Supercritical extraction from red propolis and fractionation of its hydroalcoholic and ethanolic extracts using CO$_2$ as anti-solvent

Extração supercrítica da própolis vermelha e fracionamento de seus extratos hidroalcoólicos e etanólicos usando CO2 como anti-solvente

DOI:10.34117/bjdv8n1-539

Recebimento dos originais: 07/12/2021
Aceitação para publicação: 31/01/2022

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ABSTRACT
Technologies for extraction and fractionation of phenolic compounds from a typical propolis from northeastern Brazil, called red propolis, using supercritical fluids may represent an environmentally correct alternative to the current extraction processes, as they are included in the concept of "green chemistry". The objective of this work was to extract and fractionate the phenolic compounds from red propolis: I) Using supercritical carbon dioxide (scCO₂) as solvent and ethanol as co-solvent to obtain an ethanolic extract; II) Using scCO₂ as solvent and a mixture of ethanol: water (70:30, v/v) as co-solvent to obtain a hydroalcoholic extract. After extraction, the ethanolic and hydroalcoholic extracts were fractionated using scCO₂ as antisolvent at a constant temperature of 50 ºC, subjected to four gradual pressures, in a sequential of separators operated at 200, 100 and 80 bar, at the end, atmospheric pressure (1.013 bar). The procedure was characterized according to extraction yield; total phenols, total flavonoids, antioxidant activity and color. It was found that the pressure influenced the yield and the concentration of the phenolic compounds in the extracts, demonstrating that the most efficient fractionation process occurred in the first and second separators. Overall, all extracts showed high antioxidant activity.

Keywords: Extraction techniques, isoflavones, bees, Dalbergia ecastophyllum, phenolic compounds, antioxidant activity.

RESUMO
Tecnologias para extração e fracionamento de compostos fenólicos de uma própolis típica do nordeste brasileiro, chamada própolis vermelha, utilizando fluidos supercríticos, podem representar uma alternativa ambientalmente correta aos atuais processos de extração, pois estão incluídos no conceito de "química verde". O objetivo deste trabalho foi extrair e fracionar os compostos fenólicos da própolis vermelha: I) Usando dióxido de carbono supercrítico (scCO₂) como solvente e etanol como co-solvente para obter um extrato etanolico; II) Usando scCO₂ como solvente e uma mistura de etanol: água (70:30, v/v) como co-solvente para obter um extrato hidroalcoólico. Após a extração, os extratos etanólicos e hidroalcoólicos foram fracionados utilizando scCO₂ como antisolvente a uma temperatura constante de 50 ºC, submetidos a quatro pressões graduais, em uma sequência de separadores operados a 200, 100 e 80 bar, no final, pressão atmosférica (1,013 bar). O procedimento foi caracterizado de acordo com o rendimento de extração; fenóis totais, flavonóides totais, atividade antioxidante e cor. Constatou-se que a pressão influenciou o rendimento e a concentração dos compostos fenólicos nos extratos, demonstrando que o processo de fracionamento mais eficiente ocorreu no primeiro e segundo separadores. No geral, todos os extratos mostraram alta atividade antioxidante.

Palavras-chave: Técnicas de extração, isoflavonas, abelhas, Dalbergia ecastophyllum, compostos fenólicos, atividade antioxidante.

1 INTRODUCTION
Red propolis is produced by bees from the resinous material collected from plants such as Dalbergia ecastophyllum (L) Taub. (Leguminosae). Bees use it to the defend hives, and the human population has been using it as a medicinal product for centuries.
It features an unusual chemical composition for a propolis, especially for the Brazilian ones, with isoflavones such as formononetin and biocianin (Cavendish, Santos et al. 2015). Composition and biological activities of the different types of Brazilian propolis vary accordingly to the geographical location (Machado, Silva et al. 2016) and harvest season throughout the year (de Mendonca, de Mendonca et al. 2015). Several biological properties of red propolis were reported in the scientific literature, such as antioxidant (Frozza, Garcia et al. 2013; Barbosa, Nunes et al. 2016; Monroy, Rodrigues et al. 2017; Larissa, Thais et al. 2020), anti-inflammatory (Bueno-Silva, Kawamoto et al. 2015; Bueno-Silva, Franchin et al. 2016; Franchin, Colon et al. 2016), antifungal (Freires, Queiroz et al. 2016), antimicrobial (Freires, de Alencar et al. 2016), antiproliferative (Freires, de Alencar et al. 2016), antitumour (Frozza, Garcia et al. 2013), fungicide (das Nevesa, da Silva et al. 2016), cytotoxic (Lopez, de Lourenco et al. 2015), anti-hypertensive (Teles, da Silva et al. 2015), and healing properties (Jacob, Parolia et al. 2015; Correa, Schanuel et al. 2017), as well as viability for the treatment of nerve injuries (Barbosa, Nunes et al. 2016).

Extracts from natural sources are diverse in the phenolic composition, which may also vary according to the different extraction techniques applied. Many conventional techniques were reported on the extraction of phenolic compounds from the red propolis (Daugsch, Moraes et al. 2008; Silva, Rosalen et al. 2008; Iio, Ohguchi et al. 2010; Kamiya, Nishihara et al. 2012). Recently, the technology with supercritical fluids (SFE) has been applied with propolis, as an alternative to classical methods, for obtaining distinct extracts (Lee, Chen et al. 2007; Biscaia and Ferreira 2009; Paviani, Dariva et al. 2010), including the red type (Machado, Silva et al. 2016), and it presents some advantages over conventional techniques.

Some authors compared the quality of the extracts obtained by conventional extraction with those from supercritical technology for Monroy et al. (2017) supercritical technology offered a more concentrated extracts were obtained in comparison to the latter than by conventional techniques. For Machado et al. (2016) The best results were shown for the extracts obtained through the conventional extraction method (EtOH). However, the highest concentrations of Artepillin C and p-coumaric acid were identified in the extracts from scCO₂, indicating a higher selectivity for the extraction of these compounds.

As a disadvantage of supercritical technology, the solubility of polar substances in CO₂ is low; but with a small quantity of average- or high-polarity co-solvent added to the CO₂, such as EtOH or H₂O, which are generally recognized as safe (GRAS), the
solubility of polar solutes is significantly increased. This effect has been observed by some authors with different sources (Monroy, Rodrigues et al. 2016b; Monroy, Rodrigues et al. 2016c; Corzzini, Barros et al. 2017).

To produce extracts enriched in specific compounds, they can be fractioned by using scCO$_2$ as anti-solvent in several conditions of pressure and temperature, providing their precipitation, as observed in studies with Rosmarinus officinalis L. (Rosemary) (Quintana, Villanueva-Bermejo et al. 2019), Artemisia absinthium L. (Wormwood) (Langa, Pardo et al. 2019), Eugenia uniflora L. (Pitanga, leaves) (Garmus, Kopf et al. 2019), green propolis (Monroy, Rodrigues et al. 2018), and Baccharis dracunculifolia DC. (Alecrim-do-campo) (Paula, Sousa et al. 2017).

Given this context, this study aimed to extract and enrich the phenolic compounds by fractionation of Brazilian red propolis extracts using CO$_2$ as an anti-solvent. All extracts were characterized by the total phenol content analysis, total flavonoids, and antioxidant activity by DPPH expressed in EC$_{50}$, and color by CIELAB method.

2 MATERIAL AND METHODS
2.1 RAW MATERIAL AND PRE-TREATMENT

Red propolis was acquired from the Ilha do Porto Apiary Ltd, in the town of Marechal Deodoro (AL, Brazil). It was frozen and then crushed, packaged in polypropylene bags, and then stored in a freezer at −18 °C. The methodology and equipment used for characterization of the red propolis, such as, volatiles plus moisture content, moisture content, real and apparent density were performed as described in Monroy et al. (2016a).

2.2 OBTAINING THE EXTRACTS

The extraction was conducted at the ExTrAE Laboratory, Unicamp, Brazil. For obtaining the extracts followed by fractionation, it was used the Pilot Plant Unit described by Paula et al. (2017), schematized in the Fig. 1.
In the extraction and fractionation processes, mixture of EtOH-H₂O (70:30, v/v) or absolute EtOH were used as co-solvent in the extraction step in a fixed bed at 50°C and 300 bar using approximately 30g of crushed sample and 60g of glass pearls. The fractionation step was conducted using CO₂ as anti-solvent at 50°C, in separators with pressures of 200, 100, 80 bar, and atmospheric pressure (S1 to 4), as shown in Fig. 2.
Fig. 2. Scheme of extraction and fractionation processes: \( \rho \text{CO}_2 = 1.65 \text{ g/L}, \rho \text{EtOH-H}_2\text{O (70:30, v/v)} = 0.83 \text{ g/mL}, \rho \text{EtOH-absolute} = 0.79 \text{ g/mL} \).

2.3 EXTRACTION YIELD

Extraction yield expresses the relationship between dry extract mass obtained in different separators (FRP 1 to 4), and raw material mass (dry basis), used in the extraction process (MP). This yield is based on the initial mass of the raw material used; the global yield is represented by \( X_0 \) and the individual yield is represented by \( Y_0 \), as shown in Equation 1 (Eq.1).

\[
(X_{ou}Y_0) = \frac{(1\text{FRP} \text{ to } 4\text{FRP})}{MP} \times 100 \quad (\text{Eq.1})
\]

2.4 QUALITY CONTROL OF THE EXTRACTS

The total phenolic compounds content analysis (TP), measured by the equivalent of gallic acid, was done by spectrophotometric analysis at 740 nm, according to Folin-Ciocalteu method, as described by Singleton et al. (1999). The total flavonoids analysis (TF) was based on the methodology of Jia et al. (1999), with catechin as a reference and the measure of absorbance at 510 nm. For both determinations, a blank sample was used at the same conditions, substituting the extract for the same amount of solvent. Other information with more details about the methodologies is described by Monroy et al. (2016a).

The antioxidant activity of extracts was determined by the DPPH analysis, as described by Mensor et al. (2001). Such method is based on the DPPH ability in reacting with hydrogen donors. In the presence of antioxidant substances, it receives H\(^+\), being
thus reduced, and resulting in the antioxidant activity percentage (%AA). Also, the amount needed to decrease the initial concentration of DPPH in 50% is the concentration that eliminates this percentage of free radical (EC50). Therefore, the greater the DPPH consumption by a sample, the smaller will be its EC50 and greater the antioxidant activity. Other information on this methodology is described by some authors (Martinez-Correa, Magalhaes et al. 2011; Garmus, Paviani et al. 2015; Monroy, Rodrigues et al. 2016a).

The coloration of red propolis extracts was determined by colorimetry, with the Ultra ScanVis Hunter Lab spectrophotometer (Riston, Virginia, USA), and direct reading of the parameters L* (luminosity), a* (contribution to red), and b* (contribution to yellow). Indexes (h*) and saturation (C*) were calculated as from the values of a* and b*, as shown in Equations 2 and 3 (Eq. 2 and 3).

\[ h^* = \arctan(b^*a^*) \]  
\[ C^* = [(a^*)+(b^*)]^{1/2} \]

3 RESULTS AND DISCUSSION

3.1 RED PROPOLIS CHARACTERIZATION

Table 1 presents moisture and density values that partially characterize the sample of crushed red propolis. It can be observed that the moisture content is within the standards required by Brazilian Surveillance Agency (ANVISA. 2001), which establish a maximum percentage of 8%. It is important to say that all parameters analyzed are important since they affect the extraction kinetics.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Red propolis</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volatiles + moisture (% VU)</td>
<td>5.70 ± 0.5</td>
<td>(A.O.A.C., 1997)</td>
</tr>
<tr>
<td>Moisture</td>
<td>4.10 ± 0.8</td>
<td>(A.O.C.S., 1998)</td>
</tr>
<tr>
<td>ρr (g/cm³)</td>
<td>1.25 ± 0.01</td>
<td>Helium gas pycnometry</td>
</tr>
<tr>
<td>ρa (g/cm³)</td>
<td>0.70 ± 0.01</td>
<td>(E. Uquiche, 2004)</td>
</tr>
<tr>
<td>[ ε = 1 - (ρ_a/ρ_r) ]</td>
<td>0.44± 0.01</td>
<td>(M.S. Rahman, 1996)</td>
</tr>
</tbody>
</table>

ρ_r: real and apparent density; ε: particle bed porosity.

3.2 EXTRACTION KINETICS

Fig. 3 shows the accumulated global yield (X0) and the individual yields (Y0) of the fractions obtained by selective fractionation using scCO2 as an anti-solvent. Table 2 reports the respective total yield and the mean content of total phenolic, for individuals’ separators and joined. Ethanolic and hydroalcoholic extracts were obtained in a Plant Pilot Unit with fixed bed at 300 bar and 50°C. Differences can be observed between both
cases. In the case of the hydroalcoholic extract, the higher-yielding fraction was obtained in the first separator at 200 bar – this is because the extract contains water, which has very low solubility in scCO₂. The water is precipitated in the first separator, taking most of the extracted phenols with it (more than 50%). Such behavior can be seen in other studies in the literature, such as in Paula et al. (2017) and in Monroy et al. (2018). On the other hand, ethanolic extract, according to the equation $P_c (MPa) = 0.1203. T(K) - 28.44$, of Lim et al. (1994), which correlates the critical point of an ethanol/water mixture, the ethanol, at 50°C, is completely soluble in scCO₂ with the pressure higher than 104 bar. In both cases, the fraction obtained in the separator at atmospheric pressure was 1.5 and 1.9% (around 5% of total extract), which must represent the most volatile substances of propolis since in the separator S3, almost all ethanol is separated from scCO₂. This also suggests that the CO₂ coming out the third separator (80 bar) could be re-pressurized at 200 bar and recycled in the fractionation process. It may also be noted that, in the sample characterization (Table 1), subtracting the volatiles plus moisture from moisture, 1.62% represents only volatiles from the red propolis, consistent with the 1.5 and 1.9% yields in separator S4 of both extracts. In both cases, the first and second fractions of the separators were more concentrated in phenols and flavonoids, with TP values between 330 and 395 mg GAE/g and TF values from 177 to 241 mg CE/g of the extract.

![Fig. 3. Global (X₀) and individual (Y₀) yielding’s of the fractions obtained in the separators: using EtOH-H₂O (70:30, v/v) (a) and absolute EtOH (b) as solvents.](image)

Overall, aqueous extracts (W) presented lower phenolic content than other solvents used, indicating that the prior steps have exhausted the red propolis. Similar values were found by Miguel et al. (2010), which evaluated the Canadian and Portuguese propolis, respectively. However, when Indian propolis was used, aqueous extracts were more concentrated in phenolic compounds than ethanolic extracts Miguel et al (2010).

In Fig. 4, the antioxidant activity curve is compared with the total phenols (TP) content curve. Antioxidant activities were expressed in EC₅₀ (extract concentration in
μg/mL that is capable of reacting with 50% of the radical on the DPPH solution). Thus, the smaller the EC$_{50}$, the higher the antioxidant activity of the extract analyzed. As can be seen in Fig. 4, red propolis extracts showed high antioxidant activity, with EC$_{50}$ values of 5.8 μg/mL for the hydroalcoholic extracts EtOH-H$_2$O (70:30, v/v) in the second fraction at 100 bar, followed by the absolute ethanolic extraction in the first fraction at 200 bar with an EC$_{50}$ value of 6.5 μg/mL. Extracts obtained in the different steps were as follows: 7 ± 5 (in two sequential steps, with the second extract with EtOH-H$_2$O (70:30, v/v)), EC$_{50}$ of 8 ± 4 μg/mL in only one step using co-solvent, and, finally, the extracts obtained in three steps with EtOH and H$_2$O as solvent and EC$_{50}$ of 9 ± 7 μg/mL and 14 ± 2 μg/mL, respectively.

**Fig. 4.** Relationship between antioxidant activity and total phenols content for the different red propolis extracts.
Table 2 – Extraction yield and concentration values for total phenols (TP), total flavonoids (TF), and antioxidant activity (AoA) by red propolis extracts’ DPPH.

<table>
<thead>
<tr>
<th>Extraction solvent</th>
<th>X₀</th>
<th>TP C1</th>
<th>R1</th>
<th>C2</th>
<th>R2</th>
<th>C3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Separator extractor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200 bar</td>
<td>24.0 ± 5</td>
<td>354 ± 21</td>
<td>85.0 ± 5</td>
<td>183 ± 11</td>
<td>44.0 ± 3</td>
<td>9.4</td>
</tr>
<tr>
<td>100 bar</td>
<td>4.2 ± 2.3</td>
<td>395 ± 20</td>
<td>17.0 ± 1</td>
<td>241 ± 16</td>
<td>10.1 ± 0.7</td>
<td>5.8</td>
</tr>
<tr>
<td>80 bar</td>
<td>10.0 ± 2</td>
<td>160 ± 14</td>
<td>16.0 ± 1</td>
<td>72 ± 9</td>
<td>7.2 ± 0.8</td>
<td>10.2</td>
</tr>
<tr>
<td>1.013 bar</td>
<td>1.5 ± 0.5</td>
<td>128 ± 15</td>
<td>1.9 ± 0.2</td>
<td>58 ± 9</td>
<td>0.9 ± 0.1</td>
<td>10.9</td>
</tr>
<tr>
<td>Total</td>
<td>39.7 ± 10.1</td>
<td>120.0 ± 7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>62.2</td>
</tr>
<tr>
<td><strong>Extract solvent</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200 bar</td>
<td>10.1 ± 2.1</td>
<td>330 ± 13</td>
<td>33.0 ± 1</td>
<td>177 ± 10</td>
<td>18.0 ± 1</td>
<td>6.5</td>
</tr>
<tr>
<td>100 bar</td>
<td>12.5 ± 4.9</td>
<td>386 ± 13</td>
<td>48.0 ± 2</td>
<td>201 ± 11</td>
<td>25.0 ± 1</td>
<td>9.3</td>
</tr>
<tr>
<td>80 bar</td>
<td>8.4 ± 3.2</td>
<td>135 ± 15</td>
<td>11.0 ± 1</td>
<td>65 ± 7</td>
<td>5.5 ± 0.6</td>
<td>11.7</td>
</tr>
<tr>
<td>1.013 bar</td>
<td>1.9 ± 1</td>
<td>76 ± 8</td>
<td>1.4 ± 0.1</td>
<td>46 ± 8</td>
<td>0.9 ± 0.2</td>
<td>12.2</td>
</tr>
<tr>
<td>Total</td>
<td>32.9 ± 4.5</td>
<td>94.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>49.4</td>
</tr>
</tbody>
</table>

X₀: Overall yield (%), C1 and C2 (mg/g extract); R1 and R2 (mg/g extract); C3 (EC₅₀/DPPH μg/mL).

Alencar et al. (2007) evaluated ethanolic red propolis extracts and found contents of 232 ± 22 mg/g for total phenols, of 43 ± 1 for total flavonoids, and an EC₅₀ 57 ± 3. Frozza et al. (2013) obtained 152 ± 2 mg/g of phenolic compounds in hydroalcoholic extract. Machado et al. (2016) assessed propolis extracts obtained through supercritical (scCO₂) and ethanolic (EtOH) extraction of different types of propolis (red, green, and brown) from different states of Brazil, finding a greater amount of total phenols and flavonoids, and a better antioxidant activity by ABTS in the red propolis from the state of Sergipe, with 300 mg GAE/g, 57.60 ± 0.01 mg CE/g e 98.50 ± 1.40%, respectively.

In the comparison between supercritical and ethanolic extracts, different authors found high concentrations of phenolic compounds in the extracts using ethanol as solvent, and obtained high antioxidant activity from them (Silva, Machado et al. 2017).

4 COLOR OF THE SAMPLES

Table 3 presents the mean coordinates values of extracts obtained in the diverse extraction steps, and of the red propolis extraction fractions. Red propolis extracts proved to feature the characteristics within the coordinates L*, a*, b*, hab, and Cab* of the chromatic plan. Some differences were found for the coordinates a*, b*, hab, and Cab*.

In the extracts, L* significantly decreases by the concentration of total phenols (TP). Therefore, it could be used to assess the TP concentration in red propolis extracts.
The CIELAB color system is proposed as a fast and reliable technique to evaluate propolis quality due to the relation between color and phenolic composition, or bioactivity.

Table 3 - Color parameters $L^*$, $a^*$, $b^*$, $hab$, and $Cab^*$ in the red propolis extracts undergoing various steps of extraction and in fractionated extracts.

<table>
<thead>
<tr>
<th>Extraction solvent</th>
<th>Yield</th>
<th>Parameter of the color</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP</td>
<td>$L^*$</td>
<td>$a^*$</td>
</tr>
<tr>
<td>EtOH/H$_2$O (70:30, v/v)</td>
<td>354 ± 21</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>EtOH absolute</td>
<td>395 ± 20</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>EtOH (70:30, v/v)</td>
<td>160 ± 14</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>EtOH absolute</td>
<td>128 ± 15</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>EtOH absolute</td>
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</tr>
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<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>EtOH absolute</td>
<td>76 ± 8</td>
<td>2.8 ± 0.3</td>
</tr>
</tbody>
</table>

5 CONCLUSION

The mixture ethanol:water (70:30, v/v) was effective as co-solvent of the scCO$_2$ for the phenolic compounds extraction from red propolis, obtaining high yields with high-concentration extracts of phenolic compounds. Using scCO$_2$ as anti-solvent in the fractionation of hydroalcoholic and ethanolic extracts enabled obtaining more purified extracts, with high levels of phenolic compounds and high antioxidant activity. The phenolic compounds content was correlated with the extracts’ antioxidant activity. Overall, all extracts presented low values of CE$_{50}$, showing high levels of antioxidant activities.

ACKNOWLEDGMENTS

The authors would like to thank CNPq and FAPESP (Process number 12/51317-1) for the financial support; and *Espaço da Escrita – Coordenadoria Geral da Universidade* – Unicamp – for the language services provided.
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