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Quantification of phenolic constituents of red propolis extracts of different concentrations by HPLC

Quantificação de constituintes fenólicos de extratos de própolis vermelha de diferentes concentrações por HPLC

Cuantificación de los constituyentes fenólicos de extractos de propóleos rojos de diferentes concentraciones mediante HPLC

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Abstract
Red propolis is an apicultural product of high chemical complexity from the mangrove regions of the northeastern coast of Brazil. The aim of this study was to evaluate the phenolic composition of ethanolic extracts of red propolis obtained by maceration with different concentrations and extraction cycles in order to indicate the ideal conditions to maximize the extraction of phenolic compounds. Extracts with 10, 20 and 30 g.100mL-1 were obtained and the phenolic constituents (gallic, caffeic, chlorogenic, coumaric, ferulic, catechin, kaempferol, pyrocatechin, quercetin, naringenin, daidzein, formononetin and biochanin A) were identified and quantified by HPLC. The extracts showed higher concentrations of flavonoids compared to phenolic acids. Among the flavonoids, Naringenin was found in higher concentrations followed by Formononetin, two important biomarkers of red propolis. Caffeic acid was the phenolic acid present in highest concentration. High chemical complexity was observed in the extracts, with high concentrations of compounds considered bioactive. According to what was observed, it is indicated the production of the extract with 20 grams and two cycles of extraction or with 30 grams and three cycles.
Keywords: HPLC; Bioactivity; Chemical prospection; Bee products.

Resumo
A própolis vermelha é um produto apícola de alta complexidade química oriunda de regiões de manguezais do litoral nordeste do Brasil. Objetivou-se avaliar a composição fenólica de extratos etanólicos de propolis vermelha obtidos por maceração com diferentes concentrações e ciclos de extração afim de indicar as condições ideais de acordo com a maximização de extrações de compostos fenólicos. Extratos com 10, 20 e 30 g.100mL-1 foram obtidos e os constituintes fenólicos (ácidos gálico, caféico, clorogênico, cumárico, ferúlico, catequina, kaempferol, pirocatequina, quercetina, naringenina, daidzeína, formononetina e biochanina A) foram identificados e quantificados por HPLC. Os extratos apresentaram maiores concentrações de flavonoides em relação aos ácidos fenólicos. Entre os flavonoides, foram encontrados em maiores concentrações a Naringenina seguido da Formononetina, dois biomarcadores importantes da propolis vermelha. O ácido cafeico foi o ácido fenólico presente em maior concentração. Foi observada alta

complexidade química nos extratos, com altas concentrações de compostos considerados bioativos. De acordo com o observado, indica-se a produção do extrato com 20 gramas e dois ciclos de extração ou com 30 gramas e três ciclos.
Palavras-chave: HPLC; Bioatividade; Prospecção química; Produtos apícolas.

Resumen
El propóleo rojo es un producto apícola de gran complejidad química que procede de las regiones de manglares del litoral nordeste de Brasil. El objetivo de este estudio fue evaluar la composición fenólica de los extractos etanólicos de propóleos rojos obtenidos por maceración con diferentes concentraciones y ciclos de extracción con el fin de indicar las condiciones ideales para maximizar la extracción de compuestos fenólicos. Se obtuvieron extractos con 10, 20 y 30 g.100mL-1 y se identificaron y cuantificaron por HPLC los constituyentes fenólicos (ácidos gálico, cafeico, clorogénico, cumárico, ferúlico, catequina, kaempferol, pirocatequina, quercetina, naringenina, daidzeína, formononetina y biochanina A). Los extractos presentaron mayores concentraciones de flavonoides en relación con los ácidos fenólicos. Entre los flavonoides, la naringenina, seguida de la formononetina, se encontraron en mayores concentraciones, dos importantes biomarcadores del propóleo rojo. El ácido cafeico fue el ácido fenólico presente en mayor concentración. Se observó una elevada complejidad química en los extractos, con altas concentraciones de compuestos considerados bioactivos. Según lo observado, se indica la producción del extracto con 20 gramos y dos ciclos de extracción o con 30 gramos y tres ciclos.
Palabras clave: HPLC; Bioactividad; Prospección química; Productos apícolas.

1. Introduction

Propolis is an apicultural product of high chemical complexity, produced from the extraction of gummy, resinous and balsamic substances collected by bees, from buds, flowers and plant exudates, in which bees add salivary secretions, wax and pollen for final elaboration (Anjum et al., 2019). Its main functions in the hive are protection against invaders and thermal insulation, and its composition is dependent on several factors such as flora biodiversity, climate, and seasonality of production (Andrade et al., 2017; Machado et al., 2016). These factors also imply variations in their coloration, which can range from yellow, green, brown, or red. Based on the differences in color and plant of origin, Park et al. (2000) and Park et al. (2002) divided Brazilian propolis samples into 12 different classes. The 13th type of propolis was studied by Alencar et al. (2007) in 2007. This is the red propolis that has as its botanical origin the *Dalbergia ecastophyllum*, a legume that grows abundantly in the mangrove area of the Northeast coast of Brazil.

Due to the different productive conditions present in this coastal area, red propolis can vary in terms of chemical composition according to states or micro-regions, and variations in its bioactive potential can occur (Andrade et al., 2017; Machado et al., 2016). And among the constituents that vary according to these parameters we can highlight phenolic compounds, which have been considered as one of the main biologically active constituents of propolis (Alencar et al., 2007). Studies indicate the presence of significant amounts of phenolic constituents in red propolis that allow it to be differentiated from other types of propolis (Andrade et al., 2017). Among them can be highlighted biochanin A, daidzein, formononetin, naringenin, quercetin, considered as its biomarkers (Frozza et al., 2013; López et al., 2014; Mendonça et al., 2015).

The process of obtaining propolis extract is a key step in the utilization of its bioactive constituents. Since propolis originates from plant resins, it is a product with low solubility in water and high solubility in organic solvents. There are several extraction methods for propolis, however, the most accepted and commercially applied for extraction of biologically active components is maceration using 70% ethanol as the extraction solvent (Bankova et al., 2021). However, as it is a product of high chemical complexity, it becomes necessary to evaluate individually the behavior of obtaining extracts for each type of propolis. This study aims to evaluate the phenolic composition of ethanolic extracts of red propolis obtained by maceration with different concentrations and extraction cycles in order to indicate the optimal conditions according to the maximization of phenolic compounds extractions.

2. Methodology

2.1 Acquisition of samples and extraction

The samples were obtained from an association of producers in Canavieiras, Bahia, Brazil. A small portion of propolis was taken from each hive in order to guarantee the representativeness of the sample, and a mix of samples from different producers was obtained. These samples were frozen and ground into a powder. To obtain the extracts, 10, 20 and 30 grams of samples were weighed, which are the treatments. Then 100 ml of ethanol: water 70:30 (v. v^{-1}) was added. The mixture was stirred in the same Quimis® shaker at 150 rpm and room temperature for 24h. After this period, the mixture was filtered in qualitative filter paper and from the residue obtained, the same process was performed twice more. Totaling three extraction cycles. All extracts were filtered in syringe filters with 0.22 µm membranes. Each treatment was performed in duplicate with three cycles of extraction, totaling 18 experimental units.

2.2 Reverse phase high performance liquid chromatography (HPLC)

The chromatographic experiments were performed with an HPLC-SHIMADZU DGU-20A5R system, equipped with a UV-DA detector and manual injection, composed of a C18, 5 µm, 25 cm × 4.6 mm dimensions reverse phase column (SUPELCO ANALYTICAL, SIGMA ALDRICH), with an injection volume of 20 µm. The chromatographic separation was based on the method proposed by Park et al. (2002) with modifications proposed by Lima et al. (2022). The mobile phase used was water/acetic acid (19:1, $v.v^{-1}$) (solvent A) and methanol (solvent B), with a constant flow rate of 1 $mL.min^{-1}$. The gradient started with 30% solvent B up to 40% B in 15 minutes, 50% B in 30 minutes, 60% B in 45 minutes, 70% B in 65 minutes, 80% B in 85 minutes, 90% B in 95 minutes, 100% B in 100 minutes, and 30% B in 110 minutes. The total run time was 120 minutes. The substances were determined by comparison with the spectra of the standards in the ultraviolet region from 200 to 400 nm obtained using the diode array detector. The column was kept at a constant temperature of 30 °C.

Authentic standards (Sigma Aldrich) of gallic acid, caffeic acid, chlorogenic acid, coumaric acid, ferulic acid, catechin, kaempferol, pyrocatechin, quercetin, naringenin, daidzein, formononetin, and biochanin A were used for identification and quantification. The quantification results of phenolic constituents by HPLC were expressed as $\mu g.mL^{-1}$ of propolis extract. The equations of the straight line and linearity (R) are described in Table 1.

Table 1 - Equations of the straight line, linearity (R), wavelength and retention time for each standard used in HPLC.

	Standards	Line Equation	R	Wavelenght (nm)	Retention time (min)
1	Gallic Acid	y = 7E-06x	0.9945	270	3.1
2	Caffeic Acid	y = 3E-05x	0.9997	270	6.0
3	Chlorogenic Acid	y = 1E-05x	0.9996	310	4.1
4	Coumaric Acid	y = 7E-06x	0.9989	310	10.0
5	Ferulic Acid	y = 5E-06x	0.9969	310	10.8
6	Catechin	y = 5E-05x	0.9974	270	3.5
7	Kaempferol	y = 2E-05x	0,9988	270	35.7
8	Pyrocatechin	y = 2E-05x	0,9986	270	5.7
9	Quercetin	y = 4E-06x	0,9957	270	27.5
10	Naringenin	y = 4E-05x	0.9976	310	28.0
11	Daidzein	y = 3E-05x	0.9984	310	24.7
12	Formononetin	y = 3E-05x	0.9900	310	41.8
13	Biochanin A	y = 5E-05x	0.9976	310	51.0

Source: Authors.

2.3 Statistical analysis

The results of the concentrations and extraction cycles were verified by analysis of variance (ANOVA) and submitted to the comparison of means by the Tukey test at a 5% significance level using the SAS software University Edition.

3. Results and Discussion

Table 2 shows the content of thirteen phenolic compounds quantified in the red propolis extracts in each extraction cycle. All extracts analyzed presented high concentrations of phenolic compounds, with flavovnoids being present in higher concentrations than phenolic acids. This data is interesting, since the flavovnoids group is among the phenolics associated with higher bioactive potentials (Cömert & Gökmen, 2018). Among the identified and quantified flavonoids we can also find the pricipal biomarkers of red propolis (Naringenin, Daidzein, Formononetin and Biochanin A), which give red propolis differentiated properties from other types of propolis (Frozza et al., 2013; López et al., 2014; Lucas et al., 2020; Mendonça et al., 2015; Rufatto et al., 2017; R. O. Silva et al., 2015). These biomarkers are present in high concentrations in the extracts, indicating an efficient extraction process for this type of propolis. Among the quantified flavonoids (Catechin, Kaempferol, Pyrocatechin, Quercetin, Naringenin, Daidzein, Formononetin, and Biochanin A), the ones with the highest quantified levels in all extracts were Naringenin followed by Formononetin, two important biomarkers of red propolis. For the group of phenolic acids (gallic acid, caffeic acid, chlorogenic acid, coumaric acid and ferulic acid), caffeic acid stood out as the phenolic acid present in the highest concentration.

Table 2 - Individual phenolic acids of red propolis extracts from Bahia quantified by HPLC.

Extract (g.100mL^{-1})	**Concentration (µg.mL^{-1} of propolis extract)**		
	1st Extraction	**2nd Extraction**	**3rd Extraction**
Gallic Acid			
10	5.00 Ca	0.96 Bb	0.44 Ab
20	9.25 Ba	1.39 ABb	1.20 Ab
30	14.44 Aa	2.82 Ab	1.31 Ab
Caffeic Acid			
10	15.66 Aa	0.36 Bb	0.28 Bb
20	32.43 Aa	2.25 ABb	0.53 Bb
30	191.20 Aa	5.58 Aa	2.64 Aa
Chlorogenic Acid			
10	4.20 Aa	0.19 Ca	0.02 Ba
20	9.03 Aa	0.97 Bb	0.27 Ab
30	11.71 Aa	1.68 Ab	0.38 Ac
Coumaric Acid			
10	1.91 Ba	0.01 Ba	nd Ba
20	5.48 ABa	0.16 ABb	0.04 ABb
30	9.54 Aa	0.28 Ab	0.16 Ab
Ferulic Acid			
10	2.01 Ba	0.02 Ba	nd Ba
20	6.71 Ba	0.15 Bb	0.05 ABb
30	12.04 Aa	0.60 Ab	0.20 Ab

Catechin			
10	33.13 Ca	3.94 Bb	1.12 Ab
20	68.88 Ba	8.24 ABb	4.59 Ab
30	114.76 Aa	14.96 Ab	5.50 Ac
Kaempferol			
10	95.60 Ba	5.05 Cb	0.73 Ab
20	154.80 Ba	20.29 Bb	7.40 Ab
30	274.65 Aa	35.09 Ab	11.02 Ab
Pyrocatechin			
10	6.41 Aa	0.16 Ab	0.09 Ab
20	7.99 Aa	0.26 Ab	0.26 Ab
30	15.12 Aa	0.84 Ab	0.72 Ab
Quercetin			
10	10.34 Ba	0.83 Ca	0.12 Aa
20	29.02 Aa	2.92 Bb	0.84 Ab
30	40.86 Aa	5.17 Ab	1.81 Ac
Naringenin			
10	118.41 Ba	9.14 Ca	1.50 Ba
20	354.03 Aa	39.16 Bb	6.26 Bb
30	544.67 Aa	77.43 Ab	16.78 Ac
Daidzein			
10	39.97 Aa	2.55 Bb	0.42 Ab
20	21.72 Aa	8.31 Ab	0.33 Ac
30	41.44 Aa	4.13 Bb	1.15 Ab
Formononetin			
10	121.33 Ba	7.69 Bb	1.50 Bb
20	243.04 ABa	33.71 Bb	8.79 Bb
30	472.93 Aa	7.69 Ab	30.86 Ab
Biochanin A			
10	94.61 Aa	7.31 Cb	0.78 Bb
20	177.75 Aa	32.31 Bb	4.93 Bb
30	179.77 Aa	60.93 Aa	20.59 Aa

*nd: Not detected. For each individual constituent, values in the same row, followed by identical lowercase letters and values in the same column, followed by identical uppercase letters do not differ from each other at the 5% level by Tukey's test. Source: Authors.

It is observed that the increase in concentration of the quantified phenolic constituents was proportional to the increase in concentration of the extracts, and this content decreases with each extraction cycle. Analyzing the first extraction cycle, caffeic acid, chlorogenic acid, pyrocatechin, daizein and biochanin A did not show significant differences between the extracts produced with 10, 20 and 30 grams of propolis. This suggests a possible saturation of these compounds in the solvent which could lead to a waste of bioactive molecules if further extraction cycles are not performed. Coumaric acid, ferulic acid, kaempferol and formononetin for the first cycle of extraction did not show significant differences between the treatments with 10 and 20 grams of samples. For the majority of the quantified constituents, in the first cycle the extracts produced with 30 grams of sample can be highlighted, except for the constituents that did not present differences between any of the treatments. In the second extraction cycle the idea of solvent saturation of the first cycle was confirmed for the phenolic constituents that did not differ between the

treatments of 10, 20 and 30 grams, except for pyrocatechin. During the second and third cycle, there were differences among the treatments, indicating that the residue from the first cycle still had significant concentrations of these compounds.

When comparing the phenolic compounds individually for the treatments we can observe that for gallic acid, kaempferol, pyrocatechin and daidzein the first cycle of extraction had a statistically higher content than the second and third cycles, indicating that in only one cycle these compounds were largely extracted. Similar to what was found for gallic acid, it was observed for chlorogenic acid, except for the 10 grams treatment, which did not present a significant difference among the extraction cycles. For caffeic acid, catechin and biochanin A, we also observed the behavior described above except for the extracts with 30 g, which statistically indicate the need to continue cycles of extractions since the concentrations found are still high and do not differ significantly among the three cycles.

The data observed for contents of phenolic constituents for all extracts analyzed indicate a high chemical complexity for all treatments, with a high content of phenolic constituents that are associated with several bioactive properties. These constituents already have their antioxidant (Frozza et al., 2013; Lima et al., 2022; Machado et al., 2016; Mendonça et al., 2015; Oldoni et al., 2011; Silva et al., 2017; Trusheva et al., 2006), antimicrobial (Machado et al., 2016; Regueira-Neto et al., 2017; Rufatto et al., 2018; Silva et al., 2017; Trusheva et al., 2006), anti-inflammatory (Freires et al., 2018), antitumor (Frozza et al., 2013; Mendonça et al., 2015; Rufatto et al., 2018; Silva et al., 2017) demonstrated. The content of the constituents in the first extraction cycle was similar or higher than reported by Regueira-Neto et al. (2017) for chlorogenic acid, caffeic acid, coumaric acid, and quercetin contents and than reported by Andrade et al. (2017) for caffeic acid, chlorogenic acid, gallic acid, coumaric acid, kaempferol, biochanin A, daidzein, formononetin, and naringenin for red propolis extracts.

For most phenolic compounds in the second and third extraction cycle it was still possible to quantify considerable contents of phenolic constituents. It is common to commercially produce propolis extracts in the concentration of 30 g.100 mL^{-1} with only one extraction cycle. The observed data indicate that this extraction method generates a residue that is still rich in these bioactive compounds, which generates great losses and waste for the industry. This demonstrates the importance of initially evaluating and optimizing the extraction process.

Based on the data, one can indicate the production of red propolis extracts with 20 g.100 mL^{-1} with two extraction cycles or with 30 g.100 mL^{-1} with three cycles. Evaluating the possible cost with sample amount and equipment energy, the use of the 20 g treatment with two cycles is suggested. It is worth noting that although the constituents quantified are of importance in the bioactivity of the extracts, they are only some of the constituents present, and a complete characterization is needed to indicate more precisely the best concentration and how many cycles of extraction to optimize the process.

4. Conclusion

It was possible to identify and quantify the contents of thirteen phenolic compounds in the red propolis extracts, observing significant amounts of these compounds that have demonstrated bioactive potential. High concentrations of the compounds of interest in red propolis, Naringenin, Formononetin, Biochanin A and Daidzein, considered biomarkers and with already elucidated bioactive properties, were quantified in all extracts. This study demonstrates the importance of evaluating the extraction process before industrial implementation. This initial evaluation can optimize yield and sample and energy costs. According to what was observed for the content of individual phenolic acids, it is indicated to produce the extract with 20 g.100 mL^{-1} with two extraction cycles or with 30 g.100 mL^{-1} with three cycles.

Through the data obtained, it is possible to optimize industrial processes for obtaining red propolis extracts, aiming at maximizing the extraction of phenolic constituents and minimizing losses of these compounds in the residues. The next steps taken should include the dissemination of the results obtained and the adjustment of the processes by producers.

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