

Antioxidant Activity and Composition of Propolis Obtained by Different Methods of Extraction

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Extratos etanólicos de própolis obtidos a partir de diferentes concentrações de própolis e com álcool hidratado com diferentes teores de álcool/água foram analisados. A ação antioxidante destes extratos foi determinada através da captura do radical livre 1,1-difenil-2-picril-hidrazila (DPPH•) e do poder de redução férrico (FRAP) utilizando o complexo férrico-tripiridiltriázina (Fe³⁺-TPTZ). O teor de compostos fenólicos totais e a quantificação de flavonóides também foram verificados e obtidos por espectrometria de massas com ionização por *electrospray* (ESI(-)-MS) dos extratos. Observou-se que a composição química dos extratos de própolis e sua atividade antioxidante variam com a concentração de própolis e, principalmente, com o teor de álcool/água do etanol hidratado utilizado na extração. Foi possível perceber também que a atividade antioxidante dos extratos é significativamente dependente destes parâmetros de extração e que as composições de compostos fenólicos e flavonóides também variam muito. Os valores de FRAP não apresentaram, porém, boa correlação com o teor de flavonóides.

Ethanolic extracts of propolis obtained with different concentrations of propolis and made using hydrous ethanol with various water/ethanol contents were analyzed. The antioxidant action of the extracts was determined by free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH•) scavenging and by ferric reducing ability power (FRAP), using the ferric-tripyridyl triazine (Fe³⁺-TPTZ) complex. The total phenolic and flavonoid contents were also determined, and the electrospray ionization mass spectrometry spectra (ESI(-)-MS) of the extracts were acquired. The chemical composition of propolis extracts and their antioxidant activity were observed to vary with the concentration of propolis, and especially with the water/ethanol contents of the hydrous ethanol used for extraction. The antioxidant activity of the extracts was also found to be significantly dependent on these extraction parameters, as well as on the composition of the phenolic compounds and flavonoids. FRAP values showed, however, poor correlation with the flavonoid content.

Keywords: total phenolic contents, antioxidant activity, ESI(-)-MS fingerprint, propolis extraction, flavonoids

Introduction

Propolis is a resin produced by honey bees by mixing wax, pollen, salivary secretions and collected natural resins.¹ The chemical composition of propolis is complex and varies with the vegetation visited by bees, as well as with bee

species.²⁻⁶ As propolis has characteristics that are beneficial to human health, such as antioxidant, anti-inflammatory, antifungal and anticancer activities, its chemical composition and properties have been extensively studied.⁷⁻¹⁰

The antioxidant activity of ethanolic extracts of propolis has been evaluated by free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH•) scavenging,⁸ by the ferric reducing ability power (FRAP) using the ferric-tripyridyltriazine

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(Fe³⁺-TPTZ) complex¹¹ and by oxidation of linoleic acid.⁵ Similarly, mass spectrometry analyses has been used to examine the chemical composition of propolis extracts and its correlation with type and properties.^{9,12} The compositions of total phenolic compounds by Folin-Ciocalteu method and of flavonoid by using AlCl₃ have been determined.^{11,13} It seems, however, that the correlation between the antioxidant activity and the contents of phenolic compounds and flavonoids of propolis extracts obtained with different ethanol contents and propolis concentrations has not been investigated yet.

The purpose of this study was therefore to compare the antioxidant activity of ethanolic extracts of propolis prepared using hydrous ethanol with different ethanol/water contents and different propolis concentrations. We also investigated the effect of these extraction variables on the antioxidant activity, the total phenolic compounds and flavonoid, and in the overall composition, as revealed by electrospray ionization mass spectrometry (ESI(-)-MS) fingerprints of the extracts. In addition, we have also assessed the correlation between the composition of propolis of the various extracts and their antioxidant activity.

Experimental

Collection of propolis

Propolis sample was collected from hives of *Apis mellifera* bees in the apiary of the Iguatemi Experimental Farm of the Universidade Estadual de Maringá (Maringá, Paraná State) (latitude 23°25 south and longitude 51°57 west of Greenwich, altitude of 540 m). The sample was kept in polypropylene jars at -4 °C. This sample is being certified as organic propolis by IBD-Certifications at Botucatu, São Paulo State, Brazil. This institute certifies propolis at national and international levels, which makes possible the traceability of organic propolis.

Preparation of extracts

The four extracts were obtained using two different concentrations of propolis between 5 and 30% (m/m) and two ethanol dilutions, between 60 and 96% (v/v) by turbo-

extraction for 15 min. The extracts were vacuum filtered and dealcoholized in a rotary evaporator (Buchi, model RT 210) to the limit of 15% ethanol. Then, they were spray dried in a Labmaq spray drier with the capacity of 0.5 L h⁻¹. The dried extracts were stored in amber-colored flasks with inner sealing plugs and screw caps at -4 °C. More information about the preparation method, propolis and solvent concentrations are protected by a patent at the National Institute of Industrial Property (INPI) under the number PI 0605768-3.¹⁴ Table 1 shows the labeled extracts, their respective levels (-1) for the lowest level; (+1) for the highest level) of the coded independent variables used in the experimental design and the total solid contents of the four extracts. The code of the lowest level (-1) represent the lowest concentration of propolis or the lowest ethanol contents used to prepare the extracts while the code of the highest level (+1) represent the highest concentration of propolis or the highest ethanol contents used to prepare the extracts.

DPPH radical scavenging activity

The free radical scavenging activity was measured using DPPH• as already described¹⁵ with some modifications. Briefly, various volumes of propolis extract solutions (2.0 mg mL⁻¹) were added to 2.0 mL of DPPH• methanolic solution (0.1192 mmol L⁻¹) and maintained in the dark for 30 min at room temperature. Then, absorbance was measured at 517 nm. Methanol was used instead of propolis extract solutions as a control and Trolox was used as a positive control. The results were expressed by EC₅₀ value (half maximal effective concentration), which determines the extract concentration (µg mL⁻¹) that provides 50% inhibition; the lower its value is, the greater the efficiency of the antioxidant. The scavenging capacity of the DPPH radical was calculated with equation 1 (percent inhibition of the DPPH radical).

$$\% \text{ of Inhibition DPPH}\bullet = \frac{(\text{Abs}_{\text{DPPH}\bullet} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{DPPH}\bullet}} \times 100 \quad (1)$$

The extract concentration value was plotted vs. % of inhibition of DPPH• and the EC₅₀ value was obtained by linear regression. All treatments were run in triplicate.

Table 1. Extract names, levels of coded independent variables and the total solid contents in each case of extraction

Extract names	Ethanol content (%)	Propolis concentration (% w/w)	Total solid contents (%)*
B10	-1	-1	3.6 ± 0.1
B30	+1	-1	4.5 ± 0.2
C10	-1	+1	5.5 ± 0.2
C30	+1	+1	6.8 ± 0.3

*n = 5.

Ferric reducing ability power – FRAP

The FRAP was determined as previously described¹⁶ with modifications. This method is based on a redox reaction in which the antioxidants act as reductants, and an easily reduced oxidant (Fe^{3+}) is used in stoichiometric excess, resulting in a blue ferrous complex. The absorbance at 593 nm was read using a spectrophotometer (Cary 50 – Varian) for 3.0 mL of a freshly prepared Fe^{3+} -TPTZ complex solution pre-incubated at 37 °C. This solution (FRAP reagent) was prepared by mixing acetate buffer (300 mmol L⁻¹, pH 3.6), TPTZ (10 mmol L⁻¹ in 1.0 mol L⁻¹ HCl), and FeCl_3 (20 mmol L⁻¹) at 10:1:1 (v/v/v). Different concentrations of 100 μL propolis extract solutions were added to cuvettes with 300 μL of distilled water and 3.0 mL of Fe^{3+} -TPTZ complex, totaling 1:34 dilution. The mixtures were homogenized and incubated at 37 °C for 30 min before absorbance reading at 593 nm. All treatments were run in triplicate and TroloxTM ((\pm)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was used as a positive control. The potential of the antioxidants in the propolis extract to reduce Fe^{3+} to Fe^{2+} was expressed in $\mu\text{mol Fe}^{2+} \text{ g}^{-1}$ of propolis extract using a calibration curve of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0-2000 $\mu\text{mol L}^{-1}$) ($r^2 = 0.9999$). It was assumed that the higher measured FRAP value, the higher the antioxidant content of the propolis extract that could reduce the ferric ion to ferrous ion.

Total phenolic contents

The total phenolic contents of the propolis extracts were analyzed using gallic acid as a standard by the Folin-Ciocalteu method¹⁷ with some modifications. Methanolic solutions of propolis extracts (2.5 mg mL⁻¹) were prepared and 250 μL of these solutions or of standard solutions of gallic acid or methanol as blank were added to separate test tubes. To each tube, 250 μL of Folin-Ciocalteu reagent (diluted in water 1:1), 500 μL of Na_2CO_3 saturated solution, and 4 mL of distilled water were added and mixed. The solutions were incubated in the dark at room temperature for 25 min and then centrifuged for 10 min at 3000 rpm. The sample absorbance was read against the blank at 725 nm using a spectrophotometer (Cary 50 – Varian). The total phenolic content of the extracts was determined by comparison with a calibration curve of gallic acid as a standard ($r^2 = 0.9995$) and represented as mg gallic acid equivalents (GAE) in g⁻¹ of propolis extract. The analyses were done in triplicate.

Total flavonoid contents

The total flavonoid contents in the propolis extracts were determined by the AlCl_3 method,¹⁸ with slight

modifications. Methanolic solutions of propolis extract (2.5 mg mL⁻¹) were prepared and 500 μL of solution or of quercetin standard solutions using methanol as a blank were added to separate test tubes. To each tube, 250 μL of AlCl_3 5% (m/v in methanol) and 4.25 mL of methanol were added. After 30 min, absorbance was read at 425 nm using a spectrophotometer (Cary 50 – Varian) at room temperature. The total flavonoid contents of the extracts were determined by comparison with the calibration curve of the quercetin standard ($r^2 = 0.9998$) and represented as mg quercetin equivalents g⁻¹ (mg QE g⁻¹) of propolis extract. The analyses were done in triplicate.

Electrospray ionization mass spectrometry (ESI-MS) fingerprints

For the ESI(-)-MS fingerprints of the propolis extracts, 10 mg of each extract were dissolved in 1 mL of a solution of 50% HPLC-grade methanol and 50% purified water containing 0.5% of NH_4OH . These solutions were directly infused into the ESI source by means of a syringe pump (Harvard Apparatus, Massachusetts, USA) at a flow rate of 10 $\mu\text{L min}^{-1}$. Negative ion mode fingerprints [ESI(-)-MS] were acquired using an Applied Biosystems Q-TRAP mass spectrometer (Foster City, CA, USA) in the following conditions: capillary and cone voltages of -4500 V and -30 V, respectively, desolvation temperature of 350 °C. Diagnostic ions in the different propolis extracts were identified by comparison of their ESI(-)-MS/MS dissociation patterns with those of compounds identified in previous studies.^{4,8,9,12,19} Fingerprints are shown in the m/z 150-600 range, since no important ions were observed below or above these m/z values.

Statistical analysis

The experimental design and statistical analysis were planned using the software Design Expert 7.1.3,²⁰ which used both the propolis concentration and extraction ethanol content with -1 value as the lowest value and +1 for the highest value (Table 1) for the factorial design 2². The parameters used were DPPH• test, FRAP, total phenolic contents and quantification of flavonoid. The data were expressed as mean standard deviations, one-way analysis of variance (ANOVA) and surface test from software Design Expert 7.1.3.

Chemicals

DPPH•, tripyridyltriazine, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, gallic acid, Folin-Ciocalteu reagent, Trolox and quercetin were obtained from Sigma-Aldrich. Other chemicals were analytical degree.

Results and Discussion

Determination of antioxidant activity

Table 2 shows the results of the propolis extracts and positive control obtained by analyses of scavenging free radical DPPH, FRAP, total phenolic compounds by the Folin-Ciocalteu reagent method and quantification of flavonoid using AlCl_3 . For all the responses analyzed, the linear model was significant ($P < 0.0001$).

In the analysis of DPPH•, a lower EC_{50} value indicates greater antioxidant activity, as a smaller mass of extract

is required to inhibit 50% of the DPPH radical. The best result was observed in extract C10, obtained with a higher concentration of propolis and lower ethanol content (Figure 1a). The analysis of variance revealed that the propolis concentration and the ethanol content, as well as the interaction between these two factors, were highly significant ($p < 0.0050$) for the following model: $y = + 82.6083 - 14.0083 x_1 - 24.9083 x_2 + 24.8083 x_1x_2$, where $y = \text{DPPH value}$, $x_1 = \text{ethanol content}$, $x_2 = \text{propolis concentration}$ and $x_1x_2 = \text{interaction between concentration and ethanol content}$. The antioxidant effect of the main constituents found in water extracts of propolis is greater

Table 2. Antioxidant activity and composition of propolis extracts (PE)

Extract	DPPH* ($\text{EC}_{50} \mu\text{g mL}^{-1}$)	FRAP value* ($\mu\text{mol Fe}^{2+} \text{g}^{-1} \text{PE}$)	Total phenolic content* ($\text{mg EAG g}^{-1} \text{PE}$)	Flavonoids* ($\text{mg EQ g}^{-1} \text{PE}$)
B10	160 ± 3	528 ± 0	48 ± 2	10 ± 0
C10	47 ± 1	1365 ± 2	87 ± 3	24 ± 1
C30	69 ± 1	592 ± 0	73 ± 3	26 ± 1
B30	65 ± 5	890 ± 3	76 ± 1	26 ± 1
Trolox	8 ± 0	2068 ± 10	-	-

*n = 3.

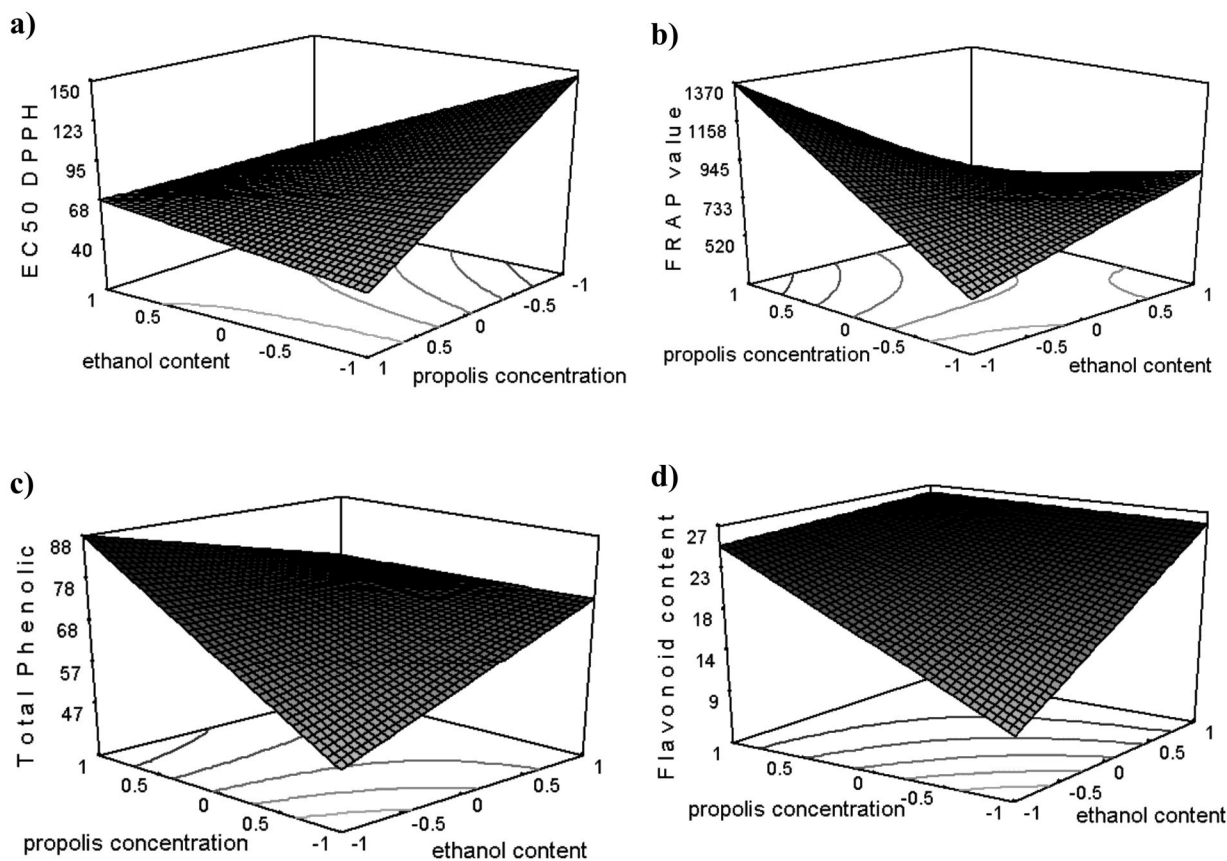


Figure 1. a) EC_{50} DPPH• ($\mu\text{g mL}^{-1}$ propolis extract), b) FRAP values ($\mu\text{mol Fe}^{2+} \text{g}^{-1}$ propolis extract), c) total phenolic compounds (mg GAE g^{-1} propolis extract), d) flavonoids (mg QE g^{-1} propolis extract) obtained for propolis extracts based on the ethanol content and propolis concentration.

than that of the ethanol extracts.²¹ Hence, higher antioxidant activity is expected for propolis extracts made by hydrous ethanol with higher water contents. In this study, the observed contribution of the ethanol content to a lower EC_{50} was 13%, while those of the effects of extract concentration and the “extract concentration \times ethanol content” interaction were both 40%. The remaining percentage likely corresponds to deviations in measurements.

In the FRAP analysis, the extract C10 also had a higher value of equivalent $\mu\text{mol Fe}^{2+} \text{ g}^{-1}$ of extract, indicating higher antioxidant activity. The analysis of variance revealed that both the concentration and ethanol content factors and the interaction between them are significant ($p < 0.0001$) for the following model: $y = + 843.6705 - 102.8131 x_1 + 134.8087 x_2 - 283.7762 x_1 x_2$, where y = FRAP value, x_1 = ethanol content, x_2 = propolis concentration and $x_1 x_2$ = the interaction between concentration and ethanol content. The “extract concentration \times ethanol content” interaction was the main contributor to the effects, with 74%, while the contributions of the extract concentration and ethanol content for a higher FRAP value were 16 and 10%, respectively (Figure 1b).

Lower antioxidant activity was observed in extract B10, both by DPPH radical scavenging and FRAP (Table 2). Likewise, this propolis extract had the lowest contents of total phenolic compounds and flavonoid, resulting from a lower concentration of propolis and a lower ethanol content used for extraction. Several studies establish a relationship between the antioxidant activity of ethanolic extracts of propolis and their contents of phenolic compounds, such as the flavonoids.^{5,22,23} The analysis of variance revealed that the propolis concentration, the ethanol content, and their interaction are significant ($P < 0.0500$) for the content of total phenolic compounds: $y = + 70.3855 + 2.4794 x_1 + 9.9669 x_2 - 10.1251 x_1 x_2$, where y = content of total phenolic compounds, x_1 = ethanol content, x_2 = propolis concentration and $x_1 x_2$ = interaction between the propolis concentration and the ethanol content. The effect of the ethanol content was only 3%, while those of the propolis concentration and the “extract concentration \times ethanol content” interaction were more important for the content of total phenolic compounds, with contributions of 46 and 48%, respectively (Figure 1c). The remaining percentage corresponds to pure error. The values of total phenolic compounds obtained in this study are within the range found in propolis, which is 31-299 mg GAE g^{-1} of ethanolic extract of propolis.⁵

The ethanol content of the hydrous ethanol used to obtain the extracts had a greater effect on the content of flavonoids (39%), whereas the propolis concentration and the interaction between these two factors had effects of 31 and

28%, respectively (Figure 1d). The remaining percentage corresponds to error. The analysis of variance showed that the main effects and the interaction between them were significant ($P < 0.0001$) for the quantification of flavonoid: $y = + 21.6882 + 4.2791 x_1 + 3.8538 x_2 - 3.6678 x_1 x_2$, where y = content of total phenolic compounds, x_1 = ethanol content, x_2 = propolis concentration and $x_1 x_2$ = interaction between the propolis concentration and the ethanol content. The flavonoid contents obtained in ethanolic extracts of propolis in this study are within the range of 2.5-176 mg QE g^{-1} found in propolis.⁵

The correlations between the method results, DPPH· and FRAP, and the contents of phenolic compounds and flavonoid are shown in Figure 2. A negative correlation is observed between the DPPH·-flavonoid content (-0.9082), DPPH·-phenolic content (-0.9338) and DPPH·-FRAP (-0.6910), since a low EC_{50} value in the DPPH· method is related to high contents of phenolic compounds and flavonoid, and a high value of FRAP and *vice-versa*. The correlation between phenolic content-flavonoid content was positive (0.8679), as well as the correlation between FRAP-phenolic content (0.8169), indicating that a large amount of phenolic content can be derived from the flavonoid contents and that the high reducing power by the FRAP method can derive from the content of phenolic compounds present in the extracts. On the other hand, the FRAP value may not be related to the flavonoid content, because the coefficient of correlation between FRAP-flavonoid content is only 0.4915. This indicates that the flavonoids in the extracts analyzed are important for the antioxidant activity by the DPPH free radical scavenging method, but not by the reducing power observed by the FRAP method. According to Nagai and co-workers,²⁴ the flavonoids in ethanolic propolis extracts behave as scavengers in the initial stage of lipid peroxidation and later react with peroxy radicals of polyunsaturated fatty acids, breaking the chain reaction; therefore, not necessarily as a reducer as in the FRAP method.

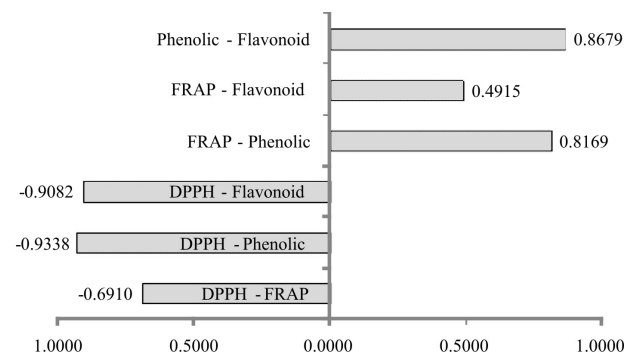


Figure 2. Correlation coefficients between the methods DPPH·, FRAP, and contents of phenolic compounds and flavonoids.

Fingerprint by mass spectrometry

These fingerprints were used for the qualitative assessment of the different methods of extraction. By comparing the four ESI(-)-MS fingerprints obtained (Figure 3), one can notice that the spectrum of the C10 propolis extract, which had the highest antioxidant activity, was similar to the spectra of extracts C30 and B30. The ion with the highest intensity in the C10 extract spectrum is that of m/z 299, which may be the phenolic

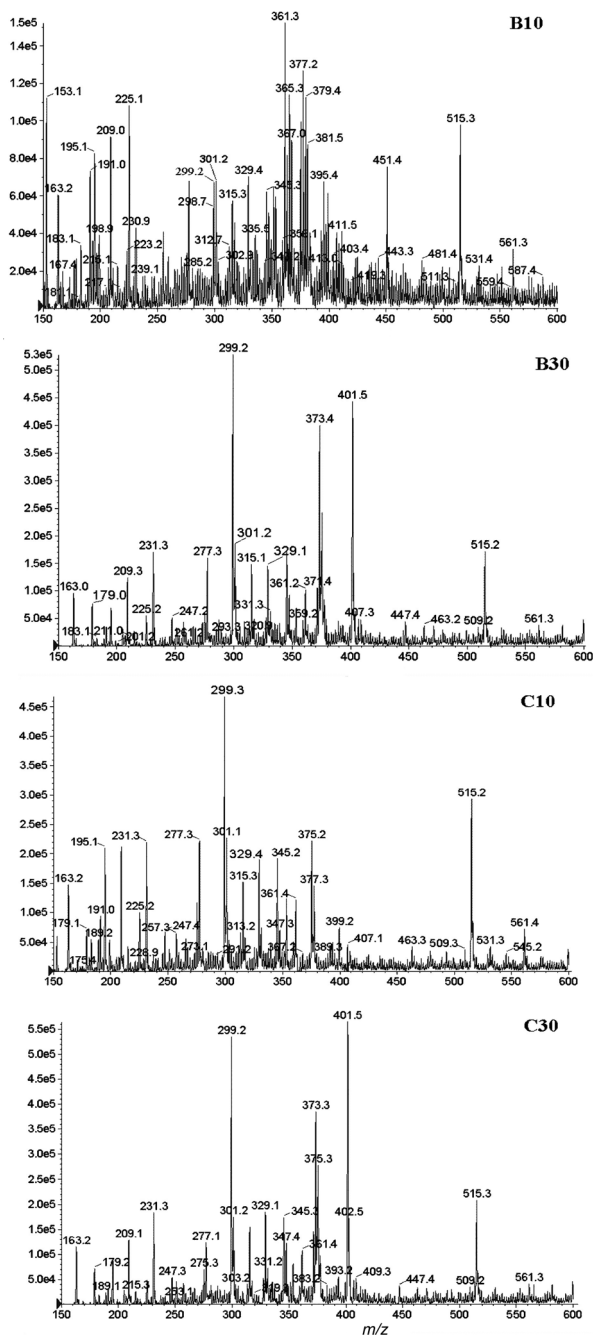


Figure 3. ESI(-)-MS fingerprints of different propolis extracts with different propolis concentrations and ethanol contents.

compound 3,5-diprenyl-4-hydroxy-cinnamic acid (DPHC) or the flavonoid kaempferide, both found in this type of propolis.^{9,19} We also found ions of m/z 163, 179, 231, 301, 329 and 515, commonly seen in ESI(-)-MS of propolis, which may correspond to *p*-coumaric acid, caffeic acid, propol or 3-prenyl-4-hydroxy-cinnamic acid (PHC), dihydrokaempferide or *e/z* communica acid, betuletol and dicaffeoylquinic acid, respectively.^{9,19} *p*-Coumaric acid, caffeic acid, propol, 3-prenyl-4-hydroxy-cinnamic acid, and dicaffeoylquinic acid are all phenolic compounds, dihydrokaempferide and betuletol are flavonoids, and *e/z* communica acid is a terpene.

An increase in the ethanol content used to obtain the propolis extracts, both from B10 to B30 and C10 to C30, resulted in the appearance of ions of m/z 373 and 401 in the spectra of B30 and C30 (Figure 3), which can be assigned to triterpenes.^{8,19} These compounds have low antioxidant activity,⁸ thereby reducing the total activity of their extracts. This is in accordance with the decrease observed in FRAP values and the increase in IC_{50} values of DPPH \cdot of extracts C10 to C30. However, extracts B10 and B30 had a distinct behavior. Possibly due to the higher intensity of the ion of m/z 299 observed in the B30 propolis extract. Studies report that mono- and diprenyl *ortho*-substituted phenolic acids (PHC and DPHC) show higher antioxidant activity in comparison with unsubstituted *p*-coumaric acid.²² Such compounds are possibly detected as the ions of m/z 231, 299 and 163, respectively (Figure 3). In addition, other studies of the effects of various parameters on the propolis extraction yield²³ also observed increased yield for compounds derived from *p*-coumaric acid with increased ethanol content, such as mono- and diprenyl *ortho*-substituted phenolic acids.

The ESI(-)-MS of extract B10, which had the lowest antioxidant activity, also had the lowest ion intensity in general. The relative intensity of the ion of m/z 299 was also lower than those of the other ions, indicating its low concentration in the extract. The ion with the highest intensity in the B10 extract was that of m/z 361, which may be attributed to 15-acetoxy-cupressic acid, found in propolis from the Paraná State.⁹

The presence of the ion of m/z 299 in the spectra of the four extracts of the same propolis characterizes it as green propolis.¹⁹ In addition, the ion of m/z 299 appears to be also responsible for the antioxidant activity of the propolis extracts analyzed, because extract B10, which showed low antioxidant activity, also had low intensity for this ion.

Conclusions

By the response surface and statistical analysis it was possible to observe that the DPPH \cdot and FRAP values are

significantly dependent of the propolis concentration and the water/ethanol content of hydrous ethanol used in the extraction. The ethanol/water content and the propolis concentration were also found to correlate with the composition of phenolic compounds and flavonoids. Likewise, similar correlation was also found with all the parameters analyzed (DPPH•, FRAP, phenolic contents and flavonoid contents) except for FRAP values, which showed poor correlation with flavonoid contents. The ESI(-)-MS fingerprints of the extracts showed substantial changes in ions intensities, indicating that the composition of propolis extracts varies with propolis concentration, especially with the water/ethanol content of the hydrous ethanol used in the extraction. The ion of m/z 299, which probably corresponds to the phenolic compound 3,5-diprenyl-4-hydroxy-cinnamic acid, was the most intense and increased with the ethanol content and propolis concentration.

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