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Determination of some phenolic acids in Algerian propolis

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Abstract. Propolis is a resinous material collected by bees from various plant exudates, rich in well-known phenolic compounds, such as phenolic acids, that are important to health. Extracts of propolis are very complex matrices that are hard to test. The purpose of this study was to characterize some of the propolis phenolics that were collected from five different districts in Algeria. The High-Performance Liquid Chromatography (HPLC), a modern quantitative method, has been adopted to identify the phenolic acids. Moreover, total phenolic content of four different phenolic acids were identified, with the most abundant being chlorogenic acid, followed by caffeic acid, gallic acid, and *p*-coumaric acid, the obtained ratios from phenolic acids being in the range of 52.193 to 148.151 μg/g, 0.043 to 7.128 mg/g, 0.328 to 0.440 mg/g and 0.328 to 0.440 mg/g, respectively. Overall, our analysis indicates that all the samples of propolis tested are healthy sources of phenolic acids and the significant differences in the concentrations of the acids were observed for propolis samples from north and south of Algeria. It is probably the effect of different conditions of the collection of the resin and secrets by bees.

Keywords: bee pollen; phenols; HPLC; antioxidant properties.

1. Introduction

Propolis is a bee product consisting mainly of plant resins and beeswax; hence its chemical composition varies because of the geographical and plant sources of these resins [1]. Since ancient times propolis has been used as a cure by humans [2].

This commodity was the object of extensive research in the last years, highlighting its biological and pharmacological properties, such as the antimicrobial [3], anti-oxidative, anti-fungal [4], antiviral [5], anti-tumoral [6, 7], anti-inflammatory [8, 9], anti-hepatotoxic [10], anti-neurodegenerative [11], anti-tuberculosis [12], and anti-HIV-1 [13].

Propolis positive medical applications have led to a greater interest in its chemical composition [14]. In "World Propolis" [15] more than 300 compounds have been identified, and many are biologically active [3]. In general, resin consisting of flavonoids and associated phenolic acids account for about half of the constituents of propolis, thus beeswax, volatiles, and pollen constitute approximately 30%, 10%, and 5%, respectively. As such flavonoids and phenolic acids have been proposed to be responsible for the biological activities of these compounds. The content of phenolic acids is therefore seen as an important index for evaluating the quality of propolis.

In general, the composition of propolis is closely related to that of bud exudates from various trees collected by bees: poplar, birch, beech, horse chestnut, alder, and various conifers [15]. Thanks to the variety of its chemical composition, propolis and pollen may be useful in the prevention of diseases involving free radicals [16].

A significant number of analytical methods for the study of propolis phenolic compounds have been used, including spectrophotometry [17], High-Performance Liquid Chromatography (HPLC) [18], Liquid Chromatography-Mass Spectrometry (LC-MS) [19], Gas Chromatography-Mass Spectrometry (GC–MS) [20]. Also, Ultra-Fast Liquid Chromatography Coupled to Tandem Mass Spectrometry (UFLC-MS / MS) was used to assess phenolic composition [21, 22]. Among these methods HPLC is one of the most commonly used techniques in bee product research [23], because it can analyze complex mixtures due to its high selectivity.

In previous research, ethyl acetate extract exhibited the highest total phenolic content and the greatest antioxidant activity compared with ethanol, chloroform,

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and butanol extracts. Many studies have shown that ethanol/water solvents are more effective in extracting phenolic compounds and exhibit greater antioxidant activity than aqueous extract [24].

Furthermore, statistical analysis showed that polyphenol content in the aqueous solutions is high, while methanol was the best extraction solvent for flavonoids. Propolis samples were collected with ethanol for the optimal extraction of total polyphenols and flavonoids [25].

Our study aimed to evaluate phenolic acids (Figure 1) from different sorts of Algerian propolis by using Folin-Ciocalteu reagent and analyzed using HPLC.

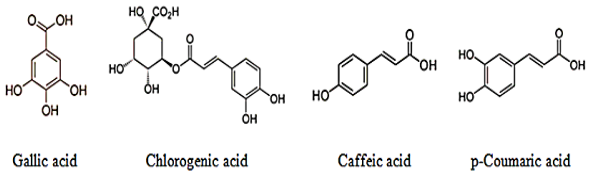

Figure 1. Chemical structures of the studied phenolic acid compounds.

2. Experimental

2.1. Chemicals

Ethanol (99%) was purchased from Sigma-Aldrich Co. 3,4,5-Trihydroxybenzoic acid (gallic acid, GA; 99%), chlorogenic acid (CGA; 97%), caffeic acid (CA; 99%), *p*-coumaric acid (98%), acetic acid (CH_3COOH; 97%), and acetonitrile (CH_3CN) HPLC grade were procured from Alfa Aesar (USA). Aluminum chloride ($AlCl_3$) and sodium carbonate (Na_2CO_3) were purchased from Prolabo (USA). The Folin-Ciocalteu reagent (FCR), methanol (MeOH), and hexane (C_6H_{14}) were obtained from BIOCHEM Chemopharma Co (FRANCE). High purity water was used for all experiments. All other reagents were of analytical grade.

2.2. Propolis origin

During the time from 2011 to 2012, five samples of dehydrated propolis were collected by beekeepers from five localities in the north and south Algeria (Table 1). The propolis was sent to the laboratory after selection and each sample was separately crushed in a commercial blender, homogenized for later analysis, and deposited in the freezer.

Table 1. Algerian propolis samples used in this study based on the date of harvest, geographical origin.

Sample code	Date of harvest	Place of production	Geographical origin
P01	2011	Tizi-Ouzou	Mountain
P02	2012	El Oued	Field
P03	2012	Jijel	Mountain
P04	2011	Tipaza	Mountain
P05	2012	Tlemcen	Mountain

2.3. Ethanolic extract of propolis (EEP)

Samples of 5 g of propolis were taken twice (30 min) at room temperature in an ultrasonic bath with 100 ml of ethanol to obtain the extract. Every of the extract was filtered over Whatman No. 4 filter paper and vaporized at 45 °C with a rotary evaporator. Until further use, the extracts thus obtained were weighed and stored in a brown bottle at 4 °C.

2.4. Determination of total phenolic content (TPC)

Total phenolic content was determined by using Folin-Ciocalteu reagent and the Kumazawa et al. method [23], which was briefly defined as 0.5 ml of Folin-Ciocalteu reagent was combined with 100 µl extract. After 3 min, 2 ml of Na_2CO_3 (20%) solution was added to the mixture. The reaction was held for 30 min in the dark, after which the absorbance was read at $\lambda = 760$ nm [26].

The calibration curve (0.03-0.3 mg/ml) was developed using gallic acid as normal. The mean of three interpretations was used and the total phenolic content was measured in mg equals of gallic acid (GAEs) (mg/100 g).

2.5. Instrumentation and chromatographic conditions

Spectrophotometric measurements were carried out on a Schimadzu Spectrophotometer (double-beam) UV-1800 fitted with quartz cuvettes of 1 cm. A high-performance liquid chromatography system, Shimadzu LC 20 AL fitted with the universal injector (Hamilton 25 µL) SPD 20A, SPD 20A (Shimadzu) UV-VIS detector was used.

2.6. Preparation of the mobile phase

The mobile phase was a blend of 0.1% acetonitrile and acetic acid. Before use, the contents of the mobile phase were filtered through a 0.45 µm membrane filter, sonicated and pumped at a flow rate of 1 ml/min from the solvent reservoir into the tank. As stated below, a linear gradient was used for the elution (Table 2).

Table 2. Gradient program for elution of phenolic acids.

Time (min)	CH_3COOH (%)	CH_3CN (%)
0	90	10
6	86	14
16	83	17
23	81	19
28	77	23
30	90	10

The column was held at room temperature, and the injection volume was 20 µl at 300 nm. Before analytic injection, the column was balanced with the mobile step for 40-50 min.

2.7. Preparation of standard solution

A stock solution of phenolic acid was prepared by dissolving 100 mg of pure phenolic acid in a 100 mL volumetric flask containing a sufficient amount of MeOH (HPLC grade) to dissolve the acid. The solution was solicited for approximately 15 min and then brought to volume by the mobile phase. Regular operating standard solutions of phenolic acids are prepared by diluting the stock solution with the mobile step accordingly. Each of these solutions (20 µl) was injected into the column three times and reported peak area and retention periods [26].

3. Results and discussion

The Folin-Ciocalteu assay is a fast and simple method that easily assess phenolic content in samples. Some of

the phenolics are rich in antioxidant production. The absolute phenolic content of propolis ethanol extract was provided in Table 3.

Table 3. Global yield and total phenolic content of propolis extract obtained.

Sample	Extraction yield (%)	Total phenolic content (mg/g)
P01	46.100	89.572±1.640
P02	11.3	28.163±0.678
P03	20.880	152.864±4.797
P04	17.020	100.525±1.131
P05	16.220	367.39±3.679

*Values are means ± SD (n = 3).

The total phenolic content was found to be higher in sample P05 (367.39 mg GAE/g) followed by sample P03 (152.864 mg GAE/g) and P04 (100.525 mg GAE/g) respectively. It has been investigated in many plant species that the total phenolic could significantly contribute to the antioxidant capacity of that species. Therefore, the higher amount of phenolic in propolis can be taken as a good indication for its higher antioxidant capacity.

In comparison to those published by Da Silva et al. [27] and Potkonjak et al. [28], these findings demonstrated that Algerian propolis had a greater phenolic content. Compared to Kumazawa et al. and Choi et al. [29], it has a reduced phenolic content.

The composition of propolis is determined by the local vegetation and the season in which it is collected [30].

HPLC chromatogram of standards is presented in Figure 2. The typical separation of phenolic acids is shown in Table 4 and expressed in retention times.

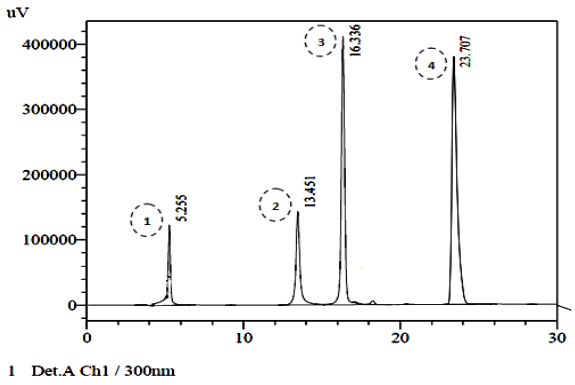

Figure 2. HPLC chromatogram for standards: 1 – gallic acid; 2 – chlorogenic acid; 3 –caffeic acid; 4 – *p*-coumaric acid.

Table 4. Standards retention times.

Peak No.	Rt (min)	Compound
1	5.25±0.18	gallic acid
2	13.62±0.09	chlorogenic acid
3	16.3±0.14	caffeic acid
4	23.70±0.05	*p*-coumaric acid

Rt: Retention time. Values are means ± SD (n = 3).

For all the ethanol extracts tested, the chromatographic profiles at 300 nm were identical in clarifying Figure 3, regardless of the sample position, consistent with the relationship between the phenolic profile and the surrounding apiary flora [31].

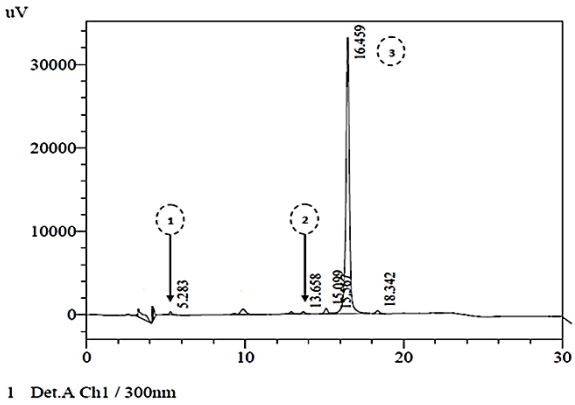

Figure 3. HPLC of propolis ethanolic extract (sample P01): 1 – gallic acid; 2 –chlorogenic acid; 3 –caffeic acid.

We have set calibration curves for norm and regression coefficients in the experimental conditions of this study (Table 5). For all compounds, the linearity of the calibration curves was very good ($R^2 > 0.99$).

Based on calibration curves and considering the dilutions made, we established the content in phenolic acids for all propolis samples.

The limit of detection was in the range of 0.1–0.2 µg/ml for phenolic acids. The phenolic acid content from the raw propolis samples determined by the HPLC method is listed in Table 6.

The concentration of gallic acid varied greatly in propolis samples: from those in which gallic acid was not detected (samples P04 from Tipaza) to other samples in which concentration of gallic acid ranged from 0.44 to 0.32 mg/g (Table 6). Caffeic acid was detected in all investigated samples of propolis from Algeria with concentrations ranging from 0.043 to 7.128 mg/g. Chlorogenic acid was detected in all investigated propolis samples from Algeria (52.193 to 148.151 µg/g). *p*-Coumaric acid was not detected in samples P01 (from Tizi-Ouzou).

Table 5. Calibration curves and regression coefficients for all standards.

Peak No.	Standard	Calibration curve	Regression coefficient (R^2)
1	Gallic acid	$y = 23616x - 7232$	0.9986
2	Chlorogenic acid	$y = 39775x - 1881$	0.9963
3	Caffeic acid	$y = 72328x$	0.9992
4	*p*-Coumaric acid	$y = 157538x$	0.9996

Table 6. Phenolic acid content in Algerian propolis.

Sample	Gallic acid (mg/g)	Caffeic acid (mg/g)	Chlorogenic acid (µg/g)	*p*-Coumaric acid (µg/g)
P01	0.440 ±0.004	7.128 ± 0.173	148.151 ± 0.32	ND
P02	0.387 ± 0.007	0.043 ± 0.009	52.193 ± 0.029	3.0849 ±0.021
P03	0.407 ± 0.023	3.805 ±0.033	101.833 ± 0.072	34.518 ±0.03
P04	ND	1.683± 0.012	94.218 ± 0.003	94.041 ±0.049
P05	0.328 ± 0.011	4.75 ± 0.019	70.911 ± 0.041	278.980 ±0.19

ND: not determined.

4. Conclusions

In conclusion, the phenolic acids from propolis extracts can be successfully isolated and classified with a gradient elution mode by reversed-phase HPLC (RP-HPLC). The proposed method allows for the simultaneous determination of the naturally occurring organic acids in a variety of propolis, providing an appropriate detection limit and repeatability limit. The significant differences in the concentrations of the acids were observed for propolis samples from north and south of Algeria. It is probably the effect of different conditions of the collection of the resin and secrets by bees.

The majority of propolis samples test results were within the permissible range of world propolis standards, indicating that there is good potential for propolis export. The five samples of Algerian propolis had a good higher phenolic content, according to all of the tests. Determination of total polyphenols by UV and HPLC might be considered important analysis methods for the evaluation of propolis content, according to the findings of this study. The findings revealed that propolis had significant health benefits for humans and that it might be used as an antioxidant source.

Conflict of interest

The authors declare no conflict of interest.

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