

Extraction, Phenolic Content and Hydrogen Peroxide Scavenging Capacity of Extracts from Some Honey Samples, Propolis and Bee Pollen

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Abstract

Honey and propolis is natural food, produced by honey bees (*Apis mellifera*) and largely used by the local population for its medicinal properties. Our work aims to extract and evaluate the hydrogen peroxide scavenging capacity of different phenolic extracts from some bee products. Phenolic compounds from honey samples, propolis, and bee pollen were extracted by methanol and subjected to radical scavenging activity towards hydrogen peroxide. The results showed the highest values for the total phenolic and total flavonoid contents in propolis and bee pollen, and a great hydrogen peroxide (H₂O₂) inhibition (IC₅₀: 0.205 - 2.210 µg/mL) with honey extracts, while sample "multiflower" is the better antioxidant, more than ascorbic acid used as control. The less scavenging activity was observed with the extract from bee pollen (IC₅₀: 39.383 µg/mL). To combat the harmful effects of free radicals, especially reactive oxygen species including hydrogen peroxide, it is important to use phenolic extracts instead of using honey as it is, and extracts from the honey of different types are excellent antioxidants compared to other bee products.

Keywords: *Apis mellifera*; honey; propolis; phenolic compounds; hydrogen peroxide.

INTRODUCTION

Honey is the nectar collected and processed from different plants by honey bees (*Apis mellifera*) and is known for its high nutritional value (An et al., 2016). It has been used in many cultures for its medicinal properties, as a remedy for burns, cataracts, ulcers and wound healing, because it exerts a soothing effect when initially applied to open wounds. According to its origin, honey can be classified in different categories: blossom honey, honeydew honey, monofloral honey, and multifloral honey that has several botanical sources (Alvarez-Suarez et al., 2014). Honey is composed of approximately 82.4% total carbohydrates (Almasaudi et al., 2017). The polyphenols constitute minor components of honey (Istasse et al., 2016).

Honey has been reported to promote numerous nutritional and biological effects, such as antioxidant, antimicrobial, antiviral, antiparasitic, anticancer, anti-inflammatory, and immunosuppressive activities (Duddukuri et al., 1997; Othman, 2012; Shahzad and Cohrs, 2012; Borsato et al., 2014; Samarghandian et al., 2017; Sowa et al., 2017; Sinha et al., 2018). However, the antioxidant properties of honey are well known because it contains a number of compounds with antioxidant properties such as polyphenols, amino acids,

ascorbic acid, and some enzymes. The most important classes of antioxidants are the phenolic acids and flavonoids (Ayoub et al., 2009). They have a promising effect on the treatment of some chronic diseases (Isla et al., 2013). A wide range of phenolic constituents in honey are known such as gallic acid, syringic acid, vanillic acid, benzoic acid, chlorogenic acid, ferulic acid, protocatechuic acid, 3-O-caffeoylquinic acid, p-hydroxybenzoic acid, 5-O-caffeoylquinic acid, caffeic acid, p-coumaric acid, ellagic acid, dihydroxybenzoic acid methyl ether, benzyl caffeate, protocatechualdehyde, gentisic acid, vanillin, sinapic acid, salicylic acid, dihydrocinnamic acid, 2,2,4-trihydroxybenzoic acid, hesperetin, naringin, naringenin, hesperidin, tricetin, myricetin, quercetin, quercitrin, quercetin 3-orhamnoside, kaempferol 3-orhamnoside, kaempferol 7-orhamnoside, luteolin, pinobanksin 5-methyl ether, naringenin, apigenin, kaempferol, methoxy-kaempferol, pinobanksin, isorhamnetin, chrysoeriol, rhamnetin, chrysin, sakuranetin, pinocembrin, galangin, kaempferide, acacetin, morin, tangeretin, catechin, genistein, ectochrysin, and other compounds such as caffeic acid phenethyl ester, Kojic acid, 5-hydroxymethylfurfural, dehydrovomifoliol, leptosin, glyoxal, methylglyoxal, 3-deoxyglucosulose (An et al., 2016; Alvarez-Suarez et al., 2014; Isla et al.,

2013; Lachman et al., 2010; Cianciosi et al., 2018; Cheung et al., 2019; Nešović et al., 2020).

Another bee product is propolis, which is a resinous substance collected by honeybees from various plant sources. This natural product has been used for thousands of years in folk medicine for several purposes and contains amino acids, terpenes, tannins, polysaccharides, phenolic acids, phenolic acid esters, cinnamic acid, caffeic acid, phenethyl caffeate, p-coumaric acid and flavonoids such as kaempferol, naringin, chrysin, and galangin. Propolis possesses several biological activities such as anti-inflammatory, immunostimulatory, antiviral, and antibacterial. Propolis extract and its active components showed strong antioxidant activities, a free radical scavenging effect, a significant inhibition of xanthine oxidase activity, ferric reducing antioxidant power, and an anti-lipoperoxidative capacity (Russo et al., 2002; Kumazawa et al., 2004; Korayem et al., 2012; Socha et al., 2015).

A large number of studies have estimated the antioxidant activities of honey by a range of methods (DPPH free radical scavenging activity, ABTS radical cation decolorization assay, FRAP assay, ORAC assay, etc.), but the step of dilution is a major drawback in antioxidant tests since it generates a toxic oxidant substance (H_2O_2), the cause of the exclusion by researchers of the antioxidant test by hydrogen peroxide scavenging activity in entire honey. So, the aim of this study was to extract phenolic and flavonoids compounds, then evaluate the antioxidant activity of obtained extracts from different types of honey against hydrogen peroxide, and to compare with two phenolic extracts from propolis and bee pollen. Also, we highlight the ability of the phenolic compounds in honey to eliminate hydrogen peroxide, and therefore the step of extraction of polyphenols is important in the context of the use of honey in the fight against oxidative stress.

MATERIALS AND METHODS

Bee products

Honey samples (Multiflower and monofloral honey: Eucalyptus, Thyme, Sidr, Citrus, and Epins honey), Propolis and Bee pollen are the local products of Tlemcen, Algeria.

The 100% natural honey samples from *Apis mellifera* were obtained directly from the beekeeper in 2019 from different vegetation sources in Tlemcen city. These honey samples were chosen for their quality, in terms of low content of water and hydroxymethylfurfural (HMF).

Extraction of phenolic compounds from honey samples

The extraction of phenolic compounds from the honey samples was performed according to the method described by An et al. (2016). The stationary phase of the glass column, Amberlite XAD-2 resin, was prepared

by soaking 100g of Amberlite XAD-2 resin in methanol. The packed column was washed successively with acidified distilled water followed by neutral distilled water. The honey sample was diluted with acidified water and loaded into the packed glass column. Sugars were eliminated with hydrochloric acid solution and water washes. Phenolics compounds were desorbed with methanol and then dried at 50°C using a Rotavapor. The dried product was protected from light and stored at 5°C until use.

Extraction of phenolic compounds from propolis and bee pollen

For extract phenolic compounds from propolis and bee pollen samples, the hydroalcoholic solutions (methanol-water) were used. For each 5g sample, 100mL of methanol 80% were added, the mixture was covered with aluminum paper and then filtered after 24h. The filtrates were dried at 50°C using a Rotavapor. The dried extracts were stored at 5°C until use.

Total phenolic contents

The total phenolic contents of honey samples, propolis, and bee pollen extracts were analyzed by the Folin-Ciocalteu method (Singleton et al., 1999) using gallic acid as a standard. The methanolic solution of each sample of honey, propolis, bee pollen, and gallic acid was prepared. The test samples and standard solutions of different concentrations were introduced into separate test tubes. Folin-Ciocalteu reagent, diluted in water (1:1), and Na_2CO_3 saturated solution were added. The tubes were vortexed, incubated in the dark at room temperature for 25 minutes and then the absorbance was read at 725nm against the blank using a spectrophotometer. The total phenolic content was determined by comparison with a calibration curve of gallic acid and represented as mg gallic acid equivalents per 100 gram (mgGAE/100g).

Total flavonoid content

The total flavonoid content was determined by the $AlCl_3$ method (Cottica et al., 2011) using rutin as standard. 250 μ L of $AlCl_3$ 5% (m/v in methanol) were added to separate test tubes containing the methanolic solution of each honey sample, propolis, bee pollen extracts, and rutin solutions of different concentrations. After 30 min incubation at room temperature, the absorbance was read at 425nm using a spectrophotometer. The total flavonoid content was determined by comparison with the calibration curve of the rutin and represented as mg rutin equivalents per 100gram (mgRE/100g).

Antioxidant activity (Hydrogen peroxide scavenging activity)

The hydrogen peroxide scavenging activity was measured by the method described by Ruch *et al.* (1989). A volume of 0.6 mL of the solution of hydrogen

peroxide (0.089 mM), prepared in phosphate buffer (0.1M, pH 7.4) was added in a series of test tubes, containing 3.4mL phenolic extract solutions (3-10µg/mL) from honey samples, propolis, bee pollen and standard (ascorbic acid). The percentage of hydrogen peroxide scavenging activity was calculated by the formula:

$$\% \text{ Scavenged } [H_2O_2] = [(AC - AT)/AC] \times 100$$

Where AC is the absorbance of the control and AT is the absorbance in the presence of the phenolic extracts from different samples (tests) and standard.

Statistical analysis

The results of experiments were expressed as (mean \pm SD; n=3), and subjected to statistical analysis at 95% confidence level (p<0.05) by student's t-test using GraphPad Prism software.

RESULTS AND DISCUSSION

The results of phenolic content and flavonoid content were reported in table 1. Total phenolic content in six honey samples was ranged from 4.36 mgGAE/100g to 26.29 mgGAE/100g honey, with the average value of 12.755 mgGAE per 100g honey. The Multiflower type of honey was the sample containing a high amount of phenolic compounds and the monofloral type "Citrus" honey was the sample with the lowest quantity of phenolic compounds compared to all honey samples. The same observation was showed in flavonoid content. The Multiflower type remains the richest honey in phenolic compounds compared to other honey. While the lowest content is also observed in the "Citrus" type. The flavonoids content obtained was from 1.95 \pm 0.352 mgRE/100g to 10.65 \pm 1.020 mgRE/100g, with an average amount of 4.607 mgRE per 100g honey. The evaluated results were in close agreement with the results reported by Meinen et al. (2014), which have reported that total phenolic content is ranged between 8.114 mg and 109 mg per 100g honey and for the total flavonoid content is ranged between 0.655 mg and 212 mg per 100g honey. This study was perfected in 30 honey samples. Boulanouar et al. (2017) have observed higher levels of phenolic compounds in two types of Algerian honey, which are 38 \pm 0.009 mgGAE/100g for the type "Zizyphus" and 86 \pm 0.008 mgGAE/100g for the type "Harmala", whereas for the flavonoids, they found contents, which belong to the range of values that we recorded, the values of flavonoids were 5 \pm 0.003 mgRE/100g and 8 \pm 0.002 mgRE/100g for "Zizyphus" and "Harmala" types, respectively. Also, the same remark is observed with the works of Khalil et al. (2012). These authors analyzed four samples of Algerian honey and registered mean values of 45.983 \pm 0.192

mg/100g for phenolic compounds and values of 5.423 \pm 0.062 mg/100g for flavonoids.

Table 1. Total Phenol content and flavonoids of honey samples, propolis and bee pollen.

Sample	Average amount of phenolic	Average amount of flavonoids
	(mgGAE/100g)	mgRE/100g
Multiflower honey	26.29 \pm 9.764 ^a	10.65 \pm 1.020 ^a
Thyme honey	8.67 \pm 2.494 ^b	2.61 \pm 0.558 ^b
Citrus honey	4.36 \pm 2.441 ^c	1.95 \pm 0.352 ^b
Eucalyptus honey	8.97 \pm 1.033 ^b	2.21 \pm 1.068 ^b
Sidr honey	14.20 \pm 3.278 ^d	5.31 \pm 0.112 ^c
Epins honey	14.04 \pm 4.260 ^d	4.91 \pm 0.232 ^c
Propolis	9161.0 \pm 6.906 ^e	2189.62 \pm 1.975 ^d
Bee pollen	4794.15 \pm 6.169 ^f	1267.38 \pm 0.912 ^e

Data are (Mean \pm SD, n = 3).

Means within a column sharing the same letter are not significantly different by Student's t-test (P < 0.05).

Total phenol and flavonoids were also compared to the values reported for the honey from different countries. Some authors have found results, approaching our range of values, such as the work of Reshma et al. (2016), Almasaudi et al. (2017) and Krpan et al. (2009). The first authors have shown that the phenolic content in 24 samples of honey was ranged between 20.2 \pm 1.2 mg/100g to 30.78 \pm 2.5 mg/100g, the second authors have observed that the high total phenolic content was 10.399 \pm 0.168 mg/100g in "Manuka" type of honey and the lowest phenolic content was 9.6 \pm 0.002 mg/100g for "Sidr" type of honey, while the third authors have ranged the total phenolic content from 3.172 mg/100g to 8.011 mg/100g honey and this later study was performed only in one type of honey, which is the "Acacia" type. In contrast, other authors have deferred high and even very high levels, for examples, for Chua et al. (2013) the total phenolic content of honey samples was in the narrow range from 110.394 to 196.500 mgGAE/100g, A-Rahaman et al. (2013) showed that the total phenolic contents for honey samples were ranged from 38.379 to 60.617 mg GAE/100g, Pontis et al. (2014) ranged the total phenolic content from 25 to 54.8 mg gallic acid per 100g of honey, while for Buba et al. (2013), the mean values of total phenolic content are around 65.31 \pm 19.50 mg gallic acid equivalent per 100g. In addition, some authors have reported very small quantities of phenolic content in honey, for example, Ferreres et al. (1994) reported 0.50 to 2.0 mg per 100g honey. For flavonoids, Pontis et al. (2014) have shown less content than obtained in our studies, the total flavonoid content was ranged from 0.9 to 4.86 mg per 100g honey.

The results obtained experimentally in table 1 show very high levels of phenolic compounds and flavonoids in propolis and bee pollen compared to those obtained in honey samples and the amount of phenolic and

flavonoids in propolis was higher than bee pollen. The quantities registered in propolis and bee pollen were 9161.0 ± 6.906 mgGAE/100g, 4794.15 ± 6.169 mgGAE/100g for total phenolic content and 2189.62 ± 1.975 mgRE/100g, 1267.38 ± 0.912 mgRE/100g for total flavonoids content, respectively. Boulanouar et al. (2017) have also observed that propolis contains more phenolic and flavonoids than honey, the respective quantities registered were 2385 ± 2.90 mgGAE/100g and 379 ± 0.54 mg RE/100g. Čeksterytė et al. (2016) have also observed high levels of phenolic compounds in bee pollen, but are less relative to the quantities we have obtained. The values of total phenolic content found were 2330 mg GAE/100g.

The antioxidant capacity of honey and bee products is due mainly to the phenolic compounds. The samples exhibited various antioxidant activity measured towards hydrogen peroxide, which is concentration-dependent (figure 1). Results show that the scavenging activity values on hydrogen peroxide of phenolic extracts from honey samples were more efficient than other bee products. Lower antioxidant activity was observed in extracts from propolis and bee pollen.

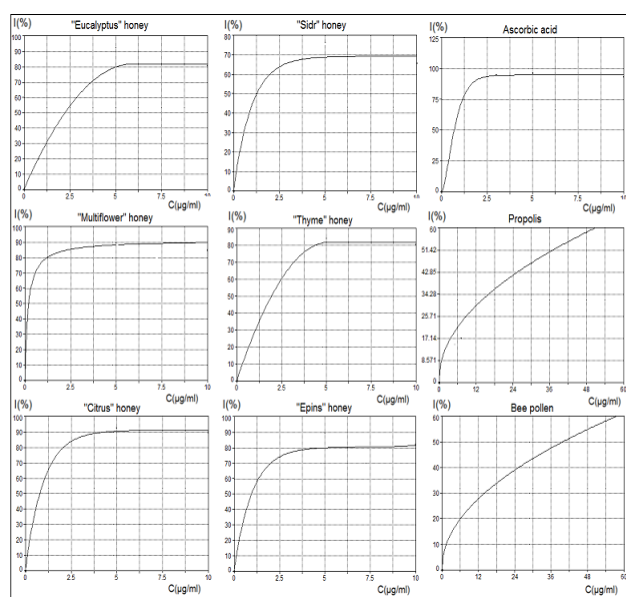


Figure 1. Hydrogen peroxide (H_2O_2) scavenging activity of phenolic extract from honeys, propolis, bee pollen, and ascorbic acid.

The antioxidant activity of honey has been reported in many scientific works (Kıran et al., 2009; Pontis et al., 2014; Reshma et al., 2016; Chua et al., 2013; A-Rahaman et al., 2013). Currently, some very limited research on antioxidant activity has been conducted on phenolic extracts of honey (Lianda et al., 2012; Bridi et al., 2017; Estevinho et al., 2008; Halagarda et al., 2020), but no research has assessed the capacity of extracts or phenolic fractions isolated from honey by the hydrogen peroxide scavenging method. Hydrogen peroxide is not very reactive, but it can be toxic because it may produce

hydroxyl radicals in the cells. Thus, removing H_2O_2 is very important in the cell. The scavenging ability of honey samples, propolis, and bee pollen was examined by comparing to that of the known antioxidants Ascorbic acid. The extract of honey type "multiflower" showed powerful activity on hydrogen peroxide and decreases its concentration more than the positive control, ascorbic acid. Antioxidant activity, expressed as IC₅₀ (Table 2) was 0.206 ± 0.0243 μ g/mL, while the standard given an IC₅₀ value of 0.694 ± 0.0238 μ g/mL. This parameter IC₅₀ defined the concentration of the sample, which scavenges 50% of hydrogen peroxide. The sample with lower IC₅₀ presents potent antioxidants. The phenolic extracts from extracts of others honey types exhibited also a good antioxidant activity on H_2O_2 . The scavenging ability is in the following order: "Multiflower" > Ascorbic acid > "Citrus" > "Epins" > "Sidr" > "Thyme" > "Eucalyptus". It has been reported that the antioxidant capacity of honey was correlated with the biochemical constituents, in particularly with phenolic compounds and flavonoids (Chua et al., 2013; Buba et al., 2013).

Table 2. Hydrogen peroxide scavenging capacity of phenolic extract from honey samples, propolis and bee pollen, expressed as IC₅₀ (μ g/mL).

Extract	IC ₅₀ (μ g/mL)
Multiflower honey	0.206 ± 0.0243^a
Thyme honey	1.928 ± 0.0209^b
Citrus honey	0.771 ± 0.0239^c
Eucalyptus honey	2.211 ± 0.0216^d
Sidr honey	1.259 ± 0.0224^e
Epins honey	0.951 ± 0.0099^f
Propolis	35.116 ± 0.4049^g
Bee pollen	39.383 ± 0.2622^h
Ascorbic acid	0.694 ± 0.0238^i

Data are (Mean \pm SD, n = 3).

Means within a column sharing the same letter are not significantly different by Student's t-test ($P < 0.05$).

Poor scavenging activity is observed with extracts from propolis (35.116 μ g/mL) and bee pollen (38.383 μ g/mL). In similar studies, the H_2O_2 scavenging assay of phenolic compounds extracted from propolis by various solvents (distilled water, absolute methanol, 50% methanol, and 70% methanol) presented different action on H_2O_2 , which IC₅₀ values were between 11.72 μ g/mL and 765.75 μ g/mL (Farooq Wali et al., 2016; Ramnath et al., 2016; Kızılpınar Temizer et al., 2017). Although these two bee products are quantitatively rich in phenolic compounds, but their extracts have a low antioxidant capacity compared to honey extracts, which suggests that the antioxidant capacity is not always dependent on the amount of phenolics and flavonoids, honeys are therefore a good source of antioxidant substances. Also, the botanical origins of honey greatly influence hydrogen peroxide scavenging capacity. The results of the IC₅₀ obtained in table 2 clearly confirm these observations.

CONCLUSION

In this present study, it had been established that phenolic extracts from all types of honey, propolis, and bee pollen contain phenolic and flavonoids compounds and possessed antioxidant property toward hydrogen peroxide, and therefore the preventive and medical application of phenolic extracts from honey, and other bee products against oxidative stress are recommended. All honey types after extraction of phenolic compounds are able to scavenge efficiently the hydrogen peroxide, and this capacity is as much greater than that observed in other bee products. Antioxidant activity against H₂O₂ in honey samples is related to botanical type, which is strongly affected by the floral origin, and therefore varies quantitatively and qualitatively with the content of antioxidant phenolic compounds. It is well shown in the results that extract from “multiflower” honey has a content of phenolic substances with good antioxidant power against hydrogen peroxide. It is important to identify and quantify the phenolic compounds in honey extract from this type.

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