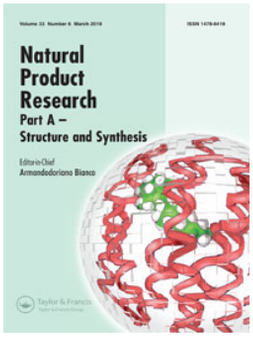

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Antioxidant and antimicrobial properties of *Casteanea sativa Miller* chestnut honey produced on Mount Etna (Sicily)

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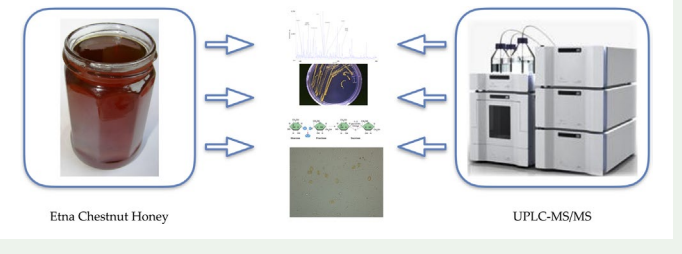
ABSTRACT

The aim of this study was the evaluation of antibacterial and antioxidant properties of Monofloral Etna *Castanea sativa Miller* honeys. *Escherichia coli* ATCC 25,922, *Pseudomonas aeruginosa* ATCC 27,853, *Enterococcus faecalis* ATCC 29,211 and *Staphylococcus aureus* ATCC 29,213 were investigated for their susceptibilities to two different honeys. Antioxidant activity was evaluated by ORAC, NO scavenger assays, FRAP and DPPH. Antioxidant activity and antibacterial properties were compared with chestnut honeys from different geographical areas and with Manuka honey. UPLC-MS/MS was used for major components characterisation.



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Etna chestnut honey; antioxidant; antibacterial; NO scavenger; UPLC-MS/MS; FRAP; DPPH

1. Introduction

Honey made by honey bees is a very complex mixture of sugars and other compounds. Honey composition and concentration of components is a function of the plants which the bees fid on (White and Landis 1980) and of the climate of the geographical area in which is produced (Kaškonienė and Venskutonis 2010; Chua et al. 2013; Schievano et al. 2013; Chua and Adnan 2014).

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Recent scientific evidence clearly demonstrated the antioxidant and the antibacterial efficacy of several honeys (Bogdanov 1997). Antioxidant properties have been related to the presence of flavonoids, phenolic acids and also enzymes (León-Ruiz et al. 2013). Phenolic compounds modulate the activity of a wide range of enzymes and cell receptors, and demonstrate antioxidant capacity against free radical species. Flavonoids are able to contrast the damage caused by free radicals and reactive oxygen species produced during normal oxygen metabolism or induced by exogenous damage. It is well established that oxidation processes involved in various chronic and degenerative diseases may be prevented with regular the intake of chemical constituents of plants (Molan 2009; Cova et al. 2015).

Many different factors seem to contribute to honey antimicrobial properties: i.e. osmolarity, acidity, enzymatic generation of hydrogen peroxide and the presence of various non-peroxide compounds derived from pollen or nectar flower (Molan 1992; Al-Waili et al. 2011). The honey pH is generally low enough to slow or prevent the growth of many species of bacteria. Glucose oxidase, which is activated by the presence of water, generates hydrogen peroxide which is usually the main antibacterial factor of honey itself (Fidaleo et al. 2011). Moreover, some honeys from specific floral sources contain various antibacterial substances such as methylglyoxal (MGO) (Mavric et al. 2008). One of the most widely used medical honeys in the world is Manuka honey. Its effectiveness is documented in the treatment of infections caused by antibiotic-susceptible and resistant pathogens (Cooper et al. 2010; Mannina et al. 2016).

Relevant studies conducted by Perna, Simonetti et al. (2013) clearly showed that *Casteanea Sativa Miller* chestnut honeys produced in southern Italy have relevant antioxidant properties, although non clearly correlated with polyphenols concentrations (as well as with flavonoids contents), when compared with other honey varieties, such as multiflorals or citrus. While several chestnut honeys of different geographical areas have been evaluated and a large variability in the chemical contents has been observed, reduced information about Sicilian chestnut honey is available.

The present study reports a qualitative/quantitative detection of some phenolic, flavonoid and sugar components, performed using UPLC-MS/MS technique, of two monofloral chestnut honeys obtained by two different producers located in different parts of the Etna Volcano (Sicily), in the area of 'Zafferana Etnea', a town in the protected National Park of Etna, where *Castanea sativa Miller* is the dominant tree (Miele 2008). Antioxidant and antimicrobial properties were evaluated by ORAC assay, NO Scavanger test, FRAP and DPPH. *Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis* and *Staphylococcus aureus* were used to evaluate antibacterial activity of the two Samples. Total Flavonoid and Phenolic Content were evaluated.

2. Results and discussion

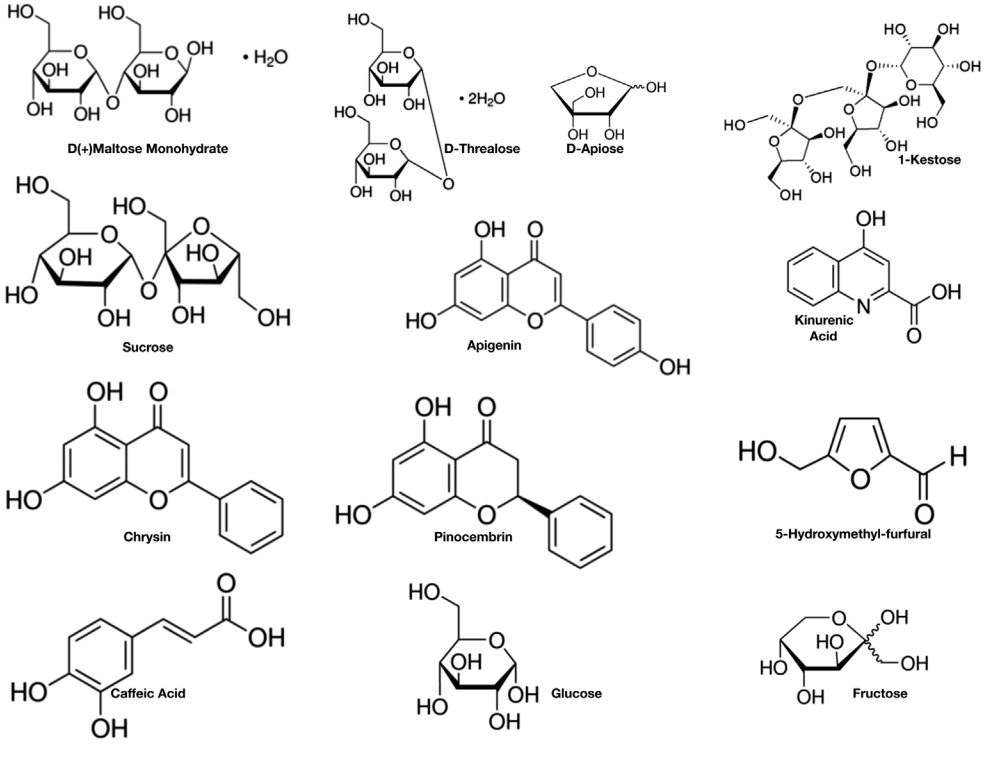
2.1. Chemical composition

We searched for and detected the concentration of the following flavonoids (apigenin, chrisin, pinocembrin), sugars (sucrose, fructose, glucose, D-apiose, threalose, maltose, 1-kestose), cinnamic acid derivatives (caffeic acid) and kynurenic acid. Such compounds were selected for their recognised antioxidant and antibacterial properties and for being present in most of the characterised honeys and were used as standards.

Two chestnut honey samples were analysed by UPLC/MS-MS (Mavric et al. 2008; Rahman et al. 2008; Oelschlaegel et al. 2012; Beitlich et al. 2014). Total ion chromatograms (TICs), using a C18 Phenomenex Luna, 5 µm, 15 × 0.1 cm column, are reported in Figure S1. Optimal separation for glucose and fructose was achieved with a different chromatographic column (Phenomenex Luna 3 µm NH2 100A, LC Column 100 × 2 mm) (Figures S2–S4). All standard compounds (Scheme 1) were identified and the results of quantitative analysis reported in Table 1A. The detected amounts of apigenin, crisin, pinocembrin and 5-hydroxymethyl-furfural resulted lower than LOQ in both samples. *Castanea Sativa Miller* chemical compositions of a southern and central Europe honeys (Italy, Spain, Poland and Slovenia) are reported and used as a reference (Bertoncelij et al. 2011; Perna, Intaglietta et al. 2013; Rodrìguez-Flores et al. 2016). For its well-known therapeutic properties, a typical Manuka honey composition is also inserted in Table 1A (Oelschlaegel et al. 2012; Beitlich et al. 2014). The two honeys (Sample 1 and Sample 2) showed only slight differences, possibly due to the different location of production both for the exposition and the level above the sea.

Sample 1 and Sample 2 showed a concentration in carbohydrates spanning from about 64% to 70%, in line with most of the chestnut honeys reported in the literature (Perna, Intaglietta et al. 2013) and only slightly higher than that of Manuka honey (about 63%). Differences have been detected in the content of D-maltose and D-apiose, with Sample 1 showing higher concentrations, 2.44 vs. 0.70%.

With regard to flavonoids, we observed that kynurenic acid concentration is about four times higher in Sample 1 with respect to Sample 2. Opposite, caffeic acid is two times higher



Scheme 1. Chemical Structure of quantified compounds.

Table 1A. Composition (%, g/100g[a] ± standard deviation), R^2, LOD and LOQ of Etna chestnut honey samples in comparison with literature data.

1A. Component	Sample 1	Sample 2	R^2	LOD	LOQ	Chestnut[b]	Manuka[c,d]
D(+)Maltose	2.44 ± 0.04	0.69 ± 0.01	0.9973	1947	6490	1.1 ± 0.08	0.53
D-Threalose	1.03 ± 0.02	0.14 ± 0.01	0.9980	1290	4330	0.1 ± 0.1	0.09
D-Apiose	3.35 ± 0.04	3.65 ± 0.03	0.9998	645	2150	–	–
1-Kestose	10.90 ± 0.06	5.70 ± 0.02	0.9969	3750	12500	–	–
Sucrose	1.53 ± 0.05	0.14 ± 0.01	0.9984	1458	4860	0.2 ± 0.3	1.02
Apigenin	<LOQ	<LOQ	0.9998	708	2360	4–55µg/100g	0.61
Kynurenic Acid	1.99 ± 0.01	0.46 ± 0.02	0.9994	936	3120	129–601ng/g	–
Chrysin	<LOQ	<LOQ	0.9995	1767	5890	24–41µg/100g	0.13
Pinocembrin	<LOQ	<LOQ	0.9983	963	3210	10–27µg/100g	0.17
5-HMF	<LOQ	<LOQ	0.9995	675	2250	–	2.1
Caffeic Acid	0.23 ± 0.01	0.44 ± 0.02	0.9993	363	1210	9.97 ± 9.22mg/kg	4.83
Glucose	21.00 ± 0.02	20.00 ± 0.12	0.9999	609	2030	25.9 ± 2.7	19.35
D(-)-Fructose	33.00 ± 0.24	35.00 ± 0.20	0.9998	603	2010	37.2 ± 2.6	38.44

[a]Unless otherwise indicated.
[b][Rodrìguez-Flores et al. (2016), Turski et al. (2016), Perna, Intaglietta et al. (2013), Perna, Simonetti et al. (2013), Oelschlaegel et al. (2012)]; LOD and LOQ were calculated from Signal to Noise measurements (ABSciex Analyst software).
[c]Data were expressed as mean ± SD of three determinations, $p < 0.05$ significantly different versus untreated control.
[d][Persano Oddo L. et al. (2000), Persano Oddo L. et al. (2004), Venugopal and Devarajan (2011), Grego E. et al. (2016), Perna, Intaglietta et al. (2013)].

in Sample 2. The very low concentration of HMF indicates a good state of preservation of both samples. This two honeys have a fructose and glucose concentration rather lower than that of other chestnut honeys, comparable to that of Manuka for glucose and lower for fructose (Table 1A).

The low content of sucrose in Sample 2 (0.14%) is comparable with that of other *Castanea Sativa* honeys but lower than that of Manuka. In sample 1 is much higher (1.53%) with respect to other chestnut honeys while comparable with Manuka.

2.2. Determination of total phenolic and flavonoid contents

The Total phenolic content of two samples was evaluated by Folin–Ciocalteau method modified by Beretta et al. (2005) while flavonoid content were determined by Dwod method implemented by Arvouet-Grand et al. The results obtained, shown in Table 1B, are in line with Italian honeys data reported in the literature (Perna, Simonetti et al. 2013).

2.3. Antioxidant activity

The antioxidant properties of the two samples were evaluated using, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and oxygen radical absorbance capacity (ORAC) assays, FRAP (ferric reducting/antioxidant power and Nitric oxide (NO)-scavenging capacities. Table 1B show the results of the two honey samples (Sample 1 and 2) (Panico et al. 2007; Roy et al. 2010). It can be seen that the antioxidant activity of both sample is superimposable. Phenolic acids and flavonoids, probably present in high concentration, strongly influences the antioxidant capacity.

Noteworthy, ORAC and NO scavenger assay values obtained for Sample 1 and Sample 2, (21.43 and 20.89 µM TE/g fw, 46.89 and 44.89%, respectively) if compared with the values

Table 1B. ORAC, NO scavenger, DPPH, FRAP, TPC, TFC and MIC values of Gram-positive and Gram-negative strains tested.

1B. Sample	TPC (mgGAE/100g)	TFC (mgQE/100g)	ORAC Units (μmol/μg)[a]	NO scav. (%)[b]	DPPH (%)[c]	FRAP[μM of (FeII) red.by 10% sol]	*S. aureus* ATCC29213	*E. faecalis* ATCC29211	*P. aeruginosa* ATCC27853	*E. coli* ATCC25922
Sample 1	14.70 ± 3.23	14.80 ± 7.58	21.43	44.89 ± 0.21[e]	75.63 ± 8.36	793.20 ± 85.12	10	5	12.5	3.1
Sample 2	16.29 ± 1.21	15.34 ± 6.03	20.89	46.89 ± 0.52[e]	77.89 ± 6.83	850.89 ± 75.21	10	10	25	25
Manuka[d]	56 – 169.67	0.25 – 3.34	21.52	–	–	–	20	10	25	25
Chestnut[f]	15.05 ± 4.14	14.16 ± 7.53	–	–	78.89 ± 9.87	894.04 ± 315.32	20	10	25	12.5

[a]Trolox = 1.00 ± 0.11 ORAC Units was used as a reference compound.
[b]Curcumin = 42 ± 0.28% was used as a reference compound.
[c]Ascorbic acid = 56.00 ± 1.28% was used as a reference compound.
[d]Data were expressed as mean ± SD of three determinations, $p < 0.05$ significantly different versus untreated control (Wang 2011).
[e][Persano Oddo L. et al. (2000), Persano Oddo L. et al. (2004), Venugopal and Devarajan (2011), Grego E. et al. (2016), Perna, Simonetti et al. (2013)].

obtained for other honeys (Cavazza et al. 2013). Manuka honey (Mavric et al. 2008; Cooper et al. 2010) has a comparable total antioxidant activity.

2.4. Antibacterial activities

The minimum inhibitory concentrations (MICs) were shown in Table 1B. The results of susceptibility test procedure (Rodrìguez-Flores et al. 2016; Turski et al. 2016; Junsei et al. 2009) for both honeys were showed according to the bacterial strain investigated. The lowest MIC values (% *v*/*v*) (Vallianou et al. 2014; Nijveldt et al. 2001) were obtained for *S. aureus* ATCC 29213. Sample 2 showed lower or similar values with respect to those showed by Sample 1. They showed also a good antibacterial activity against *E. coli, P. aeruginosa* and *E. faecalis*. While we can only speculate on the relevance of the carbohydrates concentration on antibacterial activity, we must note that neither caffeic acid nor kynurenic acid have a concentration high enough to justify the observed significative antibacterial activities.

The sensory analysis showed the typical sensory property of chestnut honey and the qualitative melissopalynological analysis identified a percentage of chestnut pollen, respectively, of 96 and 98%. In both cases, of at least 90% required by the 'descriptive sheets of italian chestnut unifloral honey', is always satisfied (Louveaux et al. 1978; Persano Oddo et al. 2000; Oddo et al. 2004).

3. Experimental

The experimental section is available online in supplementary material.

4. Conclusions

Two chestnut honeys from different producers of the Etna region have been analysed by mass spectroscopy and the major components were separated and quantitatively estimated using UPLC-MS/MS. These products showed interesting antioxidant, NO Scavengers, DPPH, FRAP and antimicrobial properties.

They also possess a high antioxidant capacity against free radical species, including reactive oxygen species and peroxynitrite radical. DPPH and FRAP values confirm a quite good antioxidant activity of Etna chestnut honey, attributable both to the high sugars concentrations and the total phenolic and flavonoid contents. A direct correlations of our data with the data reported in the literature is very difficult because conditions are different in different publications.

Noteworthy, MIC values obtained for these two products are similar or lower than those reported in the literature, using the same strains, for several different honeys (León-Ruiz et al. 2013; Cooper et al. 2010; Wallace et al. 2010), thus confirming the good antibacterial properties of the Etna honeys. Further studies are necessary for a better characterisation of the Etna chestnut honey, especially taking in account that Etna chestnut honey production is strongly dependent of the seasonal climate changes.

Disclosure statement

No potential conflict of interest was reported by the authors.

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