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Research Article

Tracing the *In Vitro* Antioxidant Activity of Some Rare Honeys Produced in the Hellenic Zone

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Abstract

Background: Honey is a complex mixture of numerous phytochemicals that possess considerable antioxidant activity.

Aim: The aim of this study was to investigate the *in vitro* antioxidant activity of some rare honey types produced in the Hellenic zone.

Methods: Fourteen honey samples were collected during two consecutive harvesting years from 5 different regions in Hellas. Honeys belonged to arbutus, asfaka, chestnut, citrus, oak, pine, sage, and thyme. The *in vitro* antioxidant activity was estimated using the 2, 2-diphenyl-1-picryl-hydrazyl spectrometric assay.

Results: The *in vitro* antioxidant activity was greatly affected by honey botanical origin. The higher *in vitro* antioxidant activity (%AA) and effective concentration (EC_{50}) was recorded for chestnut honey followed by those of oak, pine, and arbutus honeys.

Conclusion: Apart from its use as a natural sweetener, honey, especially in the form of an aqueous solution, may be used in the daily diet as a good source of water soluble antioxidants.

Introduction

It is attributed to Einstein the following quote: " If the bee disappeared of the surface of the globe, then man would have only four years of life left. No more bees, no more pollination, no more plants, no more animals, no more man". However, some others claim that this quote was spoken by Charles Darwin, or Maurice Maeterlinck or E.O. Wilson. No matter who said that, this quote helps us realize the significance of this flying insect on Earth.

Honeybees (*Apis mellifera*) produce honey via collection of the sugary secretions (floral nectar) of numerous plants or other honeydew secretions by plant sucking insects (aphid honeydew) through 3 basic actions: regurgitation, enzymatic activity and evaporation and then store it in honeycombs [1].

The basic components of honey are sugars, fructose and glucose, along with some other polysaccharides and water. It also contains small amounts of other micronutrients such as minerals, proteins, vitamins, organic acids, flavonoids, phenolic acids, enzymes and numerous other naturally occurring compounds known as "phytochemicals". Polyphenols, ascorbic acid, catalase, peroxidase, carotenoids, and products of the Maillard reaction have been reported to possess considerable *in vitro* antioxidant activity, enhancing thus, honey nutritional value [2,3].

The quantity of these components varies greatly according to the botanical and geographical origin of honey. Processing and/or handling practices, especially by beekeepers, could also affect honey composition [4,5].

The consumption of honey alone or in combination with other antioxidant beverages has been reported to significantly increase the antioxidant capacity of human serum [6]. Honey has potential therapeutic role in cancer, and several other inflammatory diseases as reported previously by other researchers [7,8]. In addition, the metabolic effects of honey, alone or in combination with other foods in type II diabetics has been also investigated [9].

Based on the above, the aim of the present work was to evaluate the *in* vitro antioxidant activity of some rare Hellenic honeys in an effort to enhance the beneficial role of honey through i.e. regular consumption, considering the great impact of honey botanical origin. To our knowledge, this is the first study in the literature enhancing the in *vitro* antioxidant activity of these rare honey types produced in the Hellenic zone.

Materials and Methods

Honey samples

Fourteen honey samples were collected: arbutus (*Arbutus unedo, Ericaceae*) from Messinia and Lakonia (5 samples); asfaka (*Phlomis fruticosa*) from Aitolokarnania (1 sample); chestnut (*Castanea Mill.*) from Messinia (2 samples); citrus (*Citrus* sp.) from Messinia (2 samples); oak (*Quercus*) from Karditsa (1 sample); pine (*Pinus* sp.) from Rhodes (1 sample); sage (*Salvia officinalis*) from Messinia (1 sample); and thyme (*Thymus* sp.) from Rhodes (1 sample), during the harvesting years 2010-2012 from professional beekeepers. All honey samples were stored in glass containers and maintained at 4 ± 1°C before analysis.

Reagents and solutions

2,2-Diphenyl-1-picrylhydrazyl [DPPH·] (99%) was purchased from Sigma-Aldrich (Germany). Methanol, acetate buffer ($CH_3COONa \times 3H_2O$), and sodium chloride were purchased from Merck (Darmstadt, Germany). All aqueous honey solutions were prepared using distilled water.

In vitro estimation of antioxidant activity (%AA) of rare Hellenic honeys

Preparation of [DPPH·] standard solutions and calibration curve: A standard solution of the free radical [DPPH·] was prepared as follows: 0.0050 g of the radical [DPPH·] were dissolved in 100 mL of methanol. The volumetric flask was wrapped in foil and then was stirred in a vortex apparatus. The solution obtained (pH=7.00, 50 mg/L) had a deep purple colour and was left in the refrigerator for 2 h in order to stabilize. A calibration curve of concentration versus absorbance of [DPPH·] was prepared by dilutions of the initial free radical solution with methanol covering a range of 10-50 mg/L. The resulting solutions were vortexed, left in the dark (until measurements were made) and absorbance was measured in a UV/VIS Spectrometer (Perkin Elmer, Lambda 25, USA) at λ= 517 nm.

The calibration curve of absorbance (y) versus concentration (x) of [DPPH·] was expressed by the following equation:

$y = 0.032x + 0.0068$; $R^2 = 0.9997$ (Eq.1) (Figure 1).

Determination of the %AA: Approximately 34 g (34.03 g) of honey were dissolved in 250 mL of distilled water [mother aqueous honey solution (w/v)]. Additionally, 1:2, 1:4, 1:10 dilutions were prepared from the mother solution in order to estimate the effective concentration of the aqueous honey solutions causing 50% inhibition of the free radical (EC_{50}). In that sense, the prepared aqueous honey solutions (mg/L) were: 6786, 23112, 57780, and 136120.

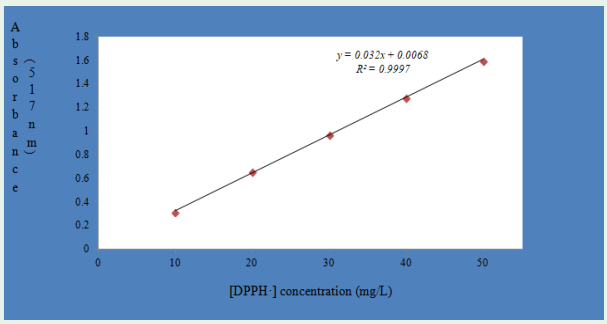

Figure 1: [DPPH•] calibration curve.

Volumes of 1.9 mL of the initial solution of [DPPH·] and 1 mL of acetate buffer 100 mM (pH=7.0) were placed in a cuvette and the absorbance of the [DPPH·] was measured at t = 0 (A_0).

Subsequently, 0.1 mL of each of the aforementioned honey solutions was added to the above medium and the absorbance was measured every 30 min (regular time periods) until the value reached a plateau (steady state, A_t). The reaction was completed in 4h.

The [DPPH·] radical scavenging activity was calculated using the following equation:

$$\%AA = A_0 - A_t / A_0 \times 100 \quad \text{(Eq.2)}$$

where A_0 is the initial absorbance of the free radical standard solution and A_t is the absorbance of remaining [DPPH·] after reaction with honey water soluble antioxidants, at steady state (t, plateau). Each analysis was run in triplicate (n=3).

Methanol and acetate buffer (2:1, v/v) were used as the blank. Parameters such as the % decrease in [DPPH·] concentration, % [DPPH·] remaining of the mixture obtained after the addition of aqueous honey solutions, when the reaction reached plateau, were estimated by Eq.1.

The [DPPH·] remaining was calculated by the formula:

$$[DPPH\cdot] \text{ remaining} = ([DPPH\bullet]t)/([DPPH\bullet]0) \times 100 \quad \text{(Eq.3)}$$

EC_{50} (mg/mL) was estimated from graphs of the % [DPPH·] remaining concentration versus concentrations of the aforementioned aqueous honey prepared dilutions: 1:1, 1:2, 1:4, 1:10 (w/v).

Statistical analysis

In order to estimate the differences between honey botanical origin and % AA or EC_{50} values, paired samples T-test was applied at the confidence level $p<0.05$. Statistical treatment of data was performed using the SPSS v.20.0 statistics software. Graphs were prepared using the Microsoft Office Excel 2007 for Windows.

Results and Discussion

Antioxidant activity (%AA) of rare Hellenic honeys

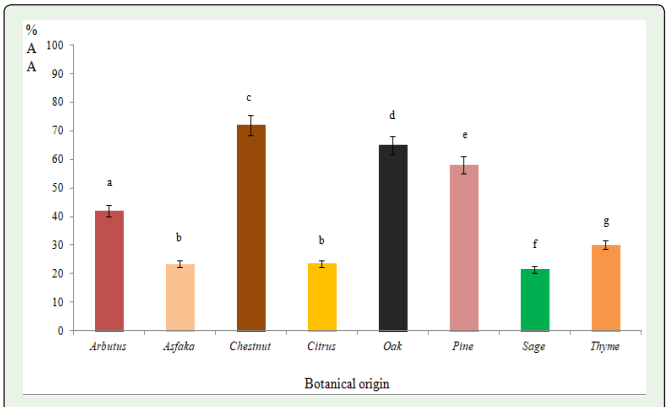

Figure 2: Antioxidant activity (%AA) of Hellenic honeys of different botanical origin. Different letters (a, b, c, d, e, f, g) indicate statistically significant differences at the confidence level p<0.05.

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Table 1: Antioxidant activity (%AA) and effective concentration (EC_{50}) of some rare Hellenic honeys.

Botanical origin	%AA	EC_{50} (mg/mL)
Arbutus	42.05±1.26	23.10±0.70
Asfaka	23.45±0.70	46.35±0.19
Chestnut	71.96±0.84	7.50±0.39
Citrus	23.56±0.71	39.82±1.19
Oak	64.97±1.95	8.31±0.25
Pine	58.03±1.74	9.13±0.27
Sage	21.50±0.65	44.06±1.32
Thyme	30.09±0.90	26.34±0.79

Every value is the average ± standard deviation of three replicates (n=3). 0.1 mL of honey aqueous solutions were used in the [DPPH·] assay. T-test in comparison of values ($p<0.05$).

The water soluble antioxidants of the rare Hellenic honeys proved to eliminate the free radical with a high degree, especially the higher honey aqueous solution (34.03 g/250 mL). The higher %AA was recorded for chestnut, followed by oak, pine and arbutus honeys (Figure 2). Analytical data involving the 8 different honey types investigated are given in Table 1.

What is remarkable is the fact that, botanical origin showed a great impact on antioxidant properties of Hellenic honeys. This is in agreement with previous work in the literature [4,5,10-12].

In general, the degree of antioxidant activity of different honey types depends on the structures of polyphenols or other phytochemicals that are naturally present in honey, as a consequence of the ability of these compounds to donate the hydrogen ion or electron for the free radicals [12,13].

This is the first free radical scavenging activity report, using the [DPPH•] assay, on these specific Hellenic honey types, especially those of arbutus, asfaka, chestnut, and oak honeys. Despite the limited honey samples collected (these were possible to be donated) the present work highlights once again the significantly ($p<0.05$) higher antioxidant activity of darkest coloured honeys compared to blossom ones along with the potential of honey water soluble antioxidants [4,5].

The effective concentration (average value, mg/mL) ranged between 7.50 and 46.35 depending on honey botanical origin ($p<0.05$). The ability of Hellenic honeys to eliminate the [DPPH·] is shown on typical graphs (Figure 3-5). It is clearly shown that, the lower the EC_{50} value the higher the antioxidant activity.

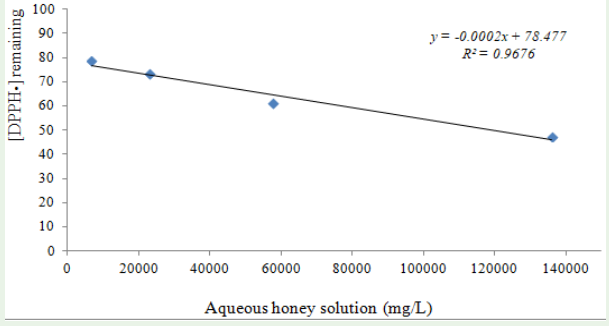

Figure 3: Ability of arbutus honey aqueous solution to eliminate the [DPPH•].

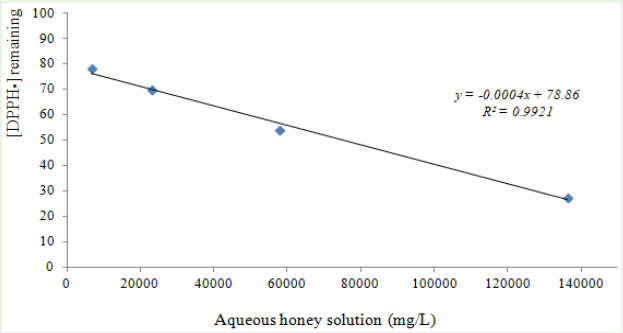

Figure 4: Ability of chestnut honey aqueous solution to eliminate the [DPPH•].

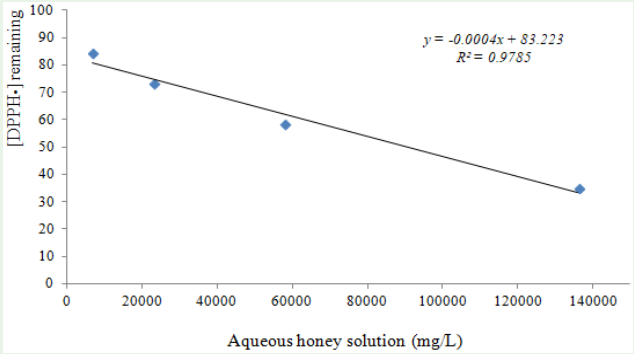

Figure 5: Ability of oak honey aqueous solution to eliminate the [DPPH•].

In a study involving Italian honeys, the reported EC_{50} values for chestnut and honeydew honeys were 7.93±0.04 and 8.48 ±0.24, respectively [4]. These values are in excellent agreement with those obtained for Hellenic chestnut and pine honeys of the present study. The same holds for the Italian light coloured honeys; dandelion, clover, and acacia, which recorded EC_{50} values of 24.39±0.07, 25.00±0.01, and 45.45±0.04, respectively. Such values are in conformity with present results regarding arbutus, thyme, and sage honeys.

A great variation in EC_{50} (mg/mL) values was reported in a study involving Brazilian honeys: 10.19±1.65 to 67.69±1.24, depending on the botanical origin of honey. Eucalyptus honeys recorded the higher antioxidant activity compared to cambara or artificial honeys [11]. However, it should not be forgotten that, bee species may affect honey overall antioxidant activity [12].

Conclusion

The present study, in a simple way, demonstrates significant variations ($p<0.05$) in antioxidant properties of Hellenic honeys of different botanical origin. Given the tremendous health benefits of natural antioxidants, floral origin should be an important factor in evaluating the potential of honey as a source of antioxidants in the daily diet. The knowledge about free radicals and antioxidants is still under investigation. It is apparent, that further research evaluating the relationship between dietary antioxidants, especially those obtained in aqueous form, and bioaccessiblity in different human bodies is mandatory. Hellenic honey may contribute to this research.

Acknowledgments

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