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Impact of Storage on Honey Antibacterial and Antioxidant Activities and their Correlation with Polyphenolic Content

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ABSTRACT

The quality of honey may be affected during storage. The purpose of this study was to assess the impact of storage of nine Algerian honey samples on its antibacterial activity and antioxidant effect. The antibacterial activity of honey samples was evaluated by both the agar well diffusion and micro-broth dilution assays. The antioxidant activity was performed by using *in vitro* scavenging assays of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric-reducing antioxidant power (FRAP). All the analyses were performed before storage and after every six months of storage in the dark at room temperature (24 ± 4°C). All the honey samples showed a decrease in antibacterial activity after six months of storage except for the honey sample 2. Indeed, there were no significant variations in the total polyphenolic content and DPPH values before and after storage, except for honey sample 6. However, there was a significant decrease in the antioxidant capacity measured by FRAP assay (P = 0.0002). There was a strong correlation between the total polyphenolic content of honey and its antioxidant activity and a moderate correlation with the antibacterial activity. The storage of honey in the dark at room temperature for 18 months influences the quality of honey. However, further investigations are required to strengthen this argument.

Keywords: Antibacterial activity, Antioxidant capacity, Honey, Polyphenolic content, Storage.

Introduction

Honey is a natural sweet product widely used around the world.[1,2] It contains approximately 200 distinct chemical compounds,[3] including fructose and glucose, water, ash, amino acids, and trace amounts of enzymes, vitamins, and other substances, such as phenolic and flavonoid compounds.[4,5] This composition attributes to honey many biological properties, such as antioxidant, anti-inflammatory, antibacterial, antiviral, anti-ulcer activities, antihyperlipidemic, antidiabetic, and anticancer properties.[6–8] Indeed, the therapeutic properties of natural honey produced by different species of bee have been documented in the world's oldest medical literature and since the ancient times, honey has been used for its antimicrobial property as well as wound-healing activity.[2,9,10] Indeed, honey contributes to create a protective wound barrier, which is due to its high viscosity and moisture content. Its immunomodulatory property is also important for wound repair. [11-14] Honey has inhibitory effects towards approximately 60 bacterial species; including multidrug-resistant bacteria that causes many fatal infections in humans.[15,16]Although the precise mode of action of honey is unknown, the factors responsible for its antimicrobial activity are well identified, including high osmolarity, low pH, hydrogen peroxide, and non-peroxide factors include lysozyme, phenolic acids, and flavonoids.[17,18]

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There is a wide variation in the antimicrobial activity of some types of natural honey, which is varied with the floral source, climate, and harvesting conditions.[12,14]In addition, honey has often been reported to have potential antioxidant activity, which is beneficial for human health.[6,19] The main components responsible for the antioxidant properties are the phenolic acids, flavonoids, vitamins, and enzymes, as well as a small amount of mineral content, particularly copper and iron.[20] However, the mechanism involved in the antioxidant effect of honey is not well known. It might be attributed to the ability of antioxidant compounds to donate electrons, scavenge free radicals, and chelate transition metal ions.[6]

Honey samples from different floral sources showed various levels of physicochemical composition and biological property.[21,22] However, variation in the composition and biological activity among honey samples from within the same floral species has also been observed.[4,23] This may be due to the geographical location of the floral source and the prevailing environmental conditions, which affect the physiology of the floral species.[24] Therefore, the quality of honey varied based on the geographical floral origin, season, and environmental factors. In addition, the quality of the honey can be affected during processing, handling, and storage.[25,26]

Algeria is an African country characterized by very diverse forest ecosystems, due to its geographical position and the important variations in climate that varied from the Mediterranean to the Saharan type.[16,24] Although these conditions offer production of honey of good quality; few studies have been conducted on Algerian honey, and no published data exist on the biological effects of most of the Algerian honey. Therefore, in this study, we aimed to evaluate the effect of storage on the variability of the antibacterial and antioxidant activity of nine Algerian honey samples and their correlation with the total polyphenolic content.

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Materials and Methods

Honey samples

Honey samples were collected in September 2019 from nine localities in the North-Eastern part of Algeria. These regions presented a rich floral diversity (Table 1). Honey samples were divided into 4 portions and stored at room temperature (24±4°C) in a dark and dry place until analyzed for the total period time of 18 months. The following honey concentrations were prepared in sterile distilled water: 2.5, 5, 10, 20, 40, 80% (w/v), and undiluted honey. Each honey sample was filtered through a 0.22 µm filter (Millipore, Nunc, Paramus, NJ, USA) to remove all bacterial contamination.

Determination of the antibacterial activity of honey samples

Honey samples were screened for their antibacterial activity, against three bacterial strains: *Staphylococcus aureus* and *Enterococcus faecalis* as Gram-positive bacteria; *Escherichia coli* as Gram-negative bacteria. The pathogenic strains were isolated from infected wounds and identified using conventional methods of microbiology (Gram staining, oxidase, catalase tests, coagulase test, and Analytical Profile Index: API20E, API STAPH, and API 20 STREP) (Biomerieux, Paris, France). An inoculum of each strain of 10^6 CFU/mL was prepared in nutrient broth (Difco, MD, USA).

Agar well diffusion assay was performed according to Molan et al. (1988). Wells of 6 mm in diameter were prepared in Mueller Hinton agar (Difco, MD, USA) plates. The plates were inoculated with bacterial suspension, and 100 µL of the test honey was added to each well. A well filled with sterile water served as a negative control. The cultures were incubated at 37°C for 24 h. The results are shown as mean values from triplicate measurements of inhibitory diameters around the wells.[16,27]

The minimum inhibitory concentration (MIC) was determined according to the assay described previously by the Clinical and Laboratory Standards Institute.[28] MIC values were determined using sterile 96-well microtitre plates (Fisher Scientific, UK). A volume of 100 µL of inoculum of a test strain was added to 100 µL of honey at different concentrations (from 2.5 to 100%) in each well. The control wells containing only broth (negative control) or only bacteria and broth (positive control) were also evaluated. The cultures were incubated at 37°C for 24 hours. Both visible growth and MIC values were recorded.

Antioxidant activity assays

Total polyphenolic content

The total phenolic content of honey samples was determined by the modified Folin–Ciocalteu colorimetric method as described previously by Chaiyasut *et al.* (2018). The absorbance was measured at 725 nm after 30 min incubation at room temperature. The total phenolic content was represented as mg gallic acid equivalents per kg of honey (GAE/kg of honey).[29]

Ferric reducing/antioxidant power (FRAP) assay

The FRAP assay was performed according to Beretta et al. (2005). This test is a direct colorimetric test; it evaluates the absorbance variation, at 593 nm, caused by the formation of blue-colored Fe^{2+}-TPTZ (2,4,6-tripyridyl-s-triazine) from colorless oxidized Fe^{3+}-TPTZ, by the action of electron-donating antioxidants. Honey samples were dissolved in double distilled water at the final concentration of 1 g/mL. A volume of 200 µL of honey solution was added to 1.8 mL of FRAP reagent (TPTZ 10 mmol/L in HCl 40 mmol/L, $FeCl_3$ 20 mmol /L, acetate buffer 0.3 mol/ L pH 3.6 in a ratio 1:1:10 v/v/v) and incubated 10 min at 37 °C. The absorbance was measured at 593 nm. The results were expressed as µmol/L of ascorbic acid equivalents per g of honey (µmol/ L of AAE/g), according to a calibration curve adequately created with pure ascorbic acid (20–700 µmol/L).[30]

1,1-diphenyl-2-picrylhydrazyl (DPPH) assay

The capacity to scavenge the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was monitored according to a method previously reported [31] with slight modifications. A volume of 0.3 mL of honey was mixed with 2.7 mL of a methanolic solution containing DPPH radicals (6.10^{-5}mol/L). The mixtures were thoroughly stirred in a vortex (2500 rpm for 1 min) and then placed in a dark room for 1 hour. The absorbance was measured at 517 nm after 15 min of incubation at 25°C. Ascorbic acid was used as a positive control. The ability to scavenge the DPPH was calculated using the following formula:

$$I\ (\%) = \left(\frac{A_{control} - A_{Sample}}{A_{control}}\right) * 100$$

Where, $A_{control}$ and A_{sample} are the absorbances of the control and sample, respectively. The concentration of honey sample required to scavenge 50% of $DPPH^{\cdot}$ (IC_{50}) was determined based on the ascorbic acid calibration curve (0–10 mg/L).

Statistical analysis

The data were presented as mean±standard deviation (m±SD). All statistical analyses were performed using the GraphPad Prism version 7.00 for Windows, (GraphPad Software, La Jolla California USA). One-way analysis of variance (ANOVA) was followed by multiple comparisons of means (Tukey's test) to distinguish homogeneous groups among honey samples. However, Dunnett's test was performed to examine the impact of storage on honey's biological properties. Statistical significance was set at $P < 0.05$. A Correlation matrix between antibacterial activity, total polyphenolic content, and antioxidant activity was established to analyze the relationship between different parameters.

Table 1: Floral and geographical origin of tested honey samples

Honey sample	Floral source	City	Geographic region
1	*Eucalyptus, Pinus*	El Taref	Extreme Mediterranean north-eastern of Algeria
2	*Quercus, Castanea*	Skikda	North eastern of Algeria
3	*Quercus, Castanea, Eucalyptus, Pinus*	Jijel	North eastern of Algeria
4	*Citrus:C.maxima, C. sinensis, C. aurantifolia, C. limon*	Blida	Central Mediterranean part of north Algeria
5	*Thymus hirtus, Ruta graviolen, Rosmarinus officinalis*	Setif	East of Algeria
6	*Ruta graviolens, Pituranthos scoparius,*	Batna	East of Algeria
7	*Thymus hirtus, Marrubium vulgare*	M'Sila	East of Algeria
8	*Rosmarinus officinalis, thymus,* Ecballium *elaterium, Lonicera caprifolium*	Tebessa	East of Algeria
9	*Ziziphus, Artemisia,*	Djelfa	Central part of north Algeria

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Results and Discussion

Antibacterial activity

The results of assessments of honey antibacterial activity against clinical bacterial strains are reported in Table 2. The nine honey samples showed a great antibacterial activity. There are no significant differences in the antibacterial activity of the different honey samples before and after storage for 6 months. However, there were significant differences before and after storage for 12 and 18 months, except for the honey sample 2 which preserved its antibacterial activity. There is a contradictory literature on the levels of the antibacterial activity of honey during storage. Some studies reported that the antibacterial activity of honey is being very stable at room temperature. In 1958, Warnecke and Duisberg reported that among the 85 test honey samples, there were 43 with high antibacterial activity, after 9 months to 2 years storage. Another study conducted by Allen et al. (1991) showed that there was no correlation between the antibacterial activity and the age of the honey. They found high antibacterial activity in old honey samples, some up to 5 years old.[32,33] Other studies have reported that honey is unstable during a long storage period. The instability of the antibacterial activity of honey was first recognized by Dold *et al*., (1937) who found that it was destroyed by heating and by exposure to light. Brudzynski and Kim (2011) have also reported that there was a high decline in the antibacterial activity of honey in the first 3–6 months of storage. These differences in findings could be related to the differences in the potency and stability of the various antibacterial factors in honey samples. Indeed, there are two types of antibacterial factors in honey. The hydrogen peroxide (H_2O_2) produced by honey glucose oxidase, which is decomposed when honey is heated or stored in the light. The second one is a non-peroxide factor such as phytochemical factors and lysozymes; these are stables to heat, light, and to the storage for a long time. In addition, non-peroxide factors are considered more significant than the peroxide factors for several researchers.[23,25,34,35] Among the nine honey samples tested in this study, only one sample has preserved its antibacterial activity after 18 months of storage at room temperature. This suggests that it has non-peroxide factors. However, the other samples could have peroxide factors.

Total polyphenolic content

The results of the total polyphenolic content are presented in Figure 1. There were significant differences between the total polyphenolic content of the nine honey samples. However, there was a slight reduction (not significant) in the total polyphenolic content after storage. This finding is also reported previously by Wang et al. (2006). However, Turkmen et al. (2006) reported that Honey quality can be affected by heat during processing or by aging during storage.[36,37] Thus, it is necessary to declare that storage in darkness at room temperature did not significantly alter the total polyphenolic content of tested honey samples.

The total polyphenolic content of the nine honey samples ranged from 54.92 to 198.79 mg of (GAE)/Kg of honey. This range is similar to the ranges typically found for European honey sample [38] and African honey samples.[30] This range is also similar to the ranges found by Meda et al. (2005), who reported that 27 Burkina Fasan honey samples have a total polyphenolic content between 42-114 mg of GAE/Kg.[39] Recently, it has been reported that Tunisian honey samples have a total polyphenolic content ranged between 63 to 119 mg of GAE/Kg of honey.[40] The range found in this study is considered high when compared with the total polyphenolic content of 124 Irish honey samples ranged from 2.59 to 81.10 mg of GAE/Kg of honey.[41]Indeed, the differences in the total polyphenolic content are related to the floral origin and geographic region of the honey samples. It is mainly responsible for the bioactive properties of honey.[16,42]

Antioxidant effect

The antioxidant properties of the honey samples were evaluated by FRAP and DPPH assays, as displayed in Figure 2. The decrease in the antioxidant capacity measured by FRAP assay is very high significant in all tested honey. However, there were no significant differences in the DPPH values before and after storage time, for the honey sample 6, which has shown an increase in DPPH values. The antioxidant activity of honey samples measured by FRAP assay (Figure 2A) varied significantly between 231.85± 1.78 µmol and 1271.42±2.13 µmol. This range is similar to those obtained for Italian (72.8 - 1501.4µmol) and Malaysian honey samples (182.2-794.67µmol), and higher than those obtained for Croatian (6.95 -142.43µmol) and Serbian honey samples (4-498µmol). The decrease in the antioxidant capacity measured by FRAP assay is very highly significant in all the tested honey. This finding is in agreement with that of Saric et al. (2012) on Croatian honey. Wang et al. (2004) have also reported that the decline in antioxidant capacity occurs during the first 6 months.[37,43] However, it is in disagreement with that of Gheldof and Engeseth (2002) who reported that there was no significant alteration of the antioxidant activity of clover honey after more than 2 years of storage. Furthermore, the antioxidant effect determined by the DPPH assay (Figure 2B) has shown that the nine honey samples have an antioxidant value which ranged from 10.02±1.85 to 74.57±0.28 mg/ml. This range is not far from those found by several other authors [6,44–46]. Indeed, there were no significant differences in the IC_{50} values before and after the storage time, except for the honey sample 6, which have shown an insignificant increase in the IC_{50} values.

Table 2: The assessment of the variation of the antibacterial activity of nine honey sample after 18 months for storage in darkness at room temperature (24±4°C)

Honey sample	Inhibitory diameters ± SD (mm)				MIC values (% w/v)			
	T_0	T_1	T_2	T_3	T_0	T_1	T_2	T_3
1	27.33 ± 03.00^a	26.24 ± 02.33^a	17.12 ± 02.00^b	09.25 ± 02.33^c	10	10	40	80
2	32.67 ± 02.33^a	31.32 ± 01.67^a	30.85 ± 01.00^a	28.82 ± 02.33^a	10	10	10	10
3	32.67 ± 04.67^a	30.25 ± 02.33^a	18.84 ± 02.33^b	08.85 ± 04.67^c	05	10	40	80
4	31.98 ± 01.33^a	30.14 ± 01.67^a	19.85 ± 01.33^b	09.68 ± 02.00^c	05	10	40	80
5	35.28 ± 01.67^a	34.82 ± 02.33^a	17.37 ± 01.33^b	10.64 ± 01.33^c	05	10	40	80
6	28.84 ± 01.33^a	23.85 ± 01.67^a	14.85 ± 01.67^b	12.12 ± 03.00^c	10	20	40	100
7	37.15 ± 02.33^a	35.78 ± 02.67^a	18.52 ± 01.33^b	04.61 ± 02.33^c	05	10	40	100
8	34.85 ± 03.33^a	31.94 ± 01.67^a	16.03 ± 01.33^b	09.85 ± 01.33^c	10	10	40	100
9	26.87 ± 01.67^a	24.11 ± 02.33^a	14.94 ± 01.67^b	10.21 ± 01.33^c	10	10	40	80

Data expressed as means of three independent experiments ± SD. T_0: before storage, T_1: after 6 months, T_2: after 12 months, T_3: after 18 months. There are no significant differences (P>0.05) between honey samples which have the same letters but there are very highly significant differences (P<0.0001) between honey samples which have different letters.

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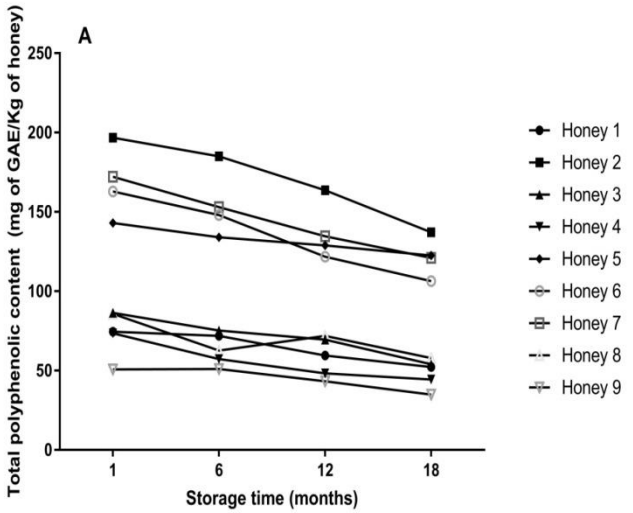

Figure 1: Changes in the total polyphenolic content in nine honey samples during 18 months of storage in darkness at room temperature (24±4°C)

These results are in contrast to those found by Saric et al. (2012) who reported that acacia honey showed a very significant increase in the IC_{50} values but the multifloral honey 6 showed an insignificant increase [43]. These differences in the results reported in the literature are due to the fact that the honey tested is not of the same floral and geographical origin. Sometimes, the honey tested is stored for a long period before use; which has a strong influence on the parameters measured.

Correlation between parameters

To assess the relationship between the total polyphenolic content, antioxidant and antibacterial activities, a Persean matrix is reported in Table 3. The results showed that there is a strong positive correlation between the total polyphenolic content of honey and its antioxidant activity (determined by FRAP and DPPH assays). Similar to our findings, Beretta et al. (2005), Bertoncelj et al. (2007) and Piljac-Žegarac et al. (2009) also reported a strong correlation between the total polyphenolic content and the antioxidant capacity (r =0.958, 0.853 and 0.918, respectively).[30,47,48] This suggests that polyphenolic contents are the major components responsible for the antioxidant effects of honey. In addition, there is a moderate correlation between the total polyphenolic content and the antibacterial activity (r=0.623), which suggests that the total polyphenolic content seems to contribute to the antibacterial activity.[49,50]

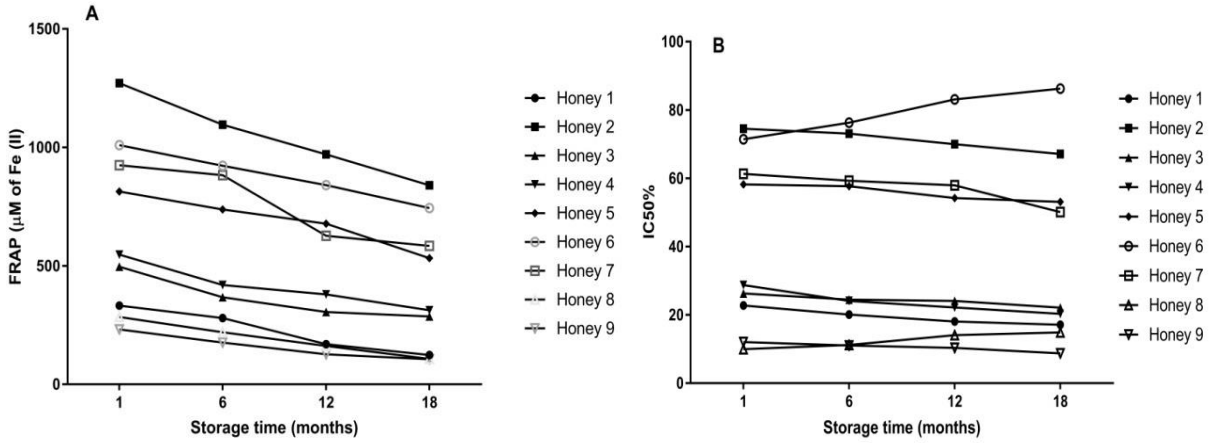

Figure 2: Changes in the antioxidant activity of the nine honey samples during storage in darkness at room temperature
(A: Variation in the antioxidant activity measured by the FRAP assay, B: Variation in the antioxidant activity measured by DPPH assays). FRAP: Ferric reducing/antioxidant power; DPPH: 1,1-diphenyl-2-picrylhydrazyl

Table 3: Correlation matrix between the different parameters

	TPC (mg (GAE/Kg)	Inhibitory diameters (mm)	FRAP (µmol)	IC_{50} (mg/mL)
TPC (GAE/Kg)	1	0.623	0.953	0.942
Inhibitory diameters (mm)	0.623	1	0.538	0.444
FRAP	0.953	0.538	1	0.977
IC_{50}	0.942	0.444	0.977	1

Conclusion

Honey produced by *Apis mellifera* has many biological activities such as antibacterial and antioxidant activities. The quality of honey depends on its composition and the conditions under which it is stored. It has been demonstrated in this study that the storage of honey for 18 months considerably influences its antibacterial activity, polyphenol content as well as antioxidant activity. In addition, there is a strong positive correlation between the total polyphenol content and the antioxidant activity of honey. However, there was a moderate correlation between the polyphenol content and the antibacterial activity.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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