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Quorum sensing inhibitory and antimicrobial activities of honeys and the relationship with individual phenolics

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ABSTRACT

Quorum sensing (QS) inhibitory activity of 29 unifloral honeys was evaluated using the bacterial model *Cromobacterium violaceum*. The tested honeys were able to inhibit the production of acyl-homoserine lactones (AHLs) produced by *C. violaceum* at 0.1 g/ml. However, chestnut and linden honey samples showed the highest inhibitory activity, while orange and rosemary were less effective in inhibiting QS. When honey samples from the same floral origin obtained from different geographical regions were compared, they showed similar QS inhibitory activity. Thus, one of the factors which influence the inhibitory activity could be derived from the floral origin, independently of the geographic location. It was observed that unifloral honey samples showed "non-peroxide" anti-QS activity, which was not linearly correlated with total and individual phenolic compounds. The obtained results showed that the preservative properties of honey could be due to both the antimicrobial properties and the QS inhibitory capacity.

1. Introduction

The antimicrobial properties attributed to honey have been related to both the physical properties of osmosis and the antibacterial properties of hydrogen peroxide and non-peroxide compounds (Taormina, Niemira, & Beuchat, 2001; Weston, 2000). Non-peroxide antibacterial activity of honey has been associated with high sugar concentration, antioxidant and proteinaceous compounds as well as other unidentified components (Mundo, Padilla-Zakour, & Worobo, 2004; Lee, Churey, & Worobo, 2008a, 2008b). Previous studies have linked the antimicrobial activity of honey and propolis to flavonoids (including flavones, flavonols, flavanones and dihydroflavonols) and other phenolics (mainly substituted cinnamic acids and their esters) (Aljadi & Yusoff, 2003; Küçük et al., 2007; Popova et al., 2007).

So far, investigations have been restricted to whether or not honey could kill or inhibit growth of bacteria. However, as previously described for other plant extracts, the antimicrobial properties of honey might only represent one face of its anti-infective potential (Adonizio, Downum, Bennett, & Mathee, 2006). The capacity of honey to inhibit the interaction between the bacterium and the food matrix may also be of interest for avoiding food spoilage. Some authors (Gram et al., 2002) defined the spoilage potential of a microorganism as the ability of a pure culture to grow and produce the metabolites associated with the spoilage of a particular product. Thus, many bacteria are able to regulate the phenotypic characteristics as a function of cell density under the control of chemical signal molecules (Gram et al., 2002). These auto-inducer molecules have been identified as oligopeptides in Gram-positive bacteria and acylated homoserine lactones (AHLs) in Gram-negative bacteria (Novick et al., 1993). The ability of bacteria to sense and respond to population density is termed "cell-to-cell communication" or "quorum sensing" (QS). In fact, many bacterial physiological functions such as luminiscence, virulence, motility, sporulation, biofilm formation, etc., are regulated by QS systems (Gram et al., 2002; Winson et al., 1995). For this reason, Rasch et al. (2005) hypothesised that if QS systems regulate bacterial mechanisms in food spoilage then, inhibition of the communication underlying the QS systems could be a good strategy to reduce or prevent the spoilage reactions.

Knowing the importance of QS during bacterial pathogenesis and spoilage, research has focused on inhibiting QS using bacterial biosensors and indicators (Choo, Rukayadi, & Hwang, 2006). Among all the possibilities to inhibit the QS activity, the use of anti-quorum sensing (anti-QS) compounds could be of great interest to avoid bacterial infections (Adonizio et al., 2006; Rice, Mcdougald, Kumar, & Kjelleberg, 2005). Such "antipathogenic" compounds, in contrast to antibacterial compounds, do not kill bacteria or stop their growth and are assumed not to lead to the development of resistant strains (Otto, 2004). Except for the halogenated furanones from the red alga *Delisea pulchra*, most of the identified anti-QS compounds of non bacterial origin have come from plant origin (Bauer & Teplitski, 2001; Choo et al., 2006; Teplitski, Robinson, & Bauer, 2000). However, little information has

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been published regarding the QS activity of honey (Rasmussen et al., 2005).

Screening for AHL production from bacterial strains has typically relied on bacteriological monitoring systems, which consist of a phenotypic response, activated through an AHL-receptor protein (Ravn, Christensen, Molin, Givskov, & Gram, 2001). This is the case of *Cromobacterium violaceum*, a Gram-negative water and soil bacterium highly sensitive to most short-chained unsubstituted AHLs (*N*-hexanoyl homoserine lactone, HHL), whose phenotypic response is the production of violacein, a water-insoluble purple pigment with antibacterial activity (McClean et al., 1997; Steindler & Venturi, 2007).

The purpose of this study was to investigate the anti-QS and antimicrobial properties of different unifloral honeys against *C. violaceum* to determine their possible use as novel QS inhibitors (QSI). Additionally, the relationship between the individual phenolic content of different unifloral honeys and their antimicrobial and anti-QS activities was also evaluated.

2. Materials and methods

2.1. Honey samples

Twenty-nine honey samples from 14 different floral origins and 15 different geographical locations were provided and certified by the Agricultural Research Council (CRA-API, Bologna, Italy) and Institute of Molecular Biology (Slovak Academy of Sciences, Bratislava, Slovakia), which received raw honey samples from different beekeepers in Italy and Slovakia, respectively. A Spanish commercial honey (Quexigal, Avila, Spain) was also included in this study. Table 1 lists the honey samples by floral and geographical origins. The botanical origins were certified by the traditional analysis method: sensorial and pollen analysis and physicochemical analyses (Truchado, Ferreres, Bortolotti, Sabbatini, & Tomás-Barberán, 2008).

During the experiments, samples were kept at 5 °C in the dark in airtight containers for less than 5 months until analysis. Prior to testing, a 66.6% (w/v) working solution was prepared diluting 2 g of each honey sample with 1 ml of sterile distilled water.

Table 1
Floral and geographical origins of honey samples.

Floral origin	Acacia	*Robinia pseudacacia*	Bologna (Italy)
	Canola	*Brassica napus*	Bologna (Italy)
	Cherry blossom	*Prunus avium*	Frossaco (Italy)
	Chestnut	*Castanea sativa*	Bologna (Italy)
	Eucalyptus	*Eucalyptus spp*	Commercial (Spain)
	Lavander	*Lavandula* ssp.	Puimoisson (France)
	Linden	*Tilia argentea*	Sebecheleby (Slovakia)
	Lucerne	*Mendicago sativa*	Bologna (Italy)
	Orange	*Citrus spp.*	Tornareccio-CH (Italy)
	Rape	*Rapessed (Brassica campestris)*	Sebechleby (Slovakia)
	Rosemary	*Rosmarinus officinalis*	Manfredonia-FG (Italy)
	Sunflower	*Helianthus annus*	Žemberovce (Slovakia)
	Taraxacum	*Taraxacum officinalis*	Bologna (Italy)
	Tilia	*Tilia* ssp.	Minerbio-BO (Italy)
Geographical origin	Chestnut – 1000	*Castanea sativa*	Bologna (Italy)
	Chestnut – 1390	*Castanea sativa*	Bologna (Italy)
	Chestnut – 1453	*Castanea sativa*	Siena (Italy)
	Chestnut – 1454	*Castanea sativa*	Arezzo (Italy)
	Chestnut – 1455	*Castanea sativa*	Pistoia (Italy)
	Chestnut – 1456	*Castanea sativa*	Arezzo (Italy)
	Chestnut – 1476	*Castanea sativa*	Bologna (Italy)
	Chestnut – 1757	*Castanea sativa*	Torino (Italy)
	Linden – SK10	*Tilia argentea*	Banská Štiavnica (Slovakia)
	Linden – SK14	*Tilia argentea*	Bratislava (Slovakia)
	Linden – SK21	*Tilia argentea*	Sebecheleby (Slovakia)
	Orange – 456	*Citrus spp.*	Tornareccio-CH (Italy)
	Orange – 826	*Citrus spp.*	Manfredonia-FG (Italy)
	Rosemary – 374	*Rosmarinus officinalis*	Tornareccio-CH (Italy)
	Rosemary – 827	*Rosmarinus officinalis*	Manfredonia-FG (Italy)
	Tilia – 100	*Tilia* ssp.	Bologna (Italy)
	Tilia – 614	*Tilia* ssp.	Bologna (Italy)
	Tilia – 892	*Tilia* ssp.	Minerbio-BO (Italy)
	Tilia – 1391	*Tilia* ssp.	Torino (Italy)
	Tilia – 1756	*Tilia* ssp.	Bologna (Italy)

2.2. Strains and culture conditions

C. violaceum wild-type strain CECT 494, obtained from the Spanish Type Culture Collection (Valencia, Spain) was used to determine QS inhibitory and antimicrobial activities. This wild type of strain produces and responds to the cognate auto-inducer molecules, acylated homoserine lactone (AHL) such as C6-AHL and C4-AHL, which makes this strain excellent for screening (Adonizio et al., 2006; McClean, Pierson, & Fuqua, 2004). This bacteriological monitor system generates a phenotypic response by the production of a purple pigment (violacein) when induced by the presence of AHLs. The bacterium was routinely grown aerobically with shaking in Luria-Bertani broth (02-385, LB broth acc. to MILLER, Scharlau Chemie, S.A. Barcelona, Spain) supplemented with 0.5% of NaCl and incubated at 30 °C for 24 h. Acylated homoserine lactone plate assays were performed using LB agar (LB broth + 07-004, bacteriological agar, Scharlau Chemie, S.A.), with a final agar concentration of 1.2%. Antibacterial activity of honey was carried out using LB agar (LB broth + bacteriological agar).

2.3. Inhibition of AHL production

The inhibitory activity of honey samples was assayed by the agar-well diffusion test. Plates were made by adding approximately 10^5 cfu/ml of an overnight culture of *C. violaceum* to the LB agar (1.2%). Wells were filled with 20 μl of different concentrations (0.1, 0.2, 0.3 and 0.4 g/ml) of each honey. Plates were incubated for 18–24 h at 30 °C prior to the determination of inhibition zone sizes by contrast camera imaging (Synoptics, Cambridge, United Kingdom). The experiment was carried out twice and there were three replicates per honey sample.

Flask-incubation assays were carried out to quantify the inhibitory activity of honey samples. The bacterium was incubated for 18 h and inoculated to $OD_{600\,nm}$ = 0.1 in Erlenmeyer flasks containing LB broth (Scharlau Chemie, S.A.) and LB supplemented with different honey samples at different concentrations (0.1, 0.2, 0.3 and 0.4 g/ml). The flasks were incubated at 30 °C in a shaking incubator. The quantification of the violacein production was carried out following the protocol described by Choo et al. (2006), where 1 ml culture from each flask was centrifuged at 13,000 rpm for 10 min to precipitate the insoluble violacein. Then, the culture supernatant was discarded and the pellet was solubilised in 1 ml of DMSO, vortexed until the violacein was homogenised, and centrifuged at 13,000 rpm for 10 min to remove the cells. Absorbance of each violacein-containing supernatant was measured at 585 nm in a UV–vis spectrophotometer (Hewlet Packard 8453). This experiment was carried out four times and there were three replicates per honey sample.

2.4. Antibacterial activity

The inhibition zones produced on lawns of the indicator strain and the inhibition of violacein production could be the result of

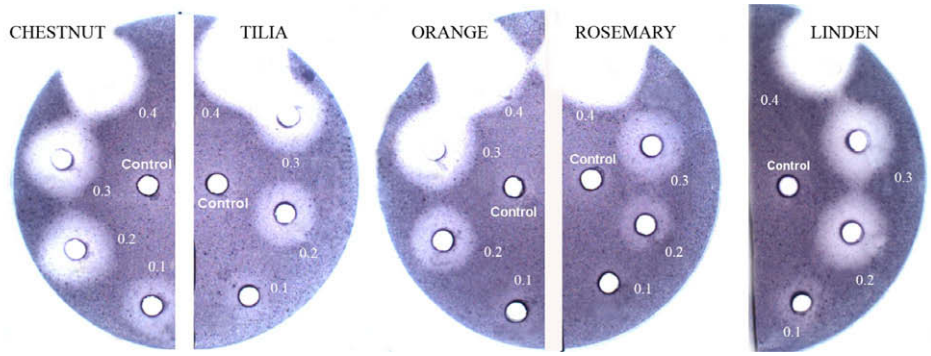

Fig. 1. Inhibition of violacein production of chestnut, tilia, orange, rosemary and linden at different concentrations (0.1, 0.2, 0.3 and 0.4 g/ml) in the agar-well diffusion test.

Table 2
Inhibition of violacein production by 15 unifloral honeys at different concentrations in the agar-well diffusion test expressed as the diameter (mm) of the inhibition zone. The results are the mean of three replicates ± standard deviation.

Honeys	Honey Concentration (g/ml)			
	0.1	0.2	0.3	0.4
Acacia	0.0 ± 0.0	28.9 ± 3.9	31.3 ± 4.7	46.9 ± 0.7
Cherry blossom	4.7 ± 4.1	28.0 ± 1.4	34.1 ± 2.1	40.6 ± 1.4
Chestnut	9.0 ± 0.6	25.0 ± 4.4	34.5 ± 2.0	38.0 ± 0.8
Canola	0.0 ± 0.0	14.2 ± 7.7	27.8 ± 3.2	46.7 ± 7.7
Eucalyptus	0.0 ± 0.0	26.1 ± 0.4	32.7 ± 2.8	42.7 ± 6.2
Lavander	2.1 ± 3.6	22.2 ± 1.5	32.7 ± 2.2	43.6 ± 1.1
Linden	5.3 ± 2.9	18.9 ± 6.1	29.9 ± 2.1	43.6 ± 0.8
Lucerne	0.0 ± 0.0	23.6 ± 0.4	34.1 ± 0.4	39.4 ± 1.8
Orange	0.0 ± 0.0	28.2 ± 1.8	36.2 ± 1.5	44.8 ± 1.2
Rape	8.9 ± 0.4	26.4 ± 0.8	36.9 ± 0.8	37.6 ± 0.8
Rhododendron	8.4 ± 1.2	30.6 ± 1.8	34.8 ± 2.0	40.1 ± 0.4
Rosemary	0.0 ± 0.0	11.4 ± 2.5	26.6 ± 2.5	53.0 ± 1.5
Sunflower	0.0 ± 0.0	21.2 ± 2.5	35.2 ± 0.4	36.6 ± 1.1
Taraxacum	0.0 ± 0.0	27.5 ± 2.7	35.7 ± 1.4	39.2 ± 3.6
Tilia	2.9 ± 1.9	16.3 ± 2.1	28.0 ± 0.7	50.6 ± 0.8

Table 3
Violacein production of 15 unifloral honeys at different concentrations in the flask-incubation assay. Values represent the absorbance at 585 nm of three replicates ± standard deviation.

Honeys	Honey concentration (g/ml)			
	0.1	0.2	0.3	0.4
Control	0.94 ± 0.10	0.94 ± 0.10	0.94 ± 0.10	0.94 ± 0.10
Acacia	0.38 ± 0.02	0.29 ± 0.05	0.06 ± 0.05	0.00 ± 0.00
Cherry blossom	0.37 ± 0.02	0.24 ± 0.06	0.04 ± 0.00	0.00 ± 0.00
Chestnut	0.19 ± 0.05	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Canola	0.39 ± 0.01	0.28 ± 0.00	0.14 ± 0.03	0.00 ± 0.00
Eucalyptus	0.28 ± 0.03	0.14 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
Lavander	0.35 ± 0.02	0.30 ± 0.06	0.03 ± 0.01	0.00 ± 0.00
Linden	0.21 ± 0.04	0.02 ± 0.00	0.02 ± 0.02	0.00 ± 0.00
Lucerne	0.37 ± 0.03	0.23 ± 0.12	0.06 ± 0.04	0.00 ± 0.00
Orange	0.43 ± 0.03	0.34 ± 0.03	0.22 ± 0.14	0.16 ± 0.06
Rape	0.39 ± 0.04	0.29 ± 0.04	0.00 ± 0.00	0.00 ± 0.00
Rhododendron	0.34 ± 0.02	0.25 ± 0.13	0.04 ± 0.02	0.00 ± 0.00
Rosemary	0.40 ± 0.05	0.36 ± 0.05	0.23 ± 0.14	0.00 ± 0.00
Sunflower	0.35 ± 0.00	0.07 ± 0.05	0.01 ± 0.00	0.00 ± 0.00
Taraxacum	0.37 ± 0.01	0.25 ± 0.02	0.04 ± 0.04	0.00 ± 0.00
Tilia	0.32 ± 0.02	0.12 ± 0.06	0.02 ± 0.07	0.00 ± 0.00

either (i) quenching of QS signals or (ii) inhibition of cell growth, although growth inhibition would produce a clear halo versus a turbid halo for the QS inhibition (Adonizio et al., 2006). Antimicrobial activity of chestnut, linden, tilia, orange and rosemary honeys against *C. violaceum* was evaluated. Ten millilitres of LB broth was

Table 4
Inhibition of violacein production of unifloral honeys from different geographical regions at different concentrations in the flask-incubation assay. Values represent the percentage of absorbance at 585 nm of three replicates ± standard deviation. (See Table 1 for sample identification).

Honeys	Honey concentration (g/ml)	
	0.1	0.2
Control	0.99 ± 0.14	0.98 ± 0.08
Chestnut – 1000	0.19 ± 0.05	0.00 ± 0.00
Chestnut – 1390	0.03 ± 0.03	0.00 ± 0.00
Chestnut – 1476	0.01 ± 0.01	0.00 ± 0.00
Chestnut – 1453	0.03 ± 0.01	0.00 ± 0.00
Chestnut – 1454	0.02 ± 0.02	0.00 ± 0.00
Chestnut – 1455	0.16 ± 0.06	0.00 ± 0.00
Chestnut –1456	0.05 ± 0.03	0.00 ± 0.00
Chestnut – 1757	0.11 ± 0.06	0.00 ± 0.00
Linden – SK10	0.31 ± 0.02	0.00 ± 0.00
Linden – SK14	0.30 ± 0.02	0.00 ± 0.00
Linden – SK21	0.38 ± 0.01	0.00 ± 0.00
Orange – 456	0.47 ± 0.02	0.20 ± 0.02
Orange – 826	0.50 ± 0.02	0.20 ± 0.02
Rosemary – 374	0.42 ± 0.09	0.28 ± 0.03
Rosemary – 827	0.46 ± 0.02	0.34 ± 0.04
Tilia – 100	0.30 ± 0.03	0.15 ± 0.06
Tilia – 614	0.21 ± 0.06	0.06 ± 0.03
Tilia – 892	0.31 ± 0.08	0.00 ± 0.00
Tilia – 1391	0.25 ± 0.12	0.01 ± 0.00
Tilia – 1756	0.25 ± 0.11	0.01 ± 0.00

Table 5
Growth inhibition of *Cromobacterium violaceum* by unifloral honeys at different concentrations with and without the addition of catalase. Values represent cfu $\log_{10}(N_0/N)$ of three replicates ± standard deviation.

Honeys	Honey concentration (g/ml)	Without catalase	With catalase
Chestnut	0.2	0.2 ± 0.2	0.3 ± 0.1
	0.3	1.0 ± 0.0	0.4 ± 0.1
	0.4	1.5 ± 0.1	0.9 ± 0.1
Linden	0.2	0.5 ± 0.5	0.3 ± 0.1
	0.3	3.2 ± 0.5	0.4 ± 0.1
	0.4	8.5 ± 0.1	8.5 ± 0.1
Orange	0.2	0.1 ± 0.0	0.2 ± 0.2
	0.3	0.3 ± 0.1	0.2 ± 0.1
	0.4	1.1 ± 0.1	0.9 ± 0.1
Rosemary	0.2	0.1 ± 0.1	0.1 ± 0.1
	0.3	1.0 ± 0.6	0.0 ± 0.1
	0.4	1.8 ± 0.0	0.9 ± 0.1
Tilia	0.2	0.1 ± 0.0	0.2 ± 0.1
	0.3	1.3 ± 0.6	0.1 ± 0.1
	0.4	1.0 ± 0.0	0.9 ± 0.1

inoculated with 10 μl of a working culture of *C. violaceum* and the amount of honey needed for each selected concentrations (0.1, 0.2,

0.3 and 0.4 g/ml). Inoculated honey solutions were mixed followed by incubation at 30 °C for 24 h. Inoculated honey samples (1 ml) were diluted in 1% sterile buffered peptone water (BPW) (AES Laboratoire, Combourg, France) (1:10 dilution). Appropriate dilutions were then spread onto LB agar (1%) (Scharlau Chemie, S.A.). Colonies were counted after 24 h incubation at 30 °C. Microbial counts were expressed as log cfu per ml.

2.5. Non-peroxide anti-QS and antibacterial activities

Selected honey samples (chestnut, linden, tilia, orange and rosemary) were diluted at 66.6%, with 0.1 M potassium phosphate buffer (pH 7) treated and untreated with catalase from bovine liver (13,500 units mg^{-1}, Sigma–Aldrich, St. Louis, MO) at ratio of 2700 units mg $solid^{-1}$ per 1 g of honey. The anti-QS and antimicrobial activities of the selected honeys treated and untreated with catalase were carried out as above described.

2.6. Phenolic composition

For the extraction of phenolic compounds, honey samples (10 g) were dissolved with five parts of water (adjusted to pH 2 with HCl) until completely fluid. This solution was flushed through an activated Sep-Pak C_{18} cartridge (Waters, Milford, MA), previously activated with methanol (10 ml) followed by water (10 ml). Then, the phenolic compounds were eluted with methanol (2 ml). The methanol extracts were filtered through a 0.45 μm filter (Millex-HV13, Millipore Corp., USA) and stored at −20 °C. Samples of 50 μl were analysed by HPLC (Agilent 1100 Series, Agilent Technologies, Waldbronn, Germany) equipped with a binary pump (G1312 A),

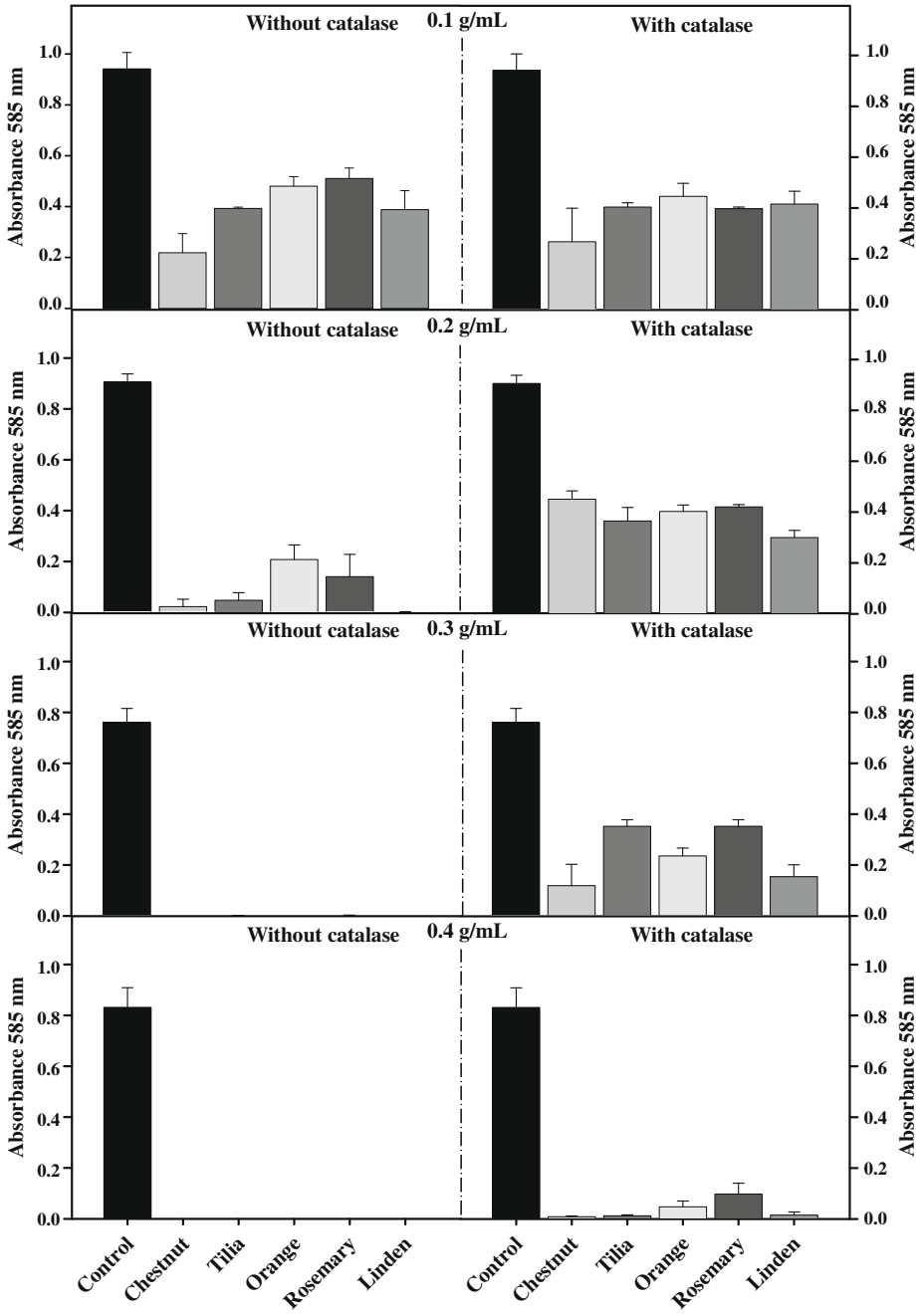

Fig. 2. Inhibition of violacein production of chestnut, tilia, orange, rosemary and linden at different concentration (0.1, 0.2, 0.3 and 0.4 g/ml) with and without the addition of catalase. Vertical bars represent means of three replicates ± standard deviation.

a degasser (G1322 A), a photodiode array diode array detector (G1315 B) and a mass detector in series (Agilent Technologies). The samples were injected by a model L-7200 autosampler. The mass detector was an ion trap spectrometer (G2445A) equipped with an electrospray ionisation (ESI) system. The nebuliser gas was nitrogen, the pressure and the flow rate of the dryer gas were set at 65 psi and 11 l min^{-1}, respectively. The full scan mass covered the range from m/z 100–1000. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycles from 0.3 up to 2 V. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. Mass spectrometry data were acquired in the negative mode. Chromatographic separations were carried out on a C_{18} LiChroCART column (Merck, Darmstadt, Germany) (RP-18, 250 × 4 mm; 5 μm particle size) protected with a 4 × 4 mm C_{18} LiChroCART guard column, with 1% formic acid (A) and methanol (B) as solvents (99.9%, HPLC grade; Merck, Darmstadt, Germany). Elution was performed with a gradient starting with 10% B in A to reach 30% B in A at 20 min, 45% B in A at 30 min, 60% B in A at 40 min, 70% B in A at 45 min, 90% B in A at 60 min and then became isocratic for 5 min. The flow rate was of 1 ml min^{-1} and chromatograms were recorded at 290, 320, 340 and 360 nm.

The phenolic compounds were identified according to their UV spectra, molecular weights, retention time and their MS–MS fragments and wherever possible, with commercially available standards. Hydroxycinnamic acid derivatives were quantified as chlorogenic acid at 320 nm, flavonols as quercetin at 340 nm, flavones as chrysin at 340 nm and flavanones as hesperetin at 290 nm. All these markers were purchased from Sigma (St. Louis MO), except chrysin from Carl Roth OGH (Karlsruhe, Germany). The results were expressed as milligrams per 100 g of honey.

3. Results and discussion

3.1. Inhibition of AHL production by honey

Fifteen unifloral honeys of different floral and geographical origins (Table 1) exerted different inhibitory activity in the agar-well diffusion test, which was concentration-dependent. The inhibition zones caused by the addition of different concentrations (0.1, 0.2, 0.3 and 0.4 g/ml) of a selection of honeys (chestnut, linden, orange, rosemary and tilia) is shown in Fig. 1. All honey samples showed inhibitory activity at 0.2 g/ml (Table 2). Similar inhibition zones were observed when higher concentrations were applied (0.3 and 0.4 g/ml). The flask-incubation assay was carried out to quantify the inhibition of AHL production by the 15 honey samples (Blosser & Gray, 2000). Compared to the control, all unifloral honeys showed a significant drop in violacein production, even at the lowest tested concentration (0.1 g/ml) (Table 3). Chestnut and linden honeys showed the highest anti-QS activity while orange and rosemary showed the lowest inhibitory activity (Table 3). When honey concentration increased, the observed differences were even more marked. In fact, *C. violaceum* was only able to produce violacein in the presence of orange honey at 0.4 g/ml.

Based on these results, 5 unifloral honeys from different geographical origins were selected (Table 1). Chestnut and linden honeys were selected as the best QSI while orange and rosemary honeys were selected as having the lowest anti-QS activity. Tilia honey was included in the assay due to its close relationship with the floral origin of linden honey. Only slight differences in the anti-QS activity of honeys from different geographical origins were observed (Table 4). The results obtained confirmed that chestnut honeys showed the highest anti-QS activity independently of the

Table 6
Flavonoids and hydroxycinnamic acids from unifloral honeys.[a]

Peaks	Honeys Flavonoids															
	[M–H]$^-$ m/z	Acacia	Canola	Cherry blossom	Chestnut	Eucalyptus	Lavander	Linden	Lucerne	Orange	Rape	Rhododendron	Rosemary	Sunflower	Taraxacum	Tilia
1	285	0.15	0.50	0.26	–	–	–	–	0.62	0.13	0.26	–	–	–	0.47	0.53
2	271	0.34	0.61	0.55	–	0.28	0.40	0.44	0.96	0.32	0.77	0.33	0.15	0.73	0.77	1.05
3	255	0.35	0.79	0.33	0.15	0.20	0.22	0.32	0.61	0.37	0.36	0.14	0.14	0.46	0.41	0.52
4	253	0.16	0.32	–	–	–	0.03	–	0.30	0.24	–	0.08	0.08	0.35	0.29	
5	317	–	–	–	–	0.27	–	–	–	–	–	–	–	–	–	–
6	301	–	–	–	–	0.15	–	–	–	–	–	–	–	–	–	–
7	301	0.03	0.10	–	–	0.22	–	0.08	0.18	0.04	0.09	0.00	0.01	0.14	0.14	0.03
8	285	–	0.18	–	–	0.25	0.03	–	–	0.02	–	0.01	–	0.35	0.04	–
9	431	0.01	0.21	–	–	–	–	–	–	–	–	–	–	0.05	–	–
10	300	0.03	0.17	0.11	–	–	0.02	0.11	0.04	0.01	0.15	0.06	0.01	0.22	0.09	0.02
11	285	0.05	0.26	0.05	–	0.10	0.03	0.07	0.29	0.05	0.27	0.03	0.04	0.11	0.12	0.28
12	269+315	0.03	0.19	0.08	–	0.07	0.09	0.17	0.23	0.06	0.11	0.01	0.02	0.16	0.17	0.19
13	284	–	0.13	0.03	–	–	0.03	0.03	0.12	0.04	0.03	0.01	0.01	0.05	0.09	0.11
14	315	0.03	0.17	0.03	0.07	0.04	0.02	0.02	0.21	0.05	0.01	–	–	0.02	0.19	0.19
15	253	0.14	0.38	0.13	0.08	0.07	0.13	0.13	0.40	0.18	0.11	0.05	0.06	0.17	0.37	0.40
16	269	0.06	0.19	0.07	0.01	0.04	0.07	0.06	0.31	0.10	0.07	–	0.03	0.14	0.18	0.24
17	267	–	0.04	0.01	–	–	–	0.01	–	0.01	0.01	–	–	0.01	0.03	0.04
	Hydroxycinnamic acid															
18	179	0.09	0.56	–	–	–	0.18	–	–	0.13	0.15	–	–	0.10	–	–
19	163	0.12	0.46	0.70	–	–	0.29	–	–	–	–	0.44	–	1.75	1.09	–
20	193	0.16	0.35	0.78	–	–	0.19	–	–	0.22	0.50	0.25	0.05	1.06	0.41	0.25
21	–[b]	0.08	0.27	–	–	0.19	–	–	0.26	0.10	0.30	–	–	0.48	0.30	–
22	–[b]	0.19	0.22	0.22	0.65	0.12	0.17	–	0.91	0.30	–	0.08	0.06	1.06	0.79	0.96
23	247	0.03	–	–	–	0.36	0.15	0.29	0.50	0.10	0.15	–	0.03	–	0.19	1.31
24	283	0.05	0.24	0.08	–	–	0.09	0.04	0.25	0.10	0.04	0.03	0.01	0.12	0.24	0.22
Total		2.10	6.34	3.43	0.81	2.36	2.14	1.77	6.19	2.57	3.38	1.52	0.70	7.54	6.38	6.34

[a] Values are mg/100 g honey. Reproducibility of the analyses was ±5%. (**1**) Isosakuranetin; (**2**) pinobanksin; (**3**) pinocembrin; (**4**) unidentified flavanone; (**5**) myricetin; (**6**) tricetin; (**7**) quercetin; (**8**) leutolin; (**9**) kaempferol-3-O-glucoside; (**10**) unidentified flavonol; (**11**) kaempferol; (**12**) apigenin + isohamnetin; (**13**) acacetin (tentatively); (**14**) methylquercetin (tentatively); (**15**) chrysin; (**16**) galangin; (**17**) tectochrysin; (**18**) caffeic acid; (**19**) *p*-coumaric acid; (**20**) ferulic acid; (**21**) and (**22**) caffeic acid derivatives; (**23**) dimethyl-allyl-caffeate; (**24**) phenyl–ethyl caffeate.

[b] The identification of two hydroxycinnamic acid derivative was not possible because of its poor ionisation in our LC–MS assay conditions.

geographical origin. When hydrogen peroxide was destroyed by the addition of the enzyme catalase, the QS inhibitory activity was lower (Fig. 2), pointing out the presence of non-peroxide anti-QS compounds in honey.

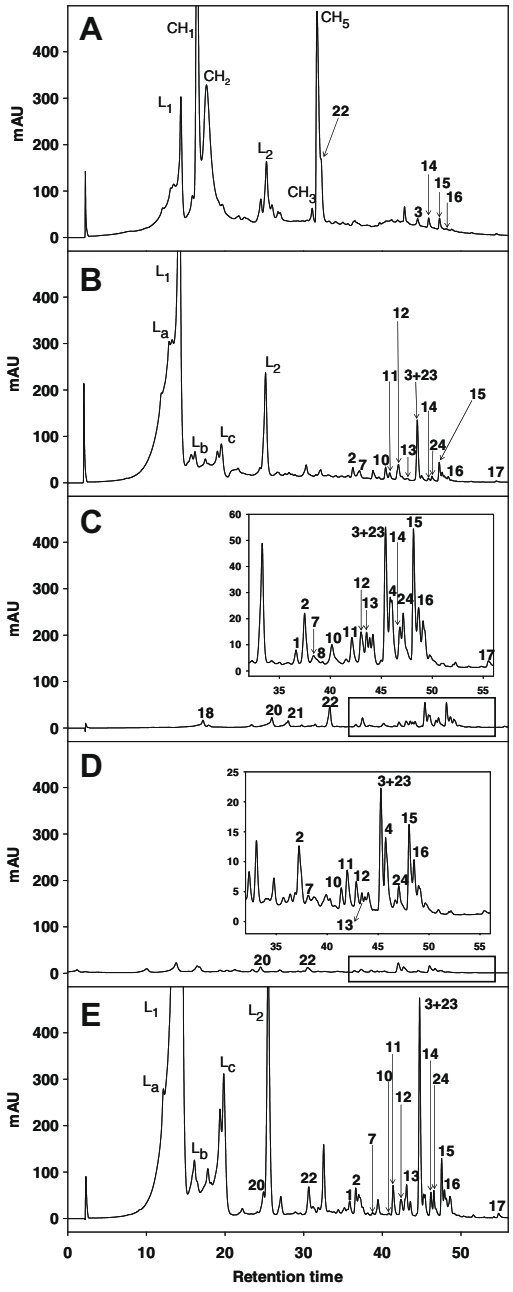

Fig. 3. HPLC chromatograms (340 nm) of phenolic compounds of (A) chestnut, (B) linden, (C) orange, (D) rosemary and (E) tilia honeys. (**1**) Isosakuranetin (4′-methoxy-5,7-dihydroxyflavanone); (**2**) pinobanksin (3,5,7-trihydroxyflavanone); (**3**) pinocembrin (5,7-dihidroxyfavanone); (**4**) (unidentified flavanone); (**5**) myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone); (**6**) tricetin (5,7,3′,4′,5′-tetrahydroxyflavone); (**7**) quercetin (3,5,7,3′,4′-pentahydroxyflavone); (**8**) luteolin (5,7,3′,4′-tetrahydroxyflavone); (**9**) kaempferol-3-O-glucoside; (**10**) (unidentified flavonol); (**11**) kaempferol (3,5,7,4′-tetrahydroxyflavone); (**12**) apigenin (5,7,4′-trihydroxyflavone) + isorhamnetin (3,5,7,4′-tetrahydroxy-3′-methoxyflavone); (**13**) acacetin (5,7 dihydroxy-4′-methoxyflavone); (**14**) methylquercetin (3,5,7,3′-tetrahydroxy-4′-methoxyflavone, tentatively); (**15**) chrysin (5,7-dihydroxyflavone); (**16**) galangin (3,5,7-trihydroxyflavone); (**17**) tectochrysin (5,hydroxyl-7-methoxyflavone); (**18**) caffeic acid; (**19**) *p*-coumaric acid; (**20**) ferulic acid; (**21**) and (**22**) hydroxycinnamic acid derivative (caffeic o ferulic acid); (**23**) dimethyl-allyl-caffeate and (**24**) phenyl–ethyl caffeate. L_1, L_2, L_a, L_b, L_c, CH_1, CH_2, CH_3, CH_5 are unknown compounds previously identified as floral markers (Tomas-Barberan et al., 2001).

3.2. Antibacterial activity

To evaluate if the inhibition of violacein production was due to the microbial growth reduction or AHL inhibition, the antimicrobial activity of 5 selected honeys was analysed. None of the tested honeys showed antimicrobial activity when applied at 0.1 g/ml (data not shown); although this concentration was enough to significantly reduce the violacein production (Tables 3 and 4). The antimicrobial activity of honey samples was concentration-dependent as moderate inhibition was observed when applied at 0.2 g/ml, but it increased significantly as honey concentrations increased (Table 5). Significant differences among the antibacterial activity of different unifloral honeys were observed. Chestnut, orange, rosemary and tilia honeys showed similar antimicrobial activity, reducing *C. violaceum* growth by approximately 1 or 2 log units at 0.4 g/ml, while linden honey reduced bacterial counts by more than 8 log units at the same concentration. When the hydrogen peroxide content of honey was eliminated by the addition of catalase, the antimicrobial activity of the honeys was only slightly reduced (Table 5).

3.3. Relationship between the anti-qs activity and the content of individual phenolic compounds

The main phenolic compounds of unifloral honeys were identified by HPLC–MS–MS, comparing retention times, UV spectrum and their MS and MS*n* fragmentation. The MS analyses showed that the flavonoid aglycones and hydroxycinnamic acid derivatives characteristic of poplar-derived propolis were present in all the tested unifloral honey samples. The aglycone $[M-H]^-$ ions in negative mode of the different phenolic compounds detected in the honey samples are shown in Table 6. HPLC chromatograms of these 15 unifloral honeys showed that they had similar, but quantitatively different, phenolic profiles (Table 6 and Fig. 3). When the phenolic profiles of 8 chestnut honeys from different geographical origins were tested they showed very relevant quantitative differences in the total phenolic content ranging from 0.6 to 1.9 mg/100 g fw. This large variation has previously been reported for unifloral honeys (Gil, Ferreres, Ortiz, Subra, & Tomás-Barberán, 1995) as honey phenolics originate mainly from propolis, and the "contamination" of honey with propolis is very variable and depends on many factors unrelated to its floral origin. There was not a linear relationship between the total or individual phenolic contents and the QS inhibitory activity or the antimicrobial capacity of honeys. Chestnut honey, which showed the highest anti-QS activity, showed one of the lowest contents of hydroxycinnamic acids, flavonols and flavanones, while tilia honey, showed both high anti-QS and total phenolic content. The HPLC chromatographs of chestnut, linden and tilia honeys showed peaks (L_1, L_2, L_a, L_b, L_c, CH_1, CH_2, CH_3, CH_5) related to their floral origin, previously identified as floral markers (Fig. 3) (Tomas-Barberan, Martos, Ferreres, Radovic, & Anklam, 2001). However, these floral markers were not found in the HPLC chromatograms of the less active orange and rosemary honeys (Fig. 3). These unidentified floral markers could be related to the antimicrobial and anti-QS activities of the honeys.

4. Discussion

The antimicrobial activity of honey and its variability according to floral origin have been widely reported (Baltrušaityte, Venskutonis, & Čeksterytė, 2007; Lee et al., 2008a; Mundo et al., 2004; Taormina et al., 2001). However, the ability of different unifloral honeys as QSI has not been studied yet. The role of QSI in food spoilage suggests that one way to prevent spoilage may be the control of QS (Ammor, Michaelidis, & Nychas, 2008). Several compounds

have been identified as QSI, although the best known are the halogenated furanones (Rasch et al., 2007). Unfortunately, these QSI are too reactive and toxic to be used in medicine, agriculture or the food industry (Bosgelmez-Tinaz, Ulusoy, Ugur, & Ceylan, 2007). Hence, there is a current need for the identification of new, non-toxic QSI compounds. Recent studies have demonstrated the potential use of different plant extracts as QSI (garlic, vanilla, pea seedlings, alfalfa, e.g.) (Bosgelmez-Tinaz et al., 2007; Choo et al., 2006; Teplitski et al., 2000).

In this study, we observed that all the 29 honey samples inhibited the AHL production, even at the lowest concentration (0.1 g/ml), although significant differences were observed according to floral origin. The anti-QS activity was concentration-dependent as the inhibition activity increased with increased honey concentration. Among all honeys, chestnut and linden samples were the strongest QSI. On the other hand, when honeys from the same floral origin but obtained from different geographical regions were compared, they showed similar anti-QS activity. Thus, it could be concluded that one of the factors which influences both the antimicrobial and anti-QS activities could be related to the floral origin, independently of the geographical region. Additionally, the antimicrobial activity of 5 different honey samples was evaluated to determine if the inhibition of AHL production was due to reduction of QS or inhibition of cell growth (Adonizio et al., 2006). The reduction in AHL production could be mainly attributed to inhibition of QS, as low concentrations of honey samples did not significantly inhibit growth of *C. violaceum*.

The variability in antimicrobial activity of different honeys has been attributed to different botanical and geographical origins and, more recently, to bee-origin metabolism products (Baltrušaityte et al., 2007; Lee et al., 2008b; Mundo et al., 2004; Weston, 2000). Furthermore, honey constituents such as sugars, volatiles, beeswax, nectar, pollen and propolis have also been characterised as responsible for the antimicrobial activity (Mundo et al., 2004; Weston, 2000). Previous studies reported that hydrogen peroxide plays a significant role in the antimicrobial activity of honey (Lee et al., 2008a, 2008b; Taormina et al., 2001). However, these studies also found that catalase-treated honeys still showed inhibitory activity, which may be attributed to non-peroxide related factors. In the present study, catalase-treated honeys showed antimicrobial and anti-QS activities, although these antipathogenic activities were lower when compared to untreated samples. This suggested that other factors of plant origin might be responsible for the "non-peroxide" antipathogenic activity of honeys as previously suggested by Weston (2000).

Popova et al. (2007) found a correlation between the concentration of total phenolics in the propolis and its antimicrobial properties. Furthermore, the phenolic content of honey samples has also been associated with antimicrobial activity (Küçük et al., 2007). However, in the present study, no relationship between either the total or individual phenolic content, and the antipathogenic activities of honeys was observed. Weston, Brocklebank, and Lu (2000) also determined that the phenolic compounds of the manuka honey were not responsible for the antibacterial activity. Thus, other non-phenolic compounds associated with floral origin could be responsible for the anti-QS activity. In fact, different non-phenolic floral markers were detected in the HPLC chromatograms of chestnut, linden and tilia honeys, which showed the highest antipathogenic activities. However, the HPLC chromatograms of orange and rosemary honeys did not show these compounds. The relationship between the type and amount of non-phenolic floral markers and the antipathogenic activities of honeys should be further studied. Therefore, it can be concluded that phenolic compounds contributed to the "non-peroxide" anti-QS activity but this contribution was relatively small as a non linear relationship was observed with the inhibitory activity.

5. Conclusions

Few studies have been conducted to investigate the potential of different compounds to reduce food spoilage by inhibiting bacteria cell-to-cell communication and consequently the spoilage mechanisms. In the present study, the ability of different unifloral honeys as QSI was demonstrated against the bacterium model *C. violaceum*. Unifloral honey samples showed "non-peroxide" anti-QS and antimicrobial activities but they were not linearly correlated with the total and individual phenolic compounds. Further research needs to be carried out to study which honey constituents, are responsible for the "non-peroxide" anti-QS activity.

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