Sardinian honeys as sources of xanthine oxidase and tyrosinase inhibitors

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Abstract Sardinian honeys obtained from different floral sources (*Arbutus*, *Asphodelus*, *Eucalyptus*, *Thistle*, and *Sulla*) were evaluated for their ability to inhibit tyrosinase and xanthine oxidase enzymes and for their antioxidant activity. Physicochemical parameters, total phenolic, and flavonoids content were also determined. Honey from *Arbutus* flowers had the highest antioxidant activity followed by *Eucalyptus* and *Thistle* ones. These three honeys showed good tyrosinase and xanthine oxidase inhibition properties. Thus, these Sardinian honeys could have a great potential as antioxidant sources for pharmaceutical and cosmetic applications.

Keywords Enzyme inhibitors · Honey · Tyrosinase · Xanthine oxidase · Antioxidant

Introduction

Honey is a natural product comprising a mixture of sugars, water, trace elements, and some chemical compounds such as phenols, flavonoids, ascorbic acid, and tocopherols and a variety of enzymes. The variety of the bioactive compounds of honey depends by several factors such as the floral source and the seasonal, geographical, and environmental conditions [1]. Different properties of honey

including antineoplastic, anti-inflammatory, antibacterial, and antioxidant properties have been reported [2, 3]. Moreover, numerous studies have demonstrated that honey is effective in reducing the risk of heart diseases, cancer, immune-system decline, cataracts, and different inflammatory processes, as reported in the National Honey Board [4].

This makes honey not only a source of nourishment but also a valuable dietary source of antioxidants, which are strongly correlated with the concentration of total phenolics. Moreover, the color of honey seems to be correlated with its antioxidant activity. In fact, dark colored honeys have a higher total phenolic content and consequently a higher antioxidant capacity [5].

Furthermore, honey is one of the most used products in traditional medicine for skin diseases [6]. In traditional Chinese and Arabic medicine, honey is used to improve the general appearance of skin and for fungal infections. Likewise, in Northern Pakistan, creamy honey is used for eyelid and face protection as well as the treatment of freckles and white spots [7, 8]. Honey is present as a component of a wide number of cosmetics like hand creams, lipsticks, and shampoos due to its moisturizing, emollient, nourishing, and antiseptic properties [9]. It has also been reported that honey possesses inhibitory activity on tyrosinase, probably due to the presence of polyphenols [10].

Tyrosinase (EC 1.14.18.1) is the key enzyme of melanogenesis, catalyzing two different reactions. In the initial step, L-tyrosine is hydroxylated to L-3,4-dihydroxyphenylalanine (L-DOPA, *o*-diphenol product) and then oxidized to the corresponding *o*-quinone. Although melanin production in human skin represents a primary defense mechanism against exposure to UV light, excessive accumulation of epidermal pigmentation can cause various

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disorders, such as melasma, age spots, flecks, ephelide, and sites of actinic damage. Therefore, tyrosinase inhibitors should be clinically useful for the treatment of dermatological disorders associated with melanin hyperpigmentation and for whitening and depigmentation after sunburns [11].

Exposure to UV radiation induces the formation of excess reactive oxygen species (ROS), which can interact with proteins, lipids, and DNA and alter cellular functions, thus causing skin aging. Melanogenesis has been reported to produce hydrogen peroxide and other ROS in melanocytes. This observation has increased interest in identifying natural antioxidants that could inhibit skin aging and melanogenesis [12, 13]. ROS are also generated by the reaction catalyzed by xanthine oxidase (XO, EC 1.1.3.22), which catalyzes the oxidation of hypoxanthine to xanthine and subsequently to uric acid. XO is responsible for oxidative damage that causes many pathological diseases, such as gout, hyperuricemia, hepatitis, carcinogenesis, and aging [14]. Several medications that inhibit XO are indicated for the treatment of hyperuricemia and gout [15].

In this context, we have analyzed the total content of polyphenolic and flavonoid compounds and the antioxidant, antityrosinase, and anti-XO properties of five Sardinian honeys of different botanical origin. To find specific compounds of these honeys, a direct RP-HPLC-DAD analysis of their polar fraction was performed. Furthermore, physicochemical parameters like moisture, pH, total acidity, electrical conductivity, and colorimetric coordinates in CIELab space were measured.

Materials and methods

All chemicals were obtained as pure commercial products from Sigma Chemical Co. (St. Louis, USA).

Honey samples

Five varieties of unifloral honey, Arbutus (*Arbutus unedo*), Asphodelus (*Asphodelus* spp.), Thistle (*Cardus f.*), Eucalyptus (*Eucalyptus* spp.), and Sulla (*Hedysarum coronarium*) were included in this study. Sardinian honeys were obtained from a local farm (Zafferano e Spezie di Sardegna srl Agricola).

After sampling, honey was stored in the dark at 4 °C until analysis in order to preserve its chemical composition.

To measure the antioxidant activity, total phenolic and flavonoid content, and inhibitory activity, each honey (5 g) was dissolved in 25 mL of distilled water and immediately tested.

Determination of physicochemical parameters of honeys

The parameters examined include moisture (g/100 g), pH, total acidity (meq/kg), electrical conductivity (mS/cm), colorimetric coordinates in CIELab space, and total polyphenol and flavonoids content. All analyses were performed in triplicate.

The moisture content was determined by refractometry [16] using a honey-type refractometer (Shibuya Optical Co., Ltd). All measurements were performed at 20 °C.

Total acidity and pH of honeys were measured according to the Codex Alimentarius Commission [17]. Briefly, each honey (5 g) was dissolved in 50 mL distilled water, and this solution was titrated with 0.05 M NaOH solution until the pH reached 8.5. The results were expressed in milliequivalent of acids per 1 kg of honey. A pH meter (Crison pH-meter Basic 20+) was used to measure the pH of each honey.

The electrical conductivity of the honeys was determined according to the International Honey Commission as measured in a 20% (w/v) solution of honey in distilled water using a Crison conductometer model 522.

The color parameters were measured in the CIELab system using a Minolta CR300 Chromameter with illuminant D65 (Osaka, Japan) by measuring lightness (L^*), redness (a^*), and yellowness (b^*) intensities. Specimens were illuminated at 45°. Two additional parameters, chroma (C^*) and hue angle (h°), were calculated according to Salvador et al. [18].

Determination of the total polyphenol and flavonoid content

The total content of polyphenols (TPC) and flavonoids (TFC) was determined as previously reported [19]. Polyphenol concentration was calculated using gallic acid as a referred standard and was expressed as mg of gallic acid equivalent (GAE) per kg of honey. Flavonoid concentration was expressed as mg of quercetin equivalent (QE) per kg of honey.

Antioxidant assays

The total free radical-scavenging capacity of the honeys was determined by the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS⁺) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods as previously reported [20, 21]. For both free radical methods, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) was used as standard reference. The activity was expressed as the concentration of the sample necessary to give a 50% reduction in the original absorbance (IC₅₀).

Xanthine oxidase inhibition assay

The inhibitory activity of honey against XO was obtained by measuring the formation of uric acid at 295 nm. The reaction mixture contained 100 mM phosphate buffer solution (pH 7.5, 689 μ L), an aqueous solution of XO from bovine milk (0.5 U/mL, Sigma Chemical Co., 50 μ L), and a sample solution or distilled water (200 μ L). Then, xanthine solution (0.82 mM) was added and the XO activity was determined at 295 nm for 3 min at 25 °C. Allopurinol was used as a standard inhibitor. The IC₅₀ value, a concentration giving 50% inhibition of XO activity, was determined by the interpolation of dose–response curves.

Tyrosinase inhibition assay

Tyrosinase inhibition assays were performed with L-DOPA as substrate according to the procedure previously described [22]. The reaction mixture contained 25 mM phosphate buffer (pH 6.8) with or without samples and L-DOPA (1.25 mM). Then, tyrosinase from mushroom (100 U/mL) was added into the mixture and the activity was determined by following the increase in absorbance at 475 nm resulting from the formation of the dopachrome product. Kojic acid was used as a positive control. The extent of inhibition by the compounds was expressed as the inhibitor concentration leading to 50% decrease in the enzyme activity (IC₅₀).

LC detection and quantitative analysis

The analyses were performed using HPLC–DAD Varian system ProStar fitted with a pump module 230, an autosampler module 410, and a ThermoSeparation diode array detector SpectroSystem UV 6000lp (ThermoSeparation, San Jose, CA) set at 210 and 280 nm. The gradient elution was performed on a Phenomenex Kinetex XB-C18 100 Å column (150 mm $\times$ 4.60 mm, 5 μ m, Chemtek Analytica, Anzola Emilia, Bologna, Italy) using 0.2 M phosphoric acid (solvent A), and acetonitrile (solvent B) was used as the mobile phase at a constant flow rate of 1.0 mL/min. The gradient (v/v) was formed as follows: 100% of solvent A for 5 min, then decreasing of solvent A to 80% in 20 min, to 50% in 40 min, to 10% in 50 min and finally increasing to 100% of solvent B until 60 min. The injection volume was 10 μ L. The obtained chromatograms and spectra were elaborated with a ChromQuest V. 4.0 data system (ThermoQuest, Rodano, Milan, Italy). The standard solutions were prepared in methanol, and the working standard solutions were prepared in ultrapure water. The calibration curves were plotted according to the external standard method, correlating the analyte peak areas with the corresponding concentrations. The honey samples were

diluted in ultrapure water in the ratio of 1:5 (w/v), vortexed, and filtered through an RC membrane syringe filter (0.45 μ m, Ø15 mm, Phenomenex) prior to the analysis.

Statistical analysis

The results were expressed as mean $\pm$ standard deviation. The ANOVA test was used to analyze the variance of data (SPSS software version 17.0 for Windows). The analysis average of the treatment using multiple comparisons was determined using Duncan's multiple range tests, and the data were compared using *p* values: *p* < 0.05 was considered statistically significant. The correlation between the parameters evaluated was obtained using Pearson's correlation coefficient (*r*).

Results and discussion

Physicochemical parameters

Honeys from different botanical origins showed different physicochemical characteristics (color, moisture, pH, electrical conductivity, and total acidity) owing to the presence of diverse types of compounds (Table 1). The CIE *L*a*b** parameters showed color variability: *L** (lightness) ranged from 22.11 (Eucalyptus) to 33.94 (Arbutus); the parameters *a** and *b** indicated that green components (negative *a** values) were present in Asphodelus and Sulla honeys. Chrome value *C** was the highest in Arbutus honey, whereas Sulla and Asphodelus honeys had the lowest values of *C**. This parameter expresses the saturation of colors, with high-saturated colors being vivid and low-saturated colors being dull [23]. The moisture of honey samples ranged from 16.24 $\pm$ 0.21 to 17.19 $\pm$ 0.26% and the pH varied from 3.65 $\pm$ 0.04 to 4.55 $\pm$ 0.02 (Table 1). The pH value is not directly related to free acidity because of the buffering action of various acids and minerals present. As shown in Table 1, the total acidity ranged from 5.61 $\pm$ 0.99 to 32.97 $\pm$ 1.41 meq/kg. Finally, the electrical conductivity of honeys varied from 0.06 $\pm$ 0.01 to 0.943 $\pm$ 0.03 mS/cm.

Total phenol and flavonoid contents

Concerning the quantification of the total phenolic compounds in Sardinian honeys, the results revealed that the TPC ranged from 200 $\pm$ 2 to 969.7 $\pm$ 8.5 mg GAE/kg (Table 2) and the honey from Arbutus had the highest TPC when compared to the others, followed by Eucalyptus. Meanwhile, the honey from Sulla showed the lowest value. The TFC of the honey samples were in the range from 10.4 $\pm$ 1.4 to 96.5 $\pm$ 3.3 mg QE/kg of honey (Table 2).

Table 1 Physicochemical parameters of Sardinian honeys

Honeys	Color			Moisture (% w/w)			Electrical conductivity (mS/cm)	pH	Total acidity (meq/kg)
	L*	a*	b*	C*	h°				
Arbutus	33.94 ± 0.01	+ 1.51 ± 0.01	+ 8.26 ± 0.01	15.09 ± 0.02	81.66° ± 0.11°	17.19 ± 0.26	0.37 ± 0.03	4.31 ± 0.02	20 ± 0.2
Asphodelus	25.4 ± 0.05	− 0.14 ± 0.03	+ 1.8 ± 0.02	3.11 ± 0.03	93.9° ± 0.1°	17.03 ± 0.05	0.06 ± 0.01	3.88 ± 0.11	5.61 ± 0.99
Eucalyptus	22.13 ± 0.01	+ 0.89 ± 0.01	+ 8.13 ± 0.01	8.18 ± 0.02	69.3° ± 0.36°	15.03 ± 0.05	0.93 ± 0.03	4.55 ± 0.02	32.97 ± 1.41
Thistle	25.23 ± 0.05	+ 0.72 ± 0.03	+ 8.68 ± 0.12	8.72 ± 0.1	85.22° ± 0.2°	16.83 ± 0.15	0.43 ± 0.01	4.12 ± 0.09	25.8 ± 0.28
Sulla	32.56 ± 0.06	− 0.53 ± 0.02	4.18 ± 0.02	3.02 ± 0.05	96.34° ± 0.24°	16.24 ± 0.21	0.12 ± 0.01	3.65 ± 0.04	15.1 ± 1.7

Results are given as the mean ± standard deviation (SD) of triplicate experiments

Table 2 Total phenol and flavonoid contents of honey samples

	Total phenol content (mg GAE/kg)	Total flavonoid content (mg QE/kg)
<i>Arbutus</i>	969.7 ± 8.5 ^e	96.5 ± 3.3 ^d
<i>Asphodelus</i>	401.5 ± 4.6 ^b	70.2 ± 4.8 ^c
<i>Eucalyptus</i>	828.8 ± 6.4 ^d	56.3 ± 2.3 ^b
<i>Thistle</i>	448.2 ± 2.6 ^c	15.1 ± 1.8 ^a
<i>Sulla</i>	200 ± 2 ^a	10.4 ± 1.4 ^a

GAE gallic acid equivalent, QE quercetin equivalent

The data are given as the mean ± standard deviation (SD) of triplicate experiments. The statistical comparison between values from the different types of honey applied using the post hoc Duncan test. Means followed by distinct letters in the same column are significantly different ($p < 0.05$)

The honey from *Arbutus*, in this case, gave the best results, showing a significantly high flavonoid content ($p < 0.05$).

Antioxidant activity

The antioxidant activities of honey from different types of pollen were evaluated using ABTS and DPPH assays (Table 3). The results of ABTS ranged from 4.2 ± 0.2 to 57.8 ± 4.4 mg/mL and of DPPH from 6.1 ± 1.2 to 54.3 ± 3.4 mg/mL. Both assays showed that honey from *Arbutus* flower had the highest antioxidant capacity compared to the others ($p < 0.05$), whereas the honeys from *Sulla* and *Asphodelus* had the lowest activity.

Inhibitory activity

All five honeys were investigated for their inhibitory activity toward XO and tyrosinase enzymes (Table 3). *Eucalyptus* honey exhibited the highest degree of inhibition of XO with IC_{50} of 38.2 ± 3.3 mg/mL followed by *Thistle* honey and *Arbutus* honey (IC_{50} values of 40.2 and 57.9 mg/mL, respectively). *Sulla* honey had the lowest

degree of inhibition (IC_{50} value of 83.9 mg/mL), whereas *Asphodelus* honey showed no inhibitory effect.

Table 3 also shows the tyrosinase inhibitory activity of honeys. Honey from *Thistle* flowers had the highest anti-tyrosinase activity (IC_{50} : 64.3 ± 1.6 mg/mL) when compared to the other types of tested honeys ($p < 0.05$) followed by *Arbutus* and *Eucalyptus* honeys (IC_{50} : 119.7 ± 5.2 and 157.7 ± 2.5 , respectively), whereas *Sulla* and *Asphodelus* honeys showed the lowest activity.

HPLC–DAD fingerprinting

HPLC–DAD fingerprinting (Fig. 1) confirmed the typical marker for *Asphodelus*, *Arbutus*, and *Thistle* unifloral honeys [24–26]. For *Eucalyptus* and *Sulla* flower unifloral honeys, there are no proposed polar marker compounds, but HPLC–DAD fingerprinting matched well with the average profiles of these unifloral honeys. Table 4 shows quantitative data for the selected marker compounds. Some amino acids were also detected. *Asphodelus* honey is characterized by a high amount of methyl syringate (174.5 ± 9.2 mg/kg), higher than the minimum level of

Table 3 Antioxidant properties and inhibitory activity of honey samples

Honeys	IC_{50}			
	ABTS (mg/mL)	DPPH (mg/mL)	Xanthine oxidase (mg/mL)	Tyrosinase (mg/mL)
<i>Arbutus</i>	4.2 ± 0.2^a	6.1 ± 1.2^a	57.9 ± 2.3^c	119.7 ± 5.2^b
<i>Asphodelus</i>	57.8 ± 4.4^d	54.3 ± 3.4^d	–	> 200
<i>Eucalyptus</i>	9.1 ± 2.5^b	21.4 ± 1.7^b	38.2 ± 3.3^a	157.7 ± 2.5^c
<i>Thistle</i>	20 ± 1.7^c	23.3 ± 0.4^b	40.2 ± 1.8^b	64.3 ± 1.6^a
<i>Sulla</i>	56.8 ± 8.3^d	45.3 ± 1.9^c	83.9 ± 3.7^d	> 200
3-Phenyl lactic acid			> 200	> 200
Lumichrome			> 200	> 200
Allopurinol			12 ± 1.7	
Kojic acid				$(0.8 \pm 0.03) \times 10^{-3}$

The data are given as the mean ± standard deviation (SD) of triplicate experiments. The statistical comparison between values from the different types of honey applied using the post hoc Duncan test. Means followed by distinct letters in the same column are significantly different ($p < 0.05$)

Fig. 1 HPLC–DAD fingerprinting of the five selected unifloral honeys at $\lambda = 280$ nm. Chromatographic conditions are described in the text. Peak identification is given in Table 4

122.6 mg/kg proposed for this unifloral honey [24]. Arbutus honey presents all the four typical markers proposed for this unifloral honey [25]: homogentisic acid (346.8 ± 17.2 mg/kg), unedone (166.2 ± 9.6 mg/kg), t,t-abscisic acid (ABA) (109.6 ± 4.2 mg/kg), and c,t-ABA (116.9 ± 4.7 mg/kg). Lumichrome and 3-phenyl lactic acid represent the chemical markers of Thistle honey and their amount in the analyzed honey (68.3 ± 2.9 and 265.6 ± 15.0 mg/kg, respectively) were on the average for this unifloral honey [26]. A small amount of kojic acid (1.7 ± 0.0 mg/kg) was identified only in Thistle honey.

This compound is a degradation product of carbohydrates and was previously found in Manuka honey [5].

In this study, Sardinian honeys were analyzed for their physicochemical characteristics and biological activities. The pH values and electrical conductivity of the honey samples were not different from each other and can be compared with honey samples from other geographical locations such as Argentina, Spain, and Italy [27]. All the samples had their acidity values within the limits of < 50 meq/kg, as specified by the international standards. Several researchers have reported different values of

Table 4 Compounds identified by targeted HPLC-DAD analysis (mg/kg)

Compound	<i>Asphodelus</i>	<i>Arbutus</i>	<i>Eucalyptus</i>	<i>Thistle</i>	<i>Sulla</i>
1 Kojic acid	nd	nd	nd	1.7 ± 0.0	nd
2 Tyrosine	130.0 ± 4.1	nd	2.5 ± 0.1	14.8 ± 0.3	7.0 ± 0.1
3 HMF	nd	nd	4.8 ± 0.1	6.7 ± 0.2	nd
4 Homogentisic acid	nd	346.8 ± 17.2	nd	nd	nd
5 Phenylalanine	348.1 ± 22.8	nd	tr	31.5 ± 1.9	8.8 ± 0.2
6 Unedone*	nd	166.2 ± 9.6	nd	nd	nd
7 3-Phenyl lactic acid	nd	nd	nd	265.6 ± 15.0	nd
8 Lumichrome	nd	nd	0.6 ± 0.0	68.3 ± 2.9	nd
9 Methyl syringate	174.5 ± 9.2	nd	nd	nd	nd
10 <i>t,t</i> -Absciscic acid	nd	109.6 ± 4.2	nd	nd	nd
11 <i>c,t</i> -Absciscic acid	nd	116.9 ± 4.7	nd	nd	nd

All measurements were performed in triplicate; the results are presented as average ± standard deviation out of n = 3 samples; nd: not detected

*Dosed as *c,t*-absciscic acid

acidities for some honeys from various locations around the world [28, 29]. All the results were within the limits allowed by the European Council Directive and the Codex Standard for Honey [17, 30]. The color of honey, in addition to the flavor and aroma, is one of the features that could allow a first classification of the honey by botanical origin. In fact, C*, which expresses the saturation of colors, was very different depending on the type of honey; *Arbutus* honey showed the highest value, whereas *Sulla* and *Asphodelus* honeys had the lowest values. Several studies reported a correlation between the color, phenolic concentration, and antioxidant capacity of unifloral and multifloral honeys. Analysis of Brazilian honeys revealed that the darkest honey samples had the highest antioxidant capacity and phenolic, flavone, and flavonol contents [31]. These data are in accordance with our results. In fact, Sardinian honeys have shown a high correlation between C*, antioxidant activity, and total phenol content. *Arbutus* honey had the highest phenolic content, antioxidant capacity, and color intensity, whereas *Sulla* honey showed the lowest phenolics content and lowest color intensity.

The phenolic and flavonoid composition of honey depends on floral sources and environmental and seasonal factors. These compounds are considered beneficial for human health due to their wide range of biological activities, including antimicrobial, anticarcinogenic, antioxidant, and skin whitening activity [32, 33].

Antityrosinase activity of honey was tested and *Thistle* honey showed the highest percentage of inhibition ($p < 0.05$) (Table 3). The results of HPLC analysis of the honeys (Table 4) revealed that the majority compounds of *Thistle* honey were 3-phenyl lactic acid and lumichrome. In literature, there is no evidence about the antityrosinase activity of lumichrome, whereas the antimelanogenic activity of 3-phenyl lactic acid in cell systems have been

reported in a previous study [34]. Therefore, we performed antityrosinase assay using commercial lumichrome and 3-phenyl lactic acid. These compounds showed no tyrosinase inhibition (Table 3). Thus, antityrosinase activity observed in *Thistle* honey cannot be attributed to lumichrome or 3-phenyl lactic acid. Even if they are present in lower concentration, kojic acid and 5-(hydroxymethyl)-2-furfural could contribute to the antityrosinase effect, as reported in the literature [35]. Furthermore, it is known that many phenol compounds have the ability to inhibit tyrosinase activity. The honeys from *Thistle*, *Arbutus*, and *Eucalyptus* had the highest concentration of phenols and highest anti-XO and antityrosinase activities.

This study revealed that these honeys could be sources of nutrition of antioxidant molecules and may be used as potential components in cosmetic products. Moreover, they could provide novel compounds useful for the treatment of various diseases such as gout or hyperpigmentation.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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