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Potential antimicrobial activity of honey phenolic compounds against *Gram positive* and *Gram negative* bacteria

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ABSTRACT

This study is focused on the potential antimicrobial activity of honey phenolic compounds against *Gram positive* and *Gram negative* bacteria. For this purpose phenolic compounds were isolated from 33 Iranian honeys obtained from different botanical and geographical origins using solid-phase extraction (SPE). Characterization of honey extracts was carried out by HPLC-DAD-ESI-QTOF/MS. Minimum inhibitory concentration (MIC) and inhibition zone were used to determine the antimicrobial capacity of honey phenolic extracts against *Gram positive* (*Staphylococcus aureus* and *Enterococcus faecalis*) and *Gram negative* bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). The results shown that *E. coli* was the most sensitive bacteria and *P. aeruginosa* the most resistant strain. Moreover, five phenolic extracts shown lower MIC values than the whole honey pointing out that honey phenolic fraction may exert antimicrobial activity by itself and be a source of bioactive compounds to develop functional ingredients.

1. Introduction

Honey is a natural sweetener produced by *Apis mellifera* honeybees using nectar collected from flowers or other parts of plants. Regarding composition, carbohydrates are the major components in honeys which represent up to 60–85% of the dry mass of honey. On the other hand, minor components are represented by proteins and free amino acids, phenolic compounds, minerals, vitamins, and lipids, which play a vital role in some activities in bees. The complexity of these minor compounds are determined by many factors such as botanical source, bee species, seasonal and environmental factors (Mahmoodi-Khaledi et al., 2016). This natural product has traditionally been used for food, but also for medicinal purposes. Indeed, many research studies have pointed out biological properties of honey, such as antioxidant, antimicrobial, antidiabetic and anticancer properties (Rao, Krishnan, Salleh, & Gan, 2016). Among these beneficial attributes for human health, antimicrobial activity of honey has been traditionally associated to the high osmolarity and acidity of this matrix (Bose, 1982) or to others minor components, mainly hydrogen peroxide and phenolic compounds and their derivatives (Russell et al., 1990; White, Subers, & Schepartz, 1963). With regard to osmolarity, reported results shown how this property cannot exert antimicrobial activity by itself (Mahmoodi-Khaledi, Kashef, Habibi-Rezaei, & Moosavi-Movahedi, 2015), but osmolarity is crucial for the formation of colloidal structure of honey that, in turn, is important for honey antibacterial activity (Brudzynski et al., 2017). In addition, neutralization of hydrogen peroxide by catalase is not always associated with diminished antibacterial activity. In others words, hydrogen peroxide is not a key antimicrobial factor in all honey types (Roshan, Rippers, Locher, & Hammer, 2016). Accordingly, antibacterial activity evoked by honey's phenolic compounds could be classified as an instance of its hydrogen peroxide independent activity.

The study of phenolic composition, including their isolation and comprehensive characterization, could have an important role to

Abbreviations: BPC, base peak chromatogram; DAD, diode array detector; HPC, high-precision calibration; HPLC, High Performance Liquid Chromatography; LLE, liquid-liquid extraction; MHA, Mueller Hinton Agar; MIC, minimal inhibitory concentration; MS, mass spectrometry; QTOF, quadrupole time of flight; RDA, Retro Diels Alder; SPE, solid-phase extraction

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elucidate its contribution to antimicrobial properties. Indeed, in most of the studies, commercial standards or whole honey were used in order to establish the antimicrobial activity (Pimentel, da Costa, Albuquerque, & Junior, 2013).

The complexity of honey matrix and phenolic fraction requires the replacement of the conventional non-specific methods by other more specific ones. HPLC coupled to mass spectrometry offers a potent analytical alternative. Mass spectrometry detection is a high sensitivity method and it has the advantages of providing precise structural information about phenolic compounds. In addition, the use of quadrupole-time-of-flight mass spectrometry (QTOF) analyzer allows for the accurate mass measurements of both MS and MS/MS ions which is essential for elemental composition assignment and, thus, for the characterization of small molecules (Cádiz-Gurrea et al., 2014).

The aim of this study was to provide a better understanding of the relationship between honey phenolic compounds and antimicrobial activity. To achieve this goal, an isolation of phenolic compounds by solid-phase extraction (SPE) from 33 Iranian honeys samples from different botanical and geographical origins was carried out. After that, a comprehensive chemical characterization of honeys phenolic extracts by HPLC-DAD-ESI-QTOF/MS was done and finally the antimicrobial activity of isolated phenolic fraction was tested against four reference strains.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical reagent grade and used as received. Methanol and diethyl ether were purchased from Panreac (Barcelona, Spain). Acetonitrile from Lab-Scan (Dublin, Ireland), and acetic acid from Fluka, Sigma-Aldrich (Steinheim, Germany). Water was purified by a Milli-Q system from Millipore (Bedford, MA, USA). Nutrient agar, Mueller–Hinton agar, and blood agar base were obtained from Merck Co. (Darmstadt, Germany). Paper disks were purchased from Difco Lab. (Sparks, MD, USA).

2.2. Honey samples and phenolic compounds extraction

The phenolic extracts used in this study were from 11 multifloral origin and 22 mono-floral honeys obtained from different geographic zones in Iran (Table 1). Honeys were kept in airtight container at 2-8 °C in the dark until phenolic extraction. Isolation of phenolic fractions was carried out by solid phase extraction (SPE, with Amberlite XAD-2 resin) (Pasini, Gardini, Marcazzan, & Caboni, 2013). Briefly, honey samples (10 g) were dissolved in acidified water (pH 2) and loaded onto the column. The cartridges were washed with 30 mL and 70 mL of acidified water (pH 2) and water, respectively in order to remove carbohydrates. After that, samples were collected by passing through 100 mL of methanol and the solvent was evaporated under vacuum. The residue was dissolved in water. Phenolic extracts were isolated by liquid-liquid extraction (LLE) using diethyl ether as solvent. The solvent was evaporated under a N_2 stream. Concerning phenolic compounds characterization, the residue was dissolved with 0.5 mL of methanol and filtered before analysis.

2.3. Instrumentation

Separation of phenolic compounds was performed in an Agilent 1200-HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, autosampler, a binary pump, and a diode-array detector (DAD) coupled to a microQTOF (Bruker Daltonik, Bremen, Germany) mass spectrometer. The QTOF mass spectrometer was equipped with an electrospray interface (ESI) (model G1607A from Agilent Technologies, Palo Alto, CA, USA).

Table 1
Geographical, botanical origin and harvesting date of 33 Iranian honeys.

Geographical origin		Botanical origin	Harvest date	Honey Sample
North	Mazandaran-Kelardasht	Chicory	February 2010	17
	Mazandaran-Tonekabon	Dog rose	March 2011	30
North East	Razavi Khorasan-Mount Binalud	Chicory	February 2010	15
South East	Kerman-Kerman	Astragal	March 2008	6
	Kermanshah-Kermanshah	Alfalfa	January 2011	23
		Coriander	January 2011	24
South	Fars-Darab	Sour orange	January 2011	27
		Sweet orange	January 2011	28
		Jujube	January 2011	29
	Fars-Shiraz	Multifloral	September 2008	4
			January 2011	25
		Eucalyptus	January 2011	26
South West	Kohgiluyeh and Boyer-Ahmad-Yasuj	Chamomile	March 2008	5
West	Hamedan-Hamedan	Persian rose	September 2008	3
		Multifloral	March 2011	32
		Thyme	August 2011	33
	Kermanshah-Kermanshah	Chicory	April 2010	18
	Markazi-Khomeyn	Barberry	May 2010	21
North West	Ardabil-Ardabil	Multifloral	September 2008	1
			April 2010	19
	East Azerbaijan-Tabriz	Licorice	September 2010	22
	Kordestan-Sanandaj	Multifloral	June 2009	14
	West Azerbaijan-Urmia	Multifloral	September 2008	2
			June 2009	8
				9
				11
				13
		White clover	June 2009	10
		Hawthorn	June 2009	12
		Locust tree	August 2009	7
	Zanjan-Zanjan	Astragal	February 2010	16
Center	Isfahan-Kashan	Black cumin	May 2010	20
		Bramble	March 2011	31

2.4. Characterization of phenolic profile by HPLC-DAD-QTOF/MS

Compounds from the honey extracts were separated using a Zorbax Eclipse Plus C_{18} column (1.8 µm, 4.6 × 150 mm) at room temperature, flow rate of 0.5 mL/min and using an injection volume of 10 µL. Gradient elution was conducted using water with 0.5% acetic acid as eluent A and acetonitrile as eluent B. The following multi-step linear gradient was applied: 0.0 min 5%B; 10.0 min 35% B; 65.0 min 95% B and 67.0 min 5% B and finally a conditioning cycle of 7 min with same initial conditions for the next analysis. The compounds separated were monitored with DAD and a mass-spectrometry detector. The use of a splitter was required for the coupling with the MS detector in order to ensure reproducible results and stable spray.

Spectra were acquired over a mass range from *m/z* 50 to 1100 operating in negative ionization mode. The operating conditions of mass spectrometer were set as follows: capillary voltage, +4.5 kV; drying gas temperature, 190 °C; drying gas flow, 9.0 L/min; nebulizing gas pressure, 29 psi; collision RF, 150 Vpp; transfer time 80 µs, and pre-pulse storage, 7 µs. Moreover, automatic MS/MS experiments were performed adjusting the collision energy values as follows: m/z 100, 20 eV; m/z 500, 30 eV; m/z 1000, 35 eV, and using nitrogen as collision gas.

External mass-spectrometer calibration was performed with sodium acetate clusters in quadratic high-precision calibration (HPC) regression

mode. The calibration solution was injected at the beginning of each run and calibrated prior to the identification. The MS and MS/MS data were processed by software DataAnalysis 4.0 (Bruker Daltonics). The threshold accepted for confirmation of elemental composition was established at 10 ppm. Besides a high mass accuracy it was consider the isotopic abundance patterns as a single further constraint removes > 95% of false candidates this gives a little list of molecular formulas.

2.5. Evaluation of antimicrobial activity of phenolic extracts

2.5.1. Preparation of honey phenolic extract dilutions

Dilutions were prepared immediately before starting each experiment by diluting dried honey phenolic extracts. The dried extracts were diluted with aqueous methanol (80:20 v/v) to reach the concentration of 6.25, 12.5, 25, 50 and 100% (w/v) and sterilized for biological survey. On each assay, cellulose acetate sterile filters (0.22 μm) were used to avoid microbiological contamination.

2.5.2. Microbial cultures

Four reference strains were used in this study, including two *Gram-negative* (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) and two *Gram-positive* (*Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 11700). After overnight growth in nutrient agar, 0.5 McFarland standard turbidity for each test bacterial cultures (1–2 x 10^8 Colony Forming Unit (CFU)/mL) was individually adjusted in sterile saline solution (0.9% w/v) which was used to antimicrobial test.

2.5.3. Disc diffusion method

Disc diffusion method was carried out using 100 μl of the suspensions of each test bacterium containing 1–2 × 10^8 CFU/mL, which were inoculated in Mueller–Hinton agar (MHA) plates. Under aseptic conditions, empty sterilized discs (6 mm diameter, Difco) were impregnated with 10 μl of the extracts at concentrations ranging from 6.25% to 100% (w/v) of the honey phenolic extracts and dispensed on the agar surface. After the incubation period at 37 °C for 24 h, the zone inhibition was measured by a transparent ruler and presented in millimeter (including disc of 6 mm in diameter). Negative controls were prepared using the same solvents employed to dissolve the phenolic extracts and standard discs of Gentamicin (10 μg/disc) were used as positive control. The MIC (minimal inhibitory concentration) considered to be the lowest concentration of the tested phenolic extracts able to inhibit growth of bacteria. All assays were made in triplicate and the average was calculated where standard deviations were less than 0.1 on average.

2.6. Statistical analysis

Base peak area obtained in HPLC-MS chromatograms was used to provide the amount of each individual compound. Data were analyzed using Origin (Version Origin Pro 8 SR0, Northampton, MA, USA) to perform one-way-analysis of variance (ANOVA) at a 95% confidence level ($p \leq 0.05$) to identify significant differences among the phenolic and other polar compound individual concentrations in the extracts obtained using SPE described above in order to establisher differences among analyzed samples and understand the relationship between phenolic extract contents and antibacterial activity.

3. Results and discussion

3.1. Characterization of honey phenolic extracts by HPLC-ESI-Q-TOF/MS

Fig. 1A includes the base peak chromatogram (BPC) of a representative honey phenolic extract analyzed by HPLC-ESI-Q-TOF/MS. All compounds are summarized in Table 2 which shows retention time, molecular ion ($[M-H]^-$), experimental *m/z*, calculated *m/z*, main MS/MS fragments and proposed compounds. The identification of the corresponding compound was based on the search of the $[M-H]^-$ deprotonated molecule together with the interpretation of its fragmentation ions provided by Q-TOF and compared with MS/MS data compiled from literature. Overall, the mass spectrometry analysis of the honey extracts allowed the identification of 46 compounds, including phenolic and non-phenolic compounds. They were classified into different chemical classes depending on their structures (organic acids; benzoic and cinnamic acids; flavonoids; terpenoids; and others). Although, Q-TOF analyzer provides useful information it was not possible to identify 4 compounds, called as unknown (Uk 1-4).

3.1.1. Organic acids

According to MS and MS/MS data and the HPLC elution profile, six organic acids were found in honey phenolic extracts. Peak 6 at *m/z* 171.0663 was characterized as cyclopentyl malonic acid. This compound presented a prominent fragment ion at *m/z* 127 which is assignable to the loss of CO_2 and other ion at *m/z* 109 resulting from the loss of H_2O.

Peak 8 was characterized as diethyl succinic acid. This compound shown a deprotonated molecule at *m/z* 173.0819. It MS/MS spectrum shown a fragment ion at *m/z* 111 $[C_7H_{11}O_1]^-$ given by the loss of carbon dioxide and subsequent water moiety. Peak 18 at the retention time 32.3 min had a $[M-H]^-$ ion at *m/z* 199.0976 with deprotonated molecular formula ($C_{10}H_{15}O_4$), and MS^2 fragmentation ion at *m/z* 155 result of the loss of carboxylic group was characterized as succinic acid monocyclohexyl ester. The presence of these organic acid derivatives could be related to the chemical transformation of several precursor previously described in honeys such as succinic and malonic acids (Suárez-Luque, Mato, Huidobro, Simal-Lozano, & Sancho, 2002).

Peak 14 gave a deprotonated molecule at *m/z* 187.0976 with molecular formula ($C_9H_{16}O_4$). Its MS^2 spectrum shown a prominent fragment ion at *m/z* 125 and other fragment ion at *m/z* 169, provided by loss of water moiety. It was identified as azelaic acid.

3.1.2. Phenolic acids

A total of seventeen phenolic acids belonging to benzoic and cinnamic acids were characterized.

3.1.2.1. Benzoic acids. Nine benzoic acid and derivatives were identified. Peak 1 had an ion at *m/z* 144 and it was tentatively proposed as quinolinol. Peak 2 with retention time 15.2 min had a $[M-H]^-$ ion at *m/z* 137.0244 was identified as *p*-hydroxybenzoic acid (*p*-HBA). This compound gave a fragment ion at *m/z* 93 (phenol), as shown in Fig. 1B. Peak 7 (*m/z* 165) was characterized as phenyllactic acid. Its MS^2 spectra generated was characterized by the product ion at *m/z* 147 which was assignable to the loss of water moiety and other ion at *m/z* 119 $[M-H-46]^-$. Peaks 4, 9, 10 and 17 were identified as syringic acid and three derivatives. Peak 4 had an ion at *m/z* 197.0455 and presented two fragment ions at *m/z* 121 and 123 indicatives of losses of carboxylic and two methyl groups. This peak was identified as syringic acid which has been found in some reports (Sergiel, Pohl, & Biesaga, 2014). Methyl syringate (10), shown an ion at *m/z* 221.0612 formed upon gain of a methyl group of syringic acid. This compound gave two fragment ions at *m/z* 196 and *m/z* 181 due to loss of one or two methyl groups, respectively, and other ion at *m/z* 153 produced by loss of carboxymethyl group. Compound 17 with deprotonated molecular formula ($C_9H_9O_4$) and *m/z* 181.0506, was associated to the loss of an oxygen atom of syringic acid and the consequent formation of an aldehyde group giving rise to the formation of syringaldehyde. All of these compounds have been reported in many studies (Jerković et al., 2015).

Compound 9 with *m/z* at 281.1394 yielded a prominent major fragment at *m/z* 221 and other 1 at *m/z* 206, due to demethylation by loses of four and five methyl groups, respectively. It was tentatively assigned to syringic acid hexyl ester. This compound has not been

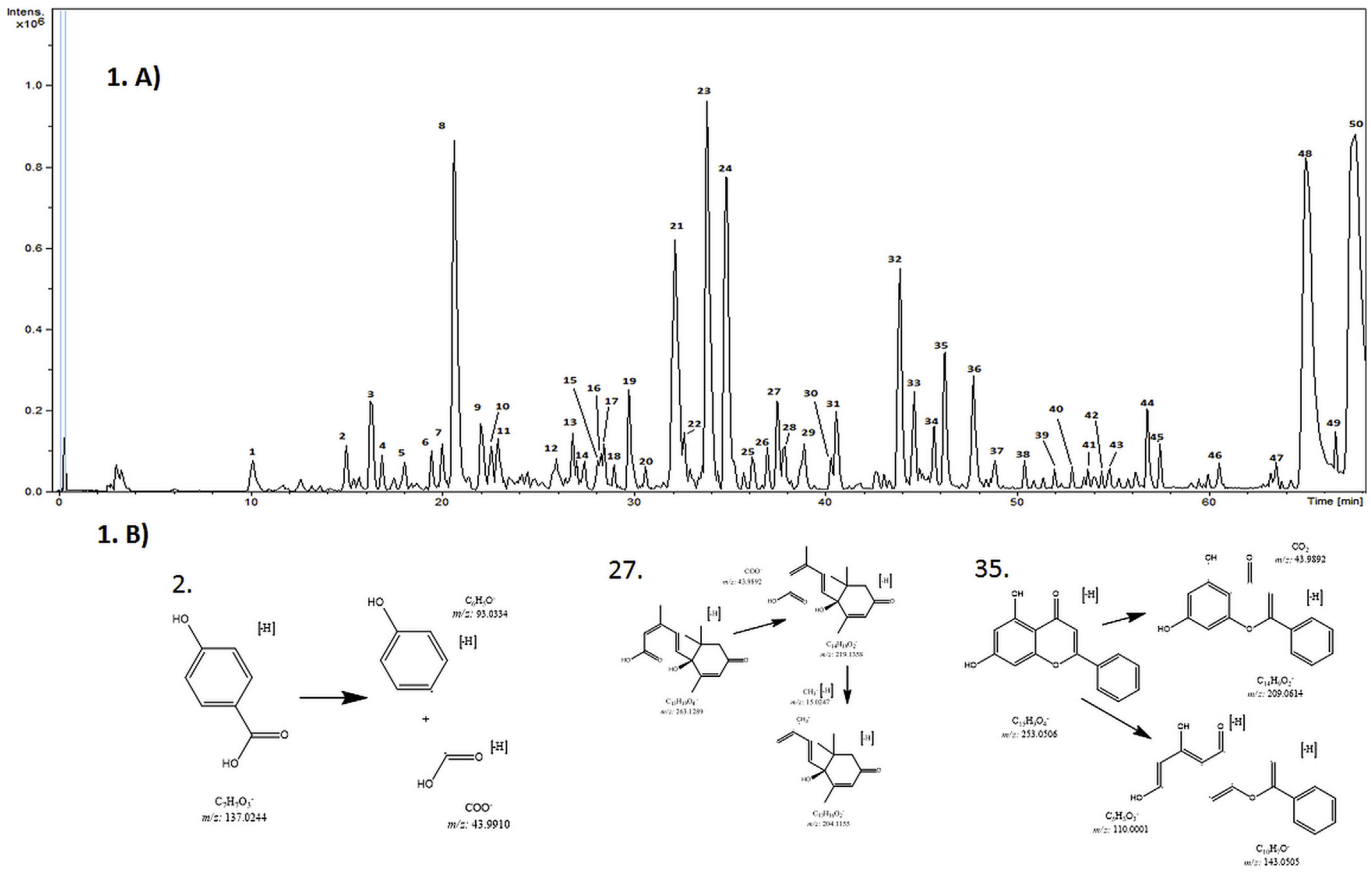


Fig. 1. Base Peak Chromatogram of representative honey phenolic extract (A) obtained by HPLC-ESI-QTOF-MS/MS. Fragmentation pattern of *p*-HBA, abscisic acid and chrysin (B).

previously reported in honeys.

Peak 24 was tentatively identified as butylhydroquinone which shown an ion at *m/z* 165.0921 and a fragment [M-H-16]$^-$ at *m/z* 149 indicating the loss of hydroxyl group. This compound was previously identified in Algerian honeys (Ouchemoukh et al., 2016).

3.1.2.2. Cinnamic acids. They were found up to eight derivative compounds of cinnamic acid. Peak 3 gave a [M-H]$^-$ ion at *m/z* 179.0350 and a characteristic fragment ion at *m/z* 135 by the release of CO_2 allowing its characterization as caffeic acid. Peak 5 was tentatively characterized as hydroxyconiferyl alcohol. It displayed a [M-H]$^-$ ion at 195.0663 and its fragmentation pattern shown three ions at *m/z* 136 [$C_8H_{10}O_2$−2H]$^-$, *m/z* 121 [$C_7H_5O_2$]$^-$ and *m/z* 119 [$C_8H_8O_1$−H]$^-$. Peaks 30, 31 and 32 were characterized as prenyl caffeate isomers since they presented the same deprotonated molecular formula ($C_{14}H_{15}O_4$) and [M-H]$^-$ at *m/z* 247.0976. All of them gave a fragment ions at *m/z* 179 (caffeic acid) and other ion at *m/z* 135, indicating the characteristic loss of CO_2 of caffeic acid.

3.1.3. Flavonoids

In this study, the method used allowed the characterization of twelve flavonoids. Most of them were presented in all Iranian honeys. Examination of MS and MS/MS spectra of honey samples revealed peak 20 gave a [M-H]$^-$ at *m/z* 271.0612 and a molecular formula ($C_{15}H_{12}O_5$). This compound was characterized as pinobanksin. Its MS/MS spectra shown a fragment ion at *m/z* 253. The loss of water is due to the presence of a hydroxyl group at C_3 of the C ring. In this way, pinobanksin-*O*-acetate (peak 33) shown a deprotonated molecular formula ($C_{17}H_{13}O_6$). This compound displayed the same fragmentation pattern pinobanksin giving two fragment ions at *m/z* 271 (pinobanksin molecule) and other ion [M-H-42-H_2O]$^-$ at *m/z* 253. These compounds were previously found in honeys with different botanical origins (Ristivojević et al., 2015).

Compound 26 and 27 gave ion species [^{1,3}A]$^-$ which corresponded to Retro Diels Alder (RDA) fragmentation pathway, underwent fragmentation by elimination of CO_2, C_2O_3 and CH_3, yielded a fragment ion [^{1,3}A]$^-$ at *m/z* 151. Compound 26 with a [M-H]$^-$ ion at *m/z* 285.0405 was characterized as luteolin and compound 27 with a [M-H]$^-$ ion at *m/z* 269.0455 with deprotonated molecular formula ($C_{15}H_9O_5$) identified as apigenin in accordance with previous report (Ristivojević et al., 2015).

Compound 35 gave the molecular ion at *m/z* 253.0506 with molecular formula $C_{15}H_{10}O_4$. In the MS/MS spectrum, the obtained ions were at *m/z* 209 [M-H−CO_2]$^-$ and *m/z* 143 [M-H−C_3O_2−C_2H_2O]$^-$ (Fig. 1B). This compound was characterized as chrysin. Compound 38 displayed a molecular formula $C_{16}H_{12}O_5$. It was identified as methoxy chrysin since it presented a MS^2 spectra with three characteristic fragment ions at *m/z* 268 [M-H−CH_3]$^-$, *m/z* 239 [M-H−CO_2]$^-$, *m/z* 211 [M-H−CO_2−CO]$^-$ (Kečkeš et al., 2013).

Compound 37, with a molecular formula $C_{16}H_{12}O_5$, presented only one prominent fragment ion at *m/z* 268 giving a fragment ion [$C_{15}H_9O_5$−H]$^-$. It was identified as acacetin.

Peak 28 gave an ion at *m/z* 299.0561 and was tentatively identified as kaempferide. This compound shown three fragment ions, the prominent 1 at *m/z* 284 produced by loss of methyl group, other ion at *m/z* 255 generated by the loss of CO_2 and finally other ion at *m/z* 227 ([M-H−CO−CO_2]$^-$).

Peaks 23 and 25 shown the same deprotonated molecular ion at *m/z* 315. Their fragmentations produced fragment ions at *m/z* 300, indicating the loss of a CH_3. According to the literature, these compounds were characterized by their elution order as rhamnetin (23) and isorhamnetin (25) (Kečkeš et al., 2013).

Table 2
Proposed compound identified in honey phenolic fraction by HPLC-ESI-QTOF- MS/MS. Number designing compound correspond to peaks as depicted in Fig.1 A.

Peak	RT	Molecular formula M-H	m/z experimental	m/z calculated	MS/MS fragments	Proposed compound
1	14.4	C_9H_6NO	144.0456	144.0455		Quinolinol
2	15.2	$C_7H_5O_3$	137.0247	137.0244	93 (100)	*p*-HBA
3	16.5	$C_9H_7O_4$	179.0360	179.0350	135 (28)	Caffeic acid
4	17.2	$C_9H_9O_5$	197.0462	197.0455	121 (100), 123 (67)	Syringic acid
5	19.7	$C_{10}H_{11}O_4$	195.0670	195.0663	136 (100), 121 (67), 119 (19)	Hydroxyconiferyl alcohol
6	20.2	$C_8H_{11}O_4$	171.0668	171.0663	127 (100), 109 (24)	Cyclopentyl malonic acid
7	20.8	$C_9H_9O_3$	165.0568	165.0557	119(100), 147 (47)	Phenyllactic acid
8	22.3	$C_8H_{13}O_4$	173.0826	173.0819	111 (100)	Diethyl succinic acid
9	23.4	$C_{15}H_{21}O_5$	281.1397	281.1394	221 (100),206(35), 131 (4)	Syringic acid hexyl ester
10	25.4	$C_{10}H_{11}O_5$	211.0617	211.0612	181 (100), 196 (21), 153 (22)	Methyl syringate
11	27.0	$C_{15}H_{19}O_4$	263.1298	263.1289	219 (37), 204 (100)	Abscisic acid isomer I
12	27.6	$C_{15}H_{19}O_4$	263.1294	263.1289	204 (100)	Abscisic acid isomer II
13	28.3	$C_{15}H_{19}O_4$	263.1294	263.1289	204(100), 219 (88)	Abscisic acid isomer III
14	28.7	$C_9H_{15}O_4$	187.0982	187.0976	125 (100)	Azelaic acid
15	29.3	$C_{15}H_{19}O_4$	263.1272	263.1289	204 (100), 219 (80)	Abscisic acid isomer IV
16	29.9	$C_{15}H_{19}O_4$	263.1298	263.1289	204(100), 219 (75), 151 (33)	Abscisic acid isomer V
17	30.8	$C_9H_9O_4$	181.0512	181.0506	137 (100), 123 (44)	Syringaldehyde
18	32.3	$C_{10}H_{15}O_4$	199.0989	199.0976	155 (100)	Succinic acid, monocyclohexyl ester
19	32.8	$C_{10}H_{17}O_3$	185.1190	185.1183	125 (100)	Royal jelly acid
20	34.0	$C_{15}H_{11}O_5$	271.0635	271.0612	253 (34), 197 (12), 125 (11)	Pinobanksin
21	35.0	$C_{10}H_{17}O_4$	201.1148	201.1132	183 (100), 139 (89)	Dihydroxy decenoic acid
22	35.4	$C_{10}H_{19}O_3$	187.1346	187.1340	127 (13), 141 (6)	Hydroxy decenoic acid
23	35.8	$C_{16}H_{11}O_7$	315.0516	315.0510	300(100), 271 (21)	Rhamnetin
24	37.1	$C_{10}H_{13}O_2$	165.0922	165.0921	149 (100)	Butylhydroquinone
25	37.6	$C_{16}H_{11}O_7$	315.0516	315.0510	300 (100), 165 (13)	Isorhamnetin
26	38.0	$C_{15}H_9O_6$	285.0412	285.0405	257 (2), 151 (1)	Luteolin
27	39.0	$C_{15}H_9O_5$	269.0461	269.0455	225 (6), 151 (12)	Apigenin
28	40.4	$C_{16}H_{11}O_6$	299.0561	299.0561	284(100), 255 (42), 227 (25)	Kaempferide
29	40.7	$C_{18}H_{31}O_5$	327.2180	327.2177	221(100), 157 (72)	UK1
30	42.7	$C_{14}H_{15}O_4$	247.0981	247.0976	135(100), 179 (54)	Prenyl caffeate isomer 1
31	44.0	$C_{14}H_{15}O_4$	247.0993	247.0976	135(100), 179 (74)	Prenyl caffeate isomer 2
32	44.6	$C_{14}H_{15}O_4$	247.0993	247.0976	135 (100), 179 (12)	Prenyl caffeate isomer 3
33	44.7	$C_{17}H_{13}O_6$	313.0726	313.0718	253 (100), 271 (8)	Pinobanksin-*O*-acetate
34	45.7	$C_{10}H_{19}O_3$	187.1346	187.1340	124(92), 130 (85), 129 (73), 89 (65), 99 (54), 101 (43),	Hydroxycapric acid
35	46.3	$C_{15}H_9O_4$	253.0514	253.0506	209 (5), 143 (3)	Chrysin
36	47.7	$C_{15}H_9O_5$	269.0464	269.0455	213 (2)	Galangin
37	48.4	$C_{16}H_{11}O_5$	283.0618	283.0612	268 (100)	Acacetin
38	48.9	$C_{16}H_{11}O_5$	283.0620	283.0612	268 (100), 239 (81), 211 (76)	Methoxy-chrysin
39	52.9	$C_{20}H_{26}NO_3$	328.1922	328.1918	273 (100), 147 (86)	UK2
40	53.7	$C_{16}H_{31}O_4$	287.2239	287.2228	269 (15), 197 (2)	Dihydroxypalmitic acid (fatty acid)
41	54.4	$C_{18}H_{31}O_4$	311.2226	311.2228	293 (15), 275 (8), 223 (100)	Hydroperoxy linoleic acid (fatty acid)
42	54.8	$C_{19}H_{23}O_2$	283.1710	283.1704	219 (62), 131 (100)	UK3
43	56.8	$C_{20}H_{25}O_4$	329.1764	329.1758	285 (100), 147 (46)	Carnosol or isomer
44	57.4	$C_{20}H_{25}O_4$	329.1764	329.1758	285(100), 237 (67)	Carnosol or isomer
45	60.5	$C_{16}H_{31}O_3$	271.2287	271.2279	253 (3), 225 (2)	Hydroxy palmitic acid (fatty acid)
46	63.4	$C_{12}H_{23}O_2$	199.1710	199.1704		Lauric acid (fatty acid)
47	64.0	$C_{25}H_{35}O_6$	431.2441	431.2439	99(100), 349 (29), 267 (21)	UK4
48	65.1	$C_{16}H_{31}O_2$	255.2351	255.2330		Myristic acid, ethyl ester (fatty acid)
49	66.6	$C_{20}H_{27}O_2$	299.2026	299.2017		Retinoic acid (vitamin)
50	67.6	$C_{18}H_{35}O_2$	283.2666	283.2643	281 (3)	Palmitic acid ethyl ester (fatty acid)

*In brackets, abundance of compound fragments.

Peak 36 was tentatively characterized as galangin. It presented a $[M\text{-}H]^-$ at *m/z* 269 and molecular formula ($C_{15}H_{10}O_5$). Its MS/MS fragmentation gave an ion at *m/z* 213 $[M\text{-}H{-}C_2O_2]^-$ which was described in previous studies (Ristivojević et al., 2015).

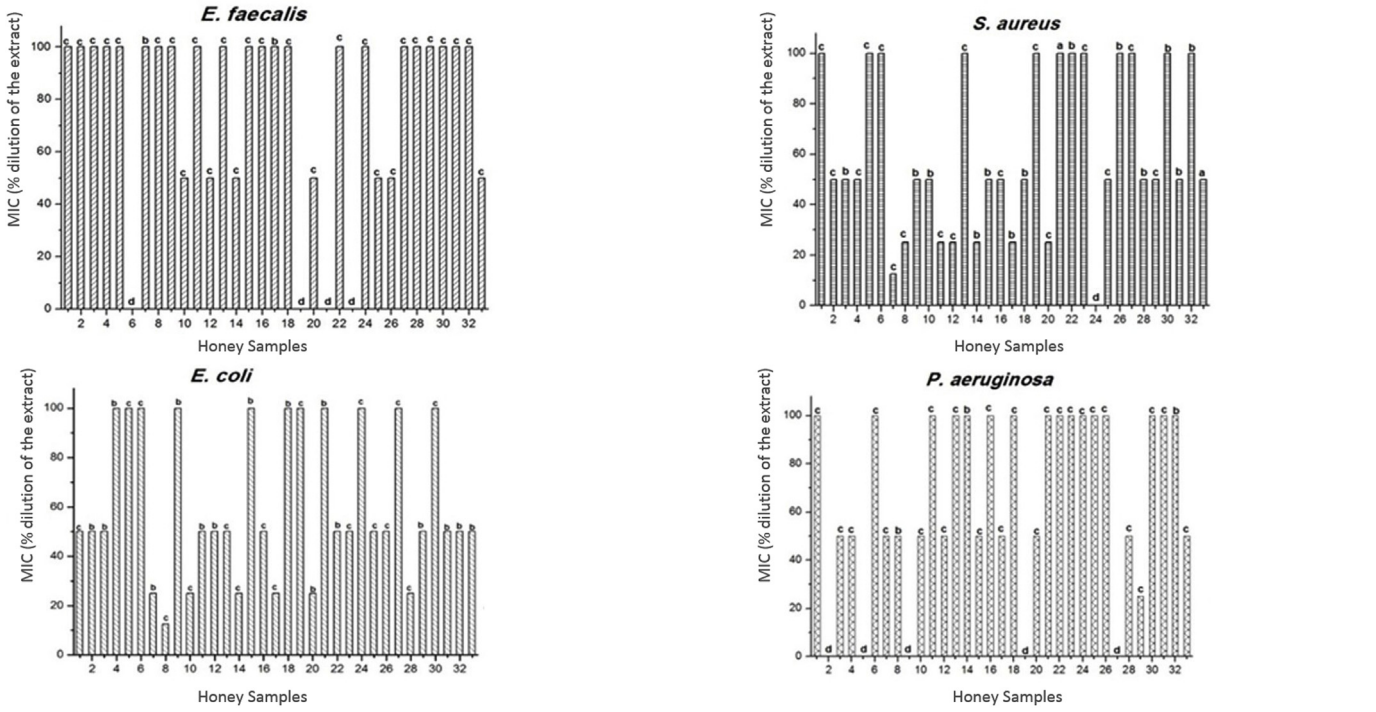
3.1.4. Terpenoids

The MS and MS/MS experimental data and the comparison with bibliography enabled the identification of 6 compounds belongs to terpenoids. Compounds 11, 12, 13, 15 and 16 were characterized as abscisic acid isomers. It is worthy to remark that the MS/MS spectra of these compounds shown the same fragmentation pattern characterizing by two fragment, one ion at *m/z* 219 and other ion at *m/z* 204, as shown in Fig. 1B. The first fragment corresponds to the loss of CO_2, the second one indicates a subsequent loss of CH_3. These evidences and according to preliminary reports allowed the assignment of these compounds as abscisic acid isomers (Jerković & Kuś, 2014).

Peaks 43 and 44 gave the same deprotonated molecule $[M–H]^-$ at *m/z* 329.1758, with molecular formula ($C_{20}H_{26}O_4$) and they were tentatively identified as carnosol isomers. Their fragmentation gave a fragment ion at *m/z* 285 resulting to the loss of CO_2.

3.1.5. Other compounds

It was possible to identify other chemical compounds which were previously reported in honey and propolis samples. Among them, it was possible identify royal jelly acids and some derivatives. They are aliphatic compounds which can be classified into both fatty and hydroxy fatty acids (Wytrychowski et al., 2013). Compound 19, presenting a $[M–H]^-$ ion at *m/z* 185.1183 with molecular formula ($C_{10}H_{18}O_3$), gave the base peak fragment ion at *m/z* 125 $[M–H{-}CO_2{-}CH_3{-}H]^-$. According to the literature, this compound was characterized as royal jelly acid. In addition, compounds 21, 22 and 34 were also identified as royal jelly acid derivatives. Compound 21 presented a deprotonated molecular formula ($C_{10}H_{17}O_4$) and an ion at *m/z* 201.1148, it gave a MS^2 spectrum at *m/z* 183 according to loss water moiety and other fragment ion at m/z 139 associated to the loss of a carboxylic group and water moiety. This compound was identified as dihydroxy decenoic acid.



Mean Zones of Inhibition are in brackets (diameter mm including disc (6 mm)):
(a) High antimicrobial activity, inhibition zone 12–15 mm; (b) Moderate antimicrobial activity, inhibition zone 10–11 mm; (c) Slight antimicrobial activity, inhibition zone 8–9 mm; (d) Inhibition, inhibition zone <7 mm.

Fig. 2. MIC and Inhibition zones of reference strains.

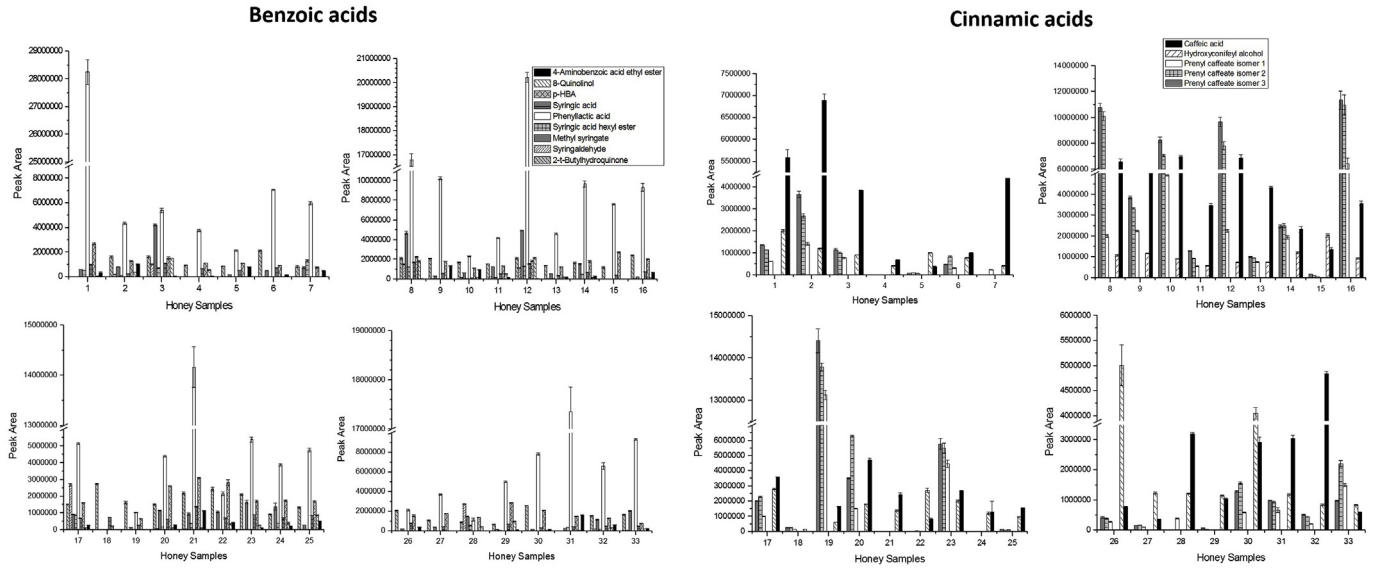


Fig. 3. Semi-quantitative composition in phenolic acids of different phenolic fractions.

Compounds 22 and 34 shown the same molecular formula ($C_{10}H_{20}O_3$) but different fragmentation pattern. The first one gave two ions at *m/z* 141 and *m/z* 127, related with the loss of carboxylic group and carboxymethyl group, respectively. This compound was identified as hydroxy decenoic acid. MS/MS spectrum of compound 34 displayed some ions, prominent ones were an ion at *m/z* 130 and other *m/z* 129 which according to the ions $[M–H–CO_2–CH_3+2H]^-$ and $[M–H–CO_2–CH_3+H]^-$, respectively. It was characterized as hydroxycapric acid. These compounds were identified in previous studies (Isidorov, Bagan, Bakier, & Swiecicka, 2015).

With regard to other fatty acids and derivatives, peaks 40, 41, 45, 46, 48 and 50 were characterized within this chemical group. Peak 40 was identified as dihydroxy palmitic acid. It shown a $[M–H]^-$ at *m/z* 287.2228 and a molecular formula ($C_{16}H_{32}O_4$). Its MS/MS spectrum presented two ions at *m/z* 269 (loss of H_2O) and *m/z* 197 $[C_{13}H_{25}O]^-$. Peak 41, which displayed a molecular formula $C_{18}H_{32}O_4$, presented a MS^2 pattern with three ions, two of them with lower abundance at *m/z* 293 $[C_{18}H_{31}O_3–H]^-$ and *m/z* 275 $[C_{18}H_{29}O_2–2H]^-$. The last ion at *m/z* 223 with the highest abundance was related to the loss of carboxylic group and 3 methylene groups $[M–COO–3CH_2–H]^-$. Peak 45 presented a $[M–H]^-$ at *m/z* 271.2279 and its MS/MS spectrum shown two fragment ions at *m/z* 253 and 225 provided by loss of hydroxyl group and

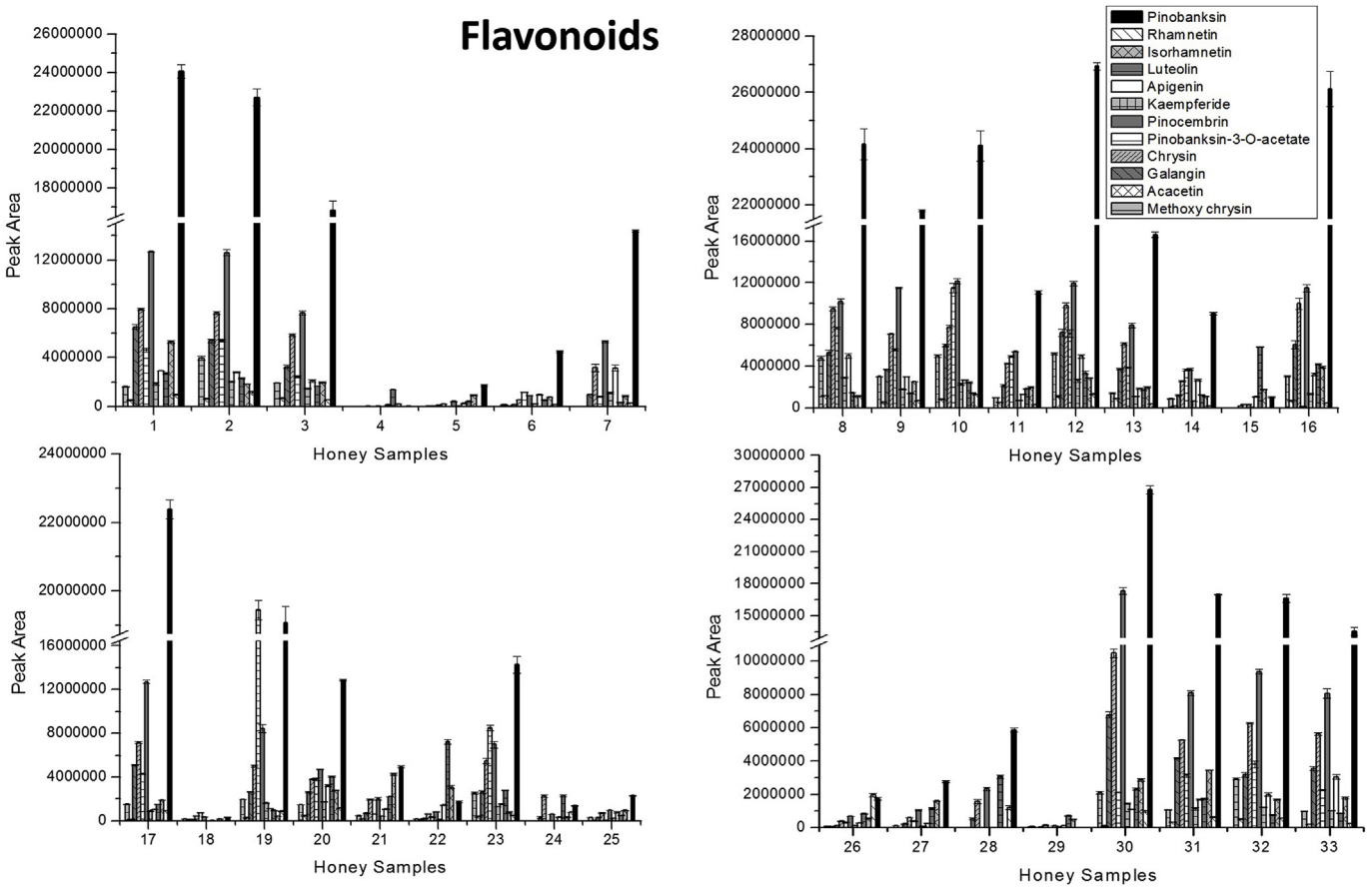


Fig. 4. Semi-quantitative composition in flavonoids of different phenolic fractions.

carboxylic group, respectively. These evidences allowed to identify this compound as hydroxypalmitic acid. Peak 46 was tentatively identified as lauric acid. Compound 48, which eluted at 65.1 min and had an ion at *m/z* 255.2330, was tentatively characterized as myristic acid. Compound 50 was characterized as palmitic acid ethyl ester. It displayed a $[M\text{-}H]^-$ at *m/z* 283.2643. It presented a fragment ion at *m/z* 281. Fatty acids and their alcohols are present in bee-stomach organic extract and their presence have previously been reported in honeys and propolis (Falcão et al., 2010).

Peak 49 was characterized as retinoic acid which presented an $[M\text{–}H]^-$ at *m/z* 299. Its MS/MS spectrum shown a fragment ion at *m/z* 241 due to the release of carboxylic and methyl group. To support this assignation, there is compiled in bibliography data which shows vitamins in different types of honey (Erejuwa, Sulaiman, & Ab Wahab, 2012).

3.2. Antimicrobial effect of honey phenolic extracts

Antimicrobial properties of honey could be attributed to the individual or synergetic effects of the high sugar osmolarity (Kwakman & Zaat, 2012), the enzymatic generation of hydrogen peroxide (Sousa et al., 2016) or the presence of other minor compounds (Güneş; Şahin, Demir, Borum, & Tosunoğlu, 2017). Indeed, several authors have reported the antimicrobial effect of whole honey and individual compounds using available commercial standards (Alvarez-Suarez et al., 2010). Nevertheless, there are not studies in which this beneficial property is evaluated in both, whole honeys and isolated minor compounds in order to establish the antimicrobial activity of phenolic fraction by itself. The antimicrobial potential of the whole honeys used in this study was analyzed in a prior study (Mahmoodi-Khaledi et al., 2016). The results shown how all honey samples affected growth of all references strains analyzed in a concentration-dependent manner. To evaluate the degree of the antimicrobial activity of honey phenolic compounds, in the present study MIC values and inhibition zones of isolated phenolic fraction from these samples was analyzed and compared with the previously reported antimicrobial activity of whole honeys. Fig. 2 indicates the MIC (%dilution of extract) and inhibition zones of each honey phenolic extract.

Bacteria strains presented different resistant potencies: *P. aeruginosa* was the most resistant strain followed by *E. faecalis*, *S. aureus,* and *E. coli*. With regard to the effect of these isolate fractions, the results pointed out that the majority of the phenolic extracts exhibited MIC values equal or higher than the whole honey. However, when *P. aeruginosa* was exposed to phenolic extract of honey number 29 exerted growth inhibition at lower concentration than whole honey. Concerning *E. faecalis*, phenolic extracts of samples number 10 and 20 shown growth inhibition at concentration 50% whereas that it needed concentrations as higher as 100% of whole honey to get the same antimicrobial activity. Phenolic extracts of honeys number 12 and 20 presented lower MIC (25%) than same whole honey extracts when were exposed to *S. aureus*. Finally, growth of *E. coli* was inhibited with phenolic extracts 8, 10 and 20 using a concentration smaller than whole.

3.3. Relationship between honey phytochemical content and antimicrobial activity

Once the chemical composition and antimicrobial activity of the different honey extracts were established, the abundance of individual phenolic and other polar compounds were used to provide semi-quantitative information for comparison purposes among honey phenolic extracts. To achieve this goal, the base peak area obtained in HPLC-MS

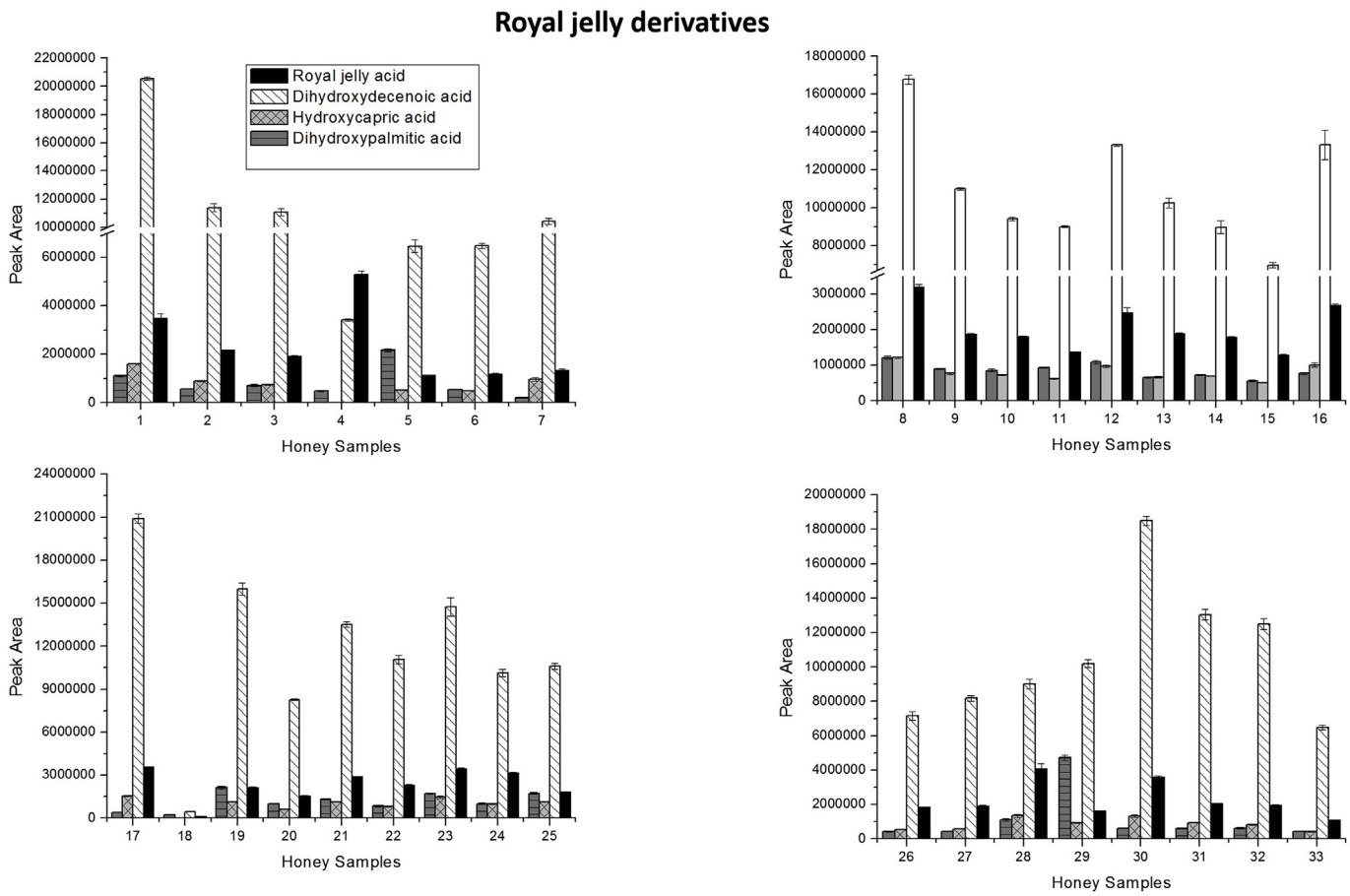


Fig. 5. Semi-quantitative composition in royal jelly and other compounds of different polar fractions.

chromatograms was used to provide the amount of each individual compound. Phenolic contents shown a large variation among the different extracts according to their geographical and botanical origin. The differences are illustrated in Figs. 3 and 4. In order to identify significant differences among the samples under study, statistical treatment was carried out (Supporting information Tables 7–10). Overall, total phenolic content were similar between all samples. Nevertheless, samples 4, 5, 6, 18, 27 shown lower amounts of phenolic compounds.

The values for the amount of benzoic acids presented variation depending on the sample. Above all, phenyllactic acid was presented in all samples as largest abundance compound. *p*-HBA was also detected in the most samples. These phenol compounds have been found in honeys (Güneş, Şahin, Demir, Borum, & Tosunoğlu, 2017). Samples 3, 8, 12 and 33, had higher peak areas of methyl syringate. This benzoic acid is the major compound in manuka honeys and its antimicrobial activity is associated to ability to form complexes and bind to soluble proteins present in honey and bacterial wall.

Cinnamic acids have previously been reported in honeys (Güneş et al., 2017) and associated to growth inhibition of *Gram positive* bacteria (Viuda-Martos, Ruiz-Navajas, Fernndez-Lpez, & Alvarez, 2008). In the analyzed samples, the peak areas of cinnamic acids were variable depending on the sample. In this sense, samples 4, 5, 6, 7 and 18 revealed lower intensities of cinnamic acids. Caffeic acid and its derivatives were the most plentiful compounds, except in sample 17.

Presence of high concentrations of some flavonoids such as rutin or chrysin have been associated to growth inhibition (Kirnpal-Kaur, Tan, Boukraa, & Gan, 2011). Pinocembrin has also been linked to growth inhibition of *S. aureus, E. coli* and *P. aeruginosa* (Pimentel et al., 2013). In analyzed honeys, only chrysin and pinocembrin were found. Chrysin was detected in samples 1, 2, 8, 10, 12, 16, 17 and 30 while pinocembrin was presented in all samples except sample 4. In the rest of samples, this was found in large amounts but lower than pinobanksin which was the major flavonoid in phenolic extracts.

In addition, royal jelly acid and its derivatives play an important role in the development of the queen honeybee (Kucharski, Maleszka, Foret, & Maleszka, 2008). The presence of 10-hydroxy-2-decenoic acid (Blum, Novak, & Taber, 1959) is related with antimicrobial effects. The most abundant compound in all samples was dihydroxy decenoic acid except in sample 4 which royal jelly acid was the major one.

Regarding phenolic extracts of honeys exhibiting higher antimicrobial activity (8, 10, 12, 20 and 29), they presented similar main compounds. In this sense, the two major benzoic acids in these samples were phenyllactic acid and *p*-HBA although peak areas of these compounds were not the highest in all analyzed honeys. Despite being associated to antimicrobial activity (Isidorov et al., 2015), obtained results in this study shown how these compounds did not exert antimicrobial activity by themselves or if they did it, they exercised it independently of their concentration. It may be explained by the phenolic extract of sample 1 had the highest amounts of phenyllactic acid and sample number 21 the highest amounts of *p*-HBA. In terms of cinnamic acids, great areas of caffeic acid and their derivatives were found. However, they were found with lower intensity in samples which exhibited more growth inhibition. As mentioned above, some flavonoids as chrysin and pinocembrin have been reported to exert antimicrobial activity by themselves. Pinocembrin was detected in samples 8, 10, 12 and 20 with great intensity except in sample 29, which was presented with less intensity. There is not report about antimicrobial effects of pinobanksin but in Iranian honeys has been found at high intensities.

Finally, royal jelly acid and its derivatives, in spite of not being

phenolic compounds, it has been reported its ability to inhibit growth of microorganism (Blum et al., 1959). In Iranian analyzed honeys, dihydroxy decenoic acid can be seen as the major compound. However, it was not found at high intensity in samples with greater antimicrobial activity (see Fig. 5).

4. Conclusions

In the present study, the antimicrobial activities of 33 honey phenolic extracts were characterized and tested against *Gram-positive* and *Gram-negative* strains. Around 50 different compounds were identified from Iranian honeys and classified into five chemical groups. MIC and inhibition zone of honey phenolic extracts were used to determine their antimicrobial effects. *P. aeruginosa* was the most resistant strain followed by *E. faecalis* and *S. aureus,* whereas *E. coli* was the weakest strain in this study. Only five samples (8, 10, 12, 20 and 29) exerted higher antimicrobial activity than whole extract. Regarding the phenolic composition, phenyllactic acid and *p*-HBA were the more abundant benzoic acids. On the other hand, cinnamic acids varied depending on the sample, being caffeic acid derivatives the most abundant ones. Furthermore, a high variety of flavonoids were found in all honey phenolic extracts presenting large compositional differences. Pinobanksin was the major flavonoid in the most samples. Royal jelly derivatives were also taking account since they could be associated with the antimicrobial power of honey. In this sense, dihydroxy decenoic acid was found in considerable amounts in all treated samples. These results have pointed out that phenolic fraction shown antimicrobial activity by itself. Therefore, given the differences in phenolic fractions, antimicrobial property could be more related to synergistic effects of phenolic compounds in samples than the antimicrobial exerted effect by an isolated compound.

Declarations of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2018.11.015.

References

Alvarez-Suarez, J. M., Tulipani, S., Díaz, D., Estevez, Y., Romandini, S., Giampieri, F., ... Battino, M. (2010). Antioxidant and antimicrobial capacity of several monofloral Cuban honeys and their correlation with color, polyphenol content and other chemical compounds. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association, 48*(8–9), 2490–2499. https://doi.org/10.1016/j.fct.2010.06.021.

Blum, M. S., Novak, A. F., & Taber, S. (1959). 10-Hydroxy-delta 2-decenoic acid, an antibiotic found in royal jelly. *Science (New York, N.Y.), 130*(3373), 452–453. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/13675771.

Bose, B. (1982). Honey or sugar in treatment of infected wounds? *The Lancet, 319*(8278), 963. https://doi.org/10.1016/S0140-6736(82)91959-6.

Brudzynski, K., Miotto, D., Kim, L., Sjaarda, C., Maldonado-Alvarez, L., & Fukś, H. (2017). Active macromolecules of honey form colloidal particles essential for honey antibacterial activity and hydrogen peroxide production. *Scientific Reports, 7*(1), 7637. https://doi.org/10.1038/s41598-017-08072-0.

Cádiz-Gurrea, M. L., Lozano-Sanchez, J., Contreras-Gámez, M., Legeai-Mallet, L., Fernández-Arroyo, S., & Segura-Carretero, A. (2014). Isolation, comprehensive characterization and antioxidant activities of Theobroma cacao extract. *Journal of Functional Foods, 10*, 485–498. https://doi.org/10.1016/j.jff.2014.07.016.

Erejuwa, O. O., Sulaiman, S. A., & Ab Wahab, M. S. (2012). Honey: A novel antioxidant. *Molecules, 17*(12), 4400–4423. https://doi.org/10.3390/molecules17044400.

Falcão, S. I., Vilas-Boas, M., Estevinho, L. M., Barros, C., Domingues, M. R. M., & Cardoso, S. M. (2010). Phenolic characterization of Northeast Portuguese propolis: Usual and unusual compounds. *Analytical and Bioanalytical Chemistry, 396*(2), 887–897. https://doi.org/10.1007/s00216-009-3232-8.

Güneş, M. E., Şahin, S., Demir, C., Borum, E., & Tosunoğlu, A. (2017). Determination of phenolic compounds profile in chestnut and floral honeys and their antioxidant and antimicrobial activities. *Journal of Food Biochemistry, 41*(3), 1–12. https://doi.org/10.1111/jfbc.12345.

Isidorov, V. A., Bagan, R., Bakier, S., & Swiecicka, I. (2015). Chemical composition and antimicrobial activity of Polish herbhoneys. *Food Chemistry, 171*, 84–88. https://doi.org/10.1016/j.foodchem.2014.08.112.

Jerković, I., & Kuś, P. M. (2014). Terpenes in honey: Occurrence, origin and their role as chemical biomarkers. *RSC Advances, 4*(60), 31710. https://doi.org/10.1039/C4RA04791E.

Jerković, I., Tuberoso, C. I. G., Baranović, G., Marijanović, Z., Kranjac, M., Svečnjak, L., et al. (2015). Characterization of summer savory (satureja hortensis L.) honey by physico-chemical parameters and chromatographic/spectroscopic techniques (GC-FID/MS, HPLC-DAD, UV/VIS and FTIR-ATR). *Croatica Chemica Acta, 88*(1), 15–22. https://doi.org/10.5562/cca2514.

Kečkeš, S., Gašić, U., Veličković, T.Ć., Milojković-Opsenica, D., Natić, M., & Tešić, Ž. (2013). The determination of phenolic profiles of Serbian unifloral honeys using ultra-high-performance liquid chromatography/high resolution accurate mass spectrometry. *Food Chemistry, 138*(1), 32–40. https://doi.org/10.1016/j.foodchem.2012.10.025.

Kirnpal-Kaur, B. S., Tan, H.-T., Boukraa, L., & Gan, S.-H. (2011). Different solid phase extraction fractions of tualang (koompassia excelsa) honey demonstrated diverse antibacterial properties against wound and enteric bacteria. *Journal of ApiProduct and ApiMedical Science, 3*(1), 59–65. https://doi.org/10.3896/IBRA.4.03.1.10.

Kucharski, R., Maleszka, J., Foret, S., & Maleszka, R. (2008). Nutritional control of reproductive status in honeybees via DNA methylation. *Science, 319*(5871), 1827–1830. https://doi.org/10.1126/science.1153069.

Kwakman, P. H. S., & Zaat, S. A. J. (2012). Antibacterial components of honey. *IUBMB Life, 64*(1), 48–55. https://doi.org/10.1002/iub.578.

Mahmoodi-Khaledi, E., Kashef, N., Habibi-Rezaei, M., & Moosavi-Movahedi, A. A. (2015). In vitro characterization of antibacterial potential of Iranian honey samples against wound bacteria. *European Food Research and Technology, 241*(3), 329–339. https://doi.org/10.1007/s00217-015-2464-4.

Mahmoodi-Khaledi, E., Lozano-Sánchez, J., Bakhouche, A., Habibi-Rezaei, M., Sadeghian, I., & Segura-Carretero, A. (2016). *Physicochemical properties and biological activities of honeys from different geographical and botanical origins in Iran.* European Food Research and Technologyhttps://doi.org/10.1007/s00217-016-2811-0.

Ouchemoukh, S., Amessis-Ouchemoukh, N., Gómez-Romero, M., Aboud, F., Giuseppe, A., Fernández-Gutiérrez, A., et al. (2016). Characterisation of phenolic compounds in Algerian honeys by RP-HPLC coupled to electrospray time-of-flight mass spectrometry. *LWT - Food Science and Technology*. https://doi.org/10.1016/j.lwt.2016.11.084.

Pasini, F., Gardini, S., Marcazzan, G. L., & Caboni, M. F. (2013). Buckwheat honeys: Screening of composition and properties. *Food Chemistry, 141*, 2802–2811.

Pimentel, R. B. de Q., da Costa, C. A., Albuquerque, P. M., & Junior, S. D. (2013). Antimicrobial activity and rutin identification of honey produced by the stingless bee Melipona compressipes manaosensis and commercial honey. *BMC Complementary and Alternative Medicine, 13*(1), 151. https://doi.org/10.1186/1472-6882-13-151.

Rao, P. V., Krishnan, K. T., Salleh, N., & Gan, S. H. (2016). Biological and therapeutic effects of honey produced by honey bees and stingless bees: A comparative review. *Brazilian Journal of Pharmacognosy, 26*(5), 657–664. https://doi.org/10.1016/j.bjp.2016.01.012.

Ristivojević, P., Trifković, J., Gašić, U., Andrić, F., Nedić, N., Tešić, Ž., ... Milojković-Opsenica, D. (2015). Ultrahigh-performance liquid chromatography and mass spectrometry (UHPLC-LTQ/Orbitrap/MS/MS) study of phenolic profile of Serbian poplar type propolis. *Phytochemical Analysis, 26*(2), 127–136. https://doi.org/10.1002/pca.2544.

Roshan, N., Rippers, T., Locher, C., & Hammer, K. A. (2016). Antibacterial activity and chemical characteristics of several Western Australian honeys compared to manuka honey and pasture honey. *Archives of Microbiology*, 1–9. https://doi.org/10.1007/s00203-016-1308-3.

Russell, K. M., Molan, P. C., Wilkins, A. L., Holland, P. T., Wilkins, A. L., & Holland, P. T. (1990). Identification of some antibacterial constituents of New Zealand manuka honey. *Journal of Agricultural and Food Chemistry, 38*(1), 10–13. https://doi.org/10.1021/jf00091a002.

Sergiel, I., Pohl, P., & Biesaga, M. (2014). Characterisation of honeys according to their content of phenolic compounds using high performance liquid chromatography/tandem mass spectrometry. *Food Chemistry, 145*, 404–408. https://doi.org/10.1016/j.foodchem.2013.08.068.

Sousa, J. M., de Souza, E. L., Marques, G., Meireles, B., de Magalhães Cordeiro, Â. T., Gullón, B., ... Magnani, M. (2016). Polyphenolic profile and antioxidant and antibacterial activities of monofloral honeys produced by Meliponini in the Brazilian semiarid region. *Food Research International, 84*, 61–68. https://doi.org/10.1016/j.foodres.2016.03.012.

Suárez-Luque, S., Mato, I., Huidobro, J. F., Simal-Lozano, J., & Sancho, M. T. (2002). Rapid determination of minority organic acids in honey by high-performance liquid chromatography. *Journal of Chromatography. A, 955*(2), 207–214. Retrieved from

http://www.ncbi.nlm.nih.gov/pubmed/12075924.
Viuda-Martos, M., Ruiz-Navajas, Y., Fernndez-Lpez, J., & Alvarez, J. A. P. (2008). Functional properties of honey, propolis, and royal jelly. *Journal of Food Science, 73*(9)https://doi.org/10.1111/j.1750-3841.2008.00966.x.
White, J. W., Jr., Subers, M. H., & Schepartz, A. I. (1963). The identification of inhibine, the antibacterial factor in honey, as hydrogen peroxide and its origin in a honey glucose-oxidase system. *Biochimica et Biophysica Acta, 73*(1), 57–70.
Wytrychowski, M., Chenavas, S., Daniele, G., Casabianca, H., Batteau, M., Guibert, S., et al. (2013). Physicochemical characterisation of French royal jelly: Comparison with commercial royal jellies and royal jellies produced through artificial bee-feeding. *Journal of Food Composition and Analysis, 29*(2), 126–133. https://doi.org/10.1016/j.jfca.2012.12.002.