 37 °C. For malondialdehyde (MDA) determination, 1 mL of 1% thiobarbituric acid (TBA) (prepared in 50 mM NaOH), 0.1 mL of 10 M NaOH and 0.5 mL of 20% H_3PO_4 were added, followed by incubation for 20 min at 85 °C. The MDA-TBA complex was extracted with 2 mL of n-butanol. The samples were then centrifuged at 3,000 rpm for 10 min. The blank was prepared without mitochondria and the control, equivalent to 100% peroxidation was prepared without antioxidant. The measurement was performed on the supernatant at 535 nm (Rodrigues et al. 2002).

3.4.3. Isolation of rat liver mitochondria

Mitochondria were isolated by differential centrifugation (Pedersen et al. 1978). Male Wistar rats weighing approximately 200 g were sacrificed by cervical dislocation; livers (10–15 g) were immediately removed, sliced in 50 mL of medium containing (mM) sucrose 250 EGTA 1 and HEPES-KOH 10, pH 7.2, and homogenized three times for 15 s at 1 min intervals in a Potter-Elvehjem homogenizer. Homogenates were centrifuged at 770 × g for 5 min and the resulting supernatant was further centrifuged at 9800 × g for 10 min. Pellets were suspended on 10 mL of medium containing (mM) sucrose 250 EGTA 0.3 and HEPES-KOH 10, pH 7.2, and centrifuged at 4500 × g for 15 min. The final mitochondrial pellet was suspended in 1 mL of medium containing (mM) sucrose 250 and HEPES-KOH 10, pH 7.2 and used within 3 h. Mitochondrial protein content was determined by the biuret reaction (Cain and Skilleter 1987).

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