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Bioactive Components and Antioxidant and Antibacterial Activities of Different Varieties of Honey: A Screening Prior to Clinical Application

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Supporting Information

ABSTRACT: This study assessed 16 different honey samples in order to select the best one for therapeutic purposes. First, a study of honey's main bioactive compounds was carried out. Then phenolic profiles were determined and specific compounds quantified using a HPLC system coupled to a mass spectrometer. Then, antioxidant activity, by three in vitro methods, and antibacterial activity against reference strains and clinical isolates were evaluated. Great variability among samples was observed regarding ascorbic acid (between 0.34 ± 0.00 and 75.8 ± 0.41 mg/100 g honey; $p < 0.001$), total phenolic compounds (between 23.1 ± 0.83 and 158 ± 5.37 mg/100 g honey; $p < 0.001$), and total flavonoid contents (between 1.65 ± 0.11 and 5.93 ± 0.21 mg/100 g honey; $p < 0.001$). Forty-nine different phenolic compounds were detected, but only 46 of them were quantified by HPLC. The concentration of phenolic compounds and the phenolic profiles varied widely among samples (between 1.06 ± 0.04 and 18.6 ± 0.73 mg/100 g honey; $p < 0.001$). Antioxidant activity also varied significantly among the samples. All honey varieties exhibited antibacterial activity against both reference and clinical strains (effective concentrations ranged between 0.05 and 0.40 g/mL depending on the honey sample and bacteria tested). Overall, samples with better combinations of bioactive properties were avocado and chestnut honeys.

KEYWORDS: *honey, bioactive compounds, antioxidant activity, antibacterial activity, phenolic profiles*

INTRODUCTION

Honey has been used as a medicinal remedy throughout the history of the human race: from ancient Egypt and the Classic civilizations (Greeks and Romans), who used honey in medicinal formulas, cosmetics, and perfumery or as embalming substance, to the Arab people of the Middle Ages, for whom honey was the basis of their pharmacy, as reflected in the Quran.[1] In modern medicine, with the advent of antibiotics and other drugs, the use of honey was abandoned, mainly due to the absence of scientific studies. However, in recent decades, several investigations have demonstrated the bioactive properties by which honey was empirically used.[2]

The miscellaneous composition of honey is responsible for the attributable numerous bioactive properties. Certain enzymes such as glucose oxidase and catalase, ascorbic acid, carotenoids, and melanoidins (Maillard reaction products) as well as phenolic acids and flavonoids are related to its antioxidant activity.[3] Antibacterial properties are associated with intrinsic characteristics such as high osmolarity and acidity and compounds such as hydrogen peroxide (H_2O_2), methyl syringate and methylglyoxal, defensin-1, nitric oxide metabolites, and phenolic acids and flavonoids.[4−7] In addition, honey may increase lymphocytic and phagocytic activity and likewise antibody production.[5]

The majority of recent studies investigating the bioactivity and the action mechanisms of honey have focused on well-characterized, standardized active manuka honey (MkH). MkH is produced from the nectar of different *Leptospermum* species, native to New Zealand and Australia. Its greater activity is related to non-peroxide activity, due to the presence of an abundant suite of phenolic compounds, such as methyl glyoxal, methyl syringate, and leptosin, that distinguish them from other types of honey.[2,8,9] However, in recent years, more and more studies are demonstrating the bioactive properties of other varieties of honey different from MkH.

Unfortunately, honey composition is rather variable, depending primarily on botanical origin,[10] conditioning its bioactive potential and hampering its further application for clinical purposes.[3,4,11] This fact highlights the importance of selecting an adequate variety of honey to carry out clinical

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assays,[7] which means a previous screening is necessary in order not only to quantify but also to determine profiles of bioactive substances, especially phenolic compounds; thus, the variation in these profiles might be responsible for the widely varying medical abilities of honey.

The working hypothesis of this study is whether distinct varieties of Spanish honey exhibit rather variable composition, especially regarding bioactive compounds, and in consequence significantly different therapeutic potential. In order to validate the veracity of this assumption, the aims of the study were (i) to quantify bioactive compounds in different honey samples (including a MkH sample as control), (ii) to identify and quantify individual phenolic compounds as major bioactive components present in honey, (iii) to determine the antioxidant activity of honey samples, and (iv) to determine their antibacterial activity against reference strains and clinical isolates. The overall goal was to compare different types of Spanish honey to select one that shows the best properties for potential therapeutic applications.

MATERIALS AND METHODS

Chemicals. Acetonitrile, acetic acid, formic acid, methanol, sodium carbonate, hydrochloric acid, and metaphosphoric acid were supplied by VWR Chemicals-Prolabo (VWR International). 2,2-Diphenyl-1-picrylhydrazyl (DPPH•), 2,6-dichloroindophenol, caffeic and gallic acids, flavonoid standards (rutin, quercetin, chrysin, and catechin), aluminum chloride, ferric chloride, and potassium ferricyanide were supplied by Sigma (St. Louis, MO, USA). All other chemicals were obtained from Merck (Darmstadt, Germany). All solvents were of analytical grade purity except for methanol, formic acid, and acetonitrile used in the identification and quantification of individual polyphenols, which were HPLC grade. Water was treated in a Milli-Q water purification system (Millipore, Molsheim, France).

Honey Samples. Fifteen samples of Spanish honey under quality brands (protected designation of origin *Miel de Granada* and *Miel de La Alcarria*, protected geographical indication *Miel de Galicia* and organic honey) with different botanical and geographical origins, as well as an MkH sample (MGO 550+; Manuka Health, Auckland, New Zealand), as a control sample, were used. Spanish honey samples, collected in two consecutive harvests, were previously characterized.[12] Table 1 summarizes the information related to botanical and geographical origin of honey samples, as well as the harvest year.

The samples were stored under dark conditions and refrigeration until analysis (few months after harvesting). They were homogenized by agitation before each determination.

Bioactive Compound Quantification. *Vitamin C Content.* Ascorbic acid (AA) content was determined following the recommended AOAC Official Titrimetric Method 967.21 for ascorbic acid in vitamin preparations and juices.[13] A 5 g portion of each sample was diluted in 5 mL of metaphosphoric acid acetic acid solution. The mixture was titrated with 2,6- dichloroindophenol dye solution. Vitamin C content was expressed in milligrams of ascorbic acid equivalents (AAE) per 100 g of honey.

Total Phenolic Content. Total phenolic content (TPC) was quantified by the Folin−Ciocalteu method according to Silici, Sagdic, and Ekici.[14] Absorbance was measured at 765 nm after 90 min of incubation at room temperature (UV−vis spectrophotometer; VWR UV-3100 PC). TPC was determined using a standard curve (y = 32.08x + 0.012; R^2 = 0.9996) of gallic acid (0−0.03 mg/mL). The results were expressed as milligrams of gallic acid equivalents per 100 g of honey.

Total Flavonoid Content. Total flavonoid content (TFC) was determined using the protocol described by Alvarez-Suarez et al.[3] A cadmium chloride solution was replaced by an aluminum chloride ($AlCl_3$) solution (10% w/v). Absorbance was measured immediately against the blank at 510 nm (UV−vis spectrophotometer; VWR UV-3100 PC). TFC was determined using a standard curve (y = 10.99x + 0.0052; R^2 = 0.9997) of (+)-catechin (0−0.03 mg/mL). The results were expressed as milligrams of catechin equivalents per 100 g of honey.

Table 1. Confirmed Botanical Origin, Year of Harvest, Quality Brand, and Geographical Origin of Spanish Honey Samples

sample identification	botanical origin	harvest year	quality brand	geographical origin
H1	multifloral	2010	PDO[a] *Miel de Granada*	Province of Granada
H1a	avocado (*Persea americana*)	2011	PDO *Miel de Granada*	Province of Granada
H2	chestnut (*Castanea sativa*)	2010	PDO *Miel de Granada*	Province of Granada
H2a	chestnut (*Castanea sativa*)	2011	PDO *Miel de Granada*	Province of Granada
H3	multifloral	2010	PDO *Miel de La Alcarria*	Province of Cuenca
H4	rosemary (*Rosmarinus officinalis*)	2010	PDO *Miel de La Alcarria*	Province of Cuenca
H4a	multifloral	2011	PDO *Miel de La Alcarria*	Province of Cuenca
H5	multifloral	2010	PGI[a] *Miel de Galicia*	Province of Pontevedra
H5a	multifloral	2011	PGI *Miel de Galicia*	Province of Pontevedra
H6	eucalyptus (*Eucalyptus* sp.)	2010	PGI *Miel de Galicia*	Province of Pontevedra
H6a	eucalyptus (*Eucalyptus* sp.)	2011	PGI *Miel de Galicia*	Province of Pontevedra
H7	multifloral	2010	certified organic honey	Province of León
H7a	thyme (*Thymus* sp.)	2011	certified organic honey	Province of León
H8	chestnut (*Castanea sativa*)	2010	certified organic honey	Province of León
H8a	multifloral	2011	certified organic honey	Province of León

[a]PDO, protected designation of origin; PGI, protected geographical indication.

Identification and Quantification of Individual Polyphenols. The identification and quantification of phenolic compounds were carried out following the protocol described by Truchado, Ferreres, and Tomás-Barberán[15] with slight modifications.

Polyphenolic Extract Preparation. Honey samples (20 g) were fully dissolved in 8 parts of acidified deionized water (adjusted to pH 2 with HCl). The solutions were centrifuged at 5000 rpm for 10 min (Eppendorf 5804R), and the supernatant was applied to a Sep-Pak Classic C_{18} cartridge (Waters, Medford, MA, USA) with a dropwise flow rate to ensure an efficient adsorption of the phenolic compounds. Phenolic content was eluted with HPLC grade methanol (2 mL). This methanolic extract was filtered through a 0.45 μm filter (Waters) and stored at −20 °C until subsequent analysis by HPLC.

Identification and Quantification of Polyphenolic Compounds. HPLC analyses were performed using an Agilent 1100 HPLC system equipped with a photodiode-array UV−vis detector and an ion-trap mass spectrometer detector in series (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was carried out on a reverse phase Poroshell120 C_{18} column (250 × 3.0 mm and 5 μm particle size) (Agilent Technologies) using water with 1% of formic acid (A) and acetonitrile (B) as mobile phases. The gradient profile was as follows: 0−20 min, 5−30% B; 20−40 min, 30−70% B; 40−45 min, 70−95% B; 46−48 min, 95−5% B; maintained at 5% for 55 min. All analyses were carried out at room temperature, with an injected volume of 20 μL and a flow rate of 1 mL/min. UV spectra were recorded from 210 to 600 nm, whereas chromatograms were monitored at 280, 320, 340, and 360 nm wavelengths.

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Table 2. Calibration Parameters for Phenolic Acids and Flavonoids Used As Standards (mg/mL) and Compound Class To Be Quantified by Each Standard

compound	linearity range	equation	R^2	LOD[a]	LOQ[a]	group to be quantified
gallic acid	0.01−0.30	$y = 49.39x$	0.999	0.02	0.05	hydroxybenzoic acids
caffeic acid	0.002−0.20	$y = 146.82x$	0.999	0.01	0.03	hydroxycinammic acids
quercetin	0.002−0.20	$y = 62.66x$	0.999	0.01	0.04	flavonols
naringenin	0.01−0.20	$y = 50.84x$	0.999	0.01	0.03	flavanonols and flavanones
chrysin	0.01−0.30	$y = 43.01x$	0.999	0.01	0.03	flavones
rutin	0.01−0.30	$y = 69.31x$	0.999	0.01	0.03	flavonol glycosides

[a]LOD, limit of detection in mg/mL; LOQ, limit of quantification in mg/mL.

Table 3. Ascorbic Acid, Total Phenolic Compounds, Total Flavonoids and Antioxidant Activity of Honey Samples (Mean SD; $n = 3$)[a]

	bioactive compounds			antioxidant activity		
honey sample	AAE[b]	TPC[c]	TFC[d]	radical scavenging activity[e]	reducing potential[f]	β-carotene bleaching inhibition[g]
H1	9.11 ± 0.61^{b}	158 ± 5.37^{a}	5.93 ± 0.21^{a}	9.25 ± 0.32^{mn}	26.3 ± 1.29^{m}	32.9 ± 1.47^{ij}
H1a	5.95 ± 0.32^{cd}	117 ± 2.74^{d}	3.30 ± 0.08^{bc}	13.8 ± 0.07^{kl}	30.3 ± 0.04^{lm}	56.9 ± 0.99^{g}
H2	3.64 ± 0.30^{f}	102 ± 1.53^{ef}	2.29 ± 0.14^{cde}	23.0 ± 0.38^{h}	55.3 ± 1.49^{i}	66.8 ± 1.76^{ef}
H2a	6.62 ± 0.05^{bc}	118 ± 3.50^{cd}	5.85 ± 0.21^{a}	9.83 ± 0.07^{lm}	43.0 ± 1.30^{j}	92.6 ± 0.58^{ab}
H3	2.41 ± 0.18^{fg}	67.9 ± 1.48^{gh}	4.06 ± 0.04^{ab}	38.0 ± 0.45^{ef}	34.7 ± 0.70^{jk}	58.4 ± 1.40^{fg}
H4	4.51 ± 0.00^{d}	23.1 ± 0.83^{l}	2.17 ± 0.11^{defg}	202 ± 5.53^{a}	215 ± 1.81^{a}	28.3 ± 1.09^{jk}
H4a	0.34 ± 0.00^{k}	27.7 ± 1.45^{kl}	2.02 ± 0.19^{efgh}	119 ± 0.02^{ab}	157 ± 1.47^{ab}	38.0 ± 0.44^{hi}
H5	1.35 ± 0.11^{hi}	67.5 ± 2.65^{gh}	1.95 ± 0.15^{fgh}	28.9 ± 0.43^{fg}	93.5 ± 0.35^{ef}	92.9 ± 0.52^{a}
H5a	0.88 ± 0.00^{ij}	56.6 ± 0.29^{hi}	1.89 ± 0.14^{gh}	28.2 ± 1.24^{g}	82.4 ± 0.95^{fg}	92.4 ± 0.28^{ab}
H6	0.34 ± 0.00^{k}	50.6 ± 1.64^{j}	1.65 ± 0.11^{h}	55.9 ± 0.35^{cd}	111 ± 1.02^{de}	71.8 ± 1.13^{de}
H6a	0.35 ± 0.00^{k}	50.5 ± 1.69^{j}	1.83 ± 0.22^{efgh}	74.1 ± 0.84^{bc}	118 ± 0.16^{cd}	82.1 ± 0.17^{bc}
H7	0.34 ± 0.00^{k}	51.3 ± 2.80^{ij}	2.25 ± 0.11^{cdef}	54.0 ± 0.81^{de}	147 ± 4.51^{bc}	68.1 ± 0.86^{ef}
H7a	75.9 ± 0.41^{a}	136 ± 2.50^{bc}	2.06 ± 0.22^{abc}	5.46 ± 0.05^{n}	54.1 ± 0.64^{i}	−1.34 ± 0.10^{k}
H8	3.61 ± 0.18^{ef}	142 ± 4.70^{ab}	2.97 ± 0.19^{bcd}	21.6 ± 0.33^{ij}	72.0 ± 0.78^{gh}	31.9 ± 1.51^{ij}
H8a	4.22 ± 0.32^{de}	114 ± 4.23^{de}	3.87 ± 0.04^{efgh}	15.1 ± 0.25^{jk}	63.7 ± 0.16^{h}	78.4 ± 0.84^{cd}
MkH	2.19 ± 0.13^{gh}	101 ± 1.92^{fg}	4.76 ± 0.26^{ab}	22.6 ± 0.50^{hi}	32.8 ± 0.33^{kl}	43.9 ± 0.91^{gh}

[a]In each column different letters mean significant differences ($p < 0.05$). [b]AAE: ascorbic acid equivalents (mg per 100 g of honey). [c]TPC: total phenolic content equivalents of gallic acid (mg per 100 g of honey). [d]TFC: total flavonoids content equivalents of catechin (mg per 100 g of honey). [e]EC_{50}: extract concentration (mg/mL) providing 50% of radical scavenging activity. [f]EC_{50}: extract concentration (mg/mL) providing 0.5 of absorbance. [g]Antioxidant activity: percentage of inhibition of β-carotene oxidation.

The HPLC system was coupled in series to an Esquire 1100 ion-trap mass spectrometer (IT) equipped with an electrospray ionization interface (ESI) (Bruker, Bremen, Germany) in negative mode. Nitrogen was used as a drying gas with a flow of 9 L/min and temperature of 350 °C and nebulizing gas at a pressure of 40 psi. The capillary voltage was set at 3500 V. Mass scan (MS) and daughter (MS−MS) spectra were recorded in the range of m/z 100−1500 with a control mass of m/z 700. The analyses were performed in duplicate.

Honey phenolic acids and flavonoids were identified according to their molecular weight (mass spectra), characteristic UV spectra, MS/MS fragmentations, and the wide information previously reported in the literature. Hydroxybenzoic acids were quantified using UV detection at 280 nm with the calibration curve obtained for gallic acid, hydroxycinnamic acids at 320 nm with the calibration curve obtained for caffeic acid, flavonols at 360 nm with the calibration curve of quercetin, flavanones at 280 nm with the calibration curve of naringenin, and flavones and flavonol glycosides at 340 nm with the calibration curves of chrysin and rutin, respectively. Calibration parameters are shown in Table 2.

Antioxidant Activity. *Radical Scavenging Activity Assay.* The radical scavenging activity (RSA) of honey samples was evaluated using the DPPH radical scavenging assay following the protocol described by Ferreira, Aires, Barreira, and Estevinho.[16] The concentration of water honey solutions tested ranged between 0 and 0.67 g/mL. Radical scavenging activity was calculated as a percentage of DPPH discoloration using the equation % RSA = $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$. The extract concentration providing 50% of radical scavenging activity (EC_{50}) was calculated by interpolation from the graph of RSA percentage against extract concentration.

Reducing Potential Assay. The ferric reduction power (RP) was evaluated using the protocol described by Ferreira, Aires, Barreira, and Estevinho.[16] The concentration of water honey solutions tested ranged between 0 and 0.11 g/mL. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated by interpolation from the graph of absorbance at 700 nm against extract concentration.

Inhibition of β-Carotene Bleaching Assay. The inhibition of β-carotene bleaching by honey samples was evaluated following the protocol described by Guerrini et al.[17] with slight modifications. A 4 mL portion of β-carotene in chloroform solution (0.2 mg/mL) was pipetted into a round-bottom flask containing 80 μL of linoleic acid and 800 μL of Tween 40 as emulsifier. The mixture was shaken, and chloroform was removed at 40 °C under vacuum. A 200 mL portion of distilled water, previously swamped in O_2, was added to the flask, which was vigorously shaken. Aliquots of 4.8 mL of this emulsion were transferred into different test tubes containing 0.2 mL of 300 mg/mL water−honey solutions. The tubes were shaken and incubated in darkness at 55 °C. The absorbance was measured at 470 nm (VWR UV-3100 PC) at the moment of emulsion addition and after 120 min. An emulsion without β-carotene was used as a control. The antioxidant activity (AA) expressed as a percentage of inhibition of β-carotene oxidation was calculated using the equation $AA = [100(DR_C - DR_S)]/DR_C$, where $DR_C = \ln(a/b)/120$ is the percentage of degradation of β-carotene in the control and $DR_S = \ln(a/b)/120$ is the percentage degradation of β-carotene in honey

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Table 4. Peak Numbers, Target Compounds, Average Expected Retention Times (R_t), and UV and MS Spectra of the Different Phenolic Compounds Identified in Honey Samples

peak no.	compound name	R_t (min)	UV_{max} (nm)	$[M - H]^-$ (m/z)	$-MS^n$ (m/z)
1	UI 1	8.45	306 sh, 316, 328 sh	188	144
2	UI 2	10.29	318 sh, 330	188	144
3	kynurenic acid	10.77	308, 332, 335 sh, 340 sh	188	144
4	caffeic acid	11.59	238, 296 sh, 322	179	161, 135
5	leptosperin	11.84	266, 296 sh	581	323, 211
6	quercetin-3-*O*-hex (1→2) hex[a]	13.92	259, 265 sh, 299 sh, 355	625	445, 301
7	8-methoxykaempferol-3-*O*- hex (1→2) hex[a]	14.87		639	624, 459, 315
8	kaempferol-3-*O*-hex (1→2) hex[a]	15.39	265, 296 sh, 349	609	447, 429, 285
9	*trans*-cinnamic acid	15.75	276	147	119, 103
10	8-*O*-methoxykaempferol-3-*O*-neoh[a]	15.97	310 sh, 324, 362 sh	623	608, 459, 315
11	quercertin-3-*O*-rutinoside	16.25	258, 260 sh, 291 sh, 349	609	301
12	ellagic acid	16.50	253, 367	301	301, 257, 229
13	kaempferol-3-*O*-neoh[a]	16.62	248, 262 sh, 298 sh, 326	593	429, 285
14	4-methoxyphenyllactic acid	16.70	274	195	177, 149
15	UI 3	16.73	298 sh, 309, 319 sh	144	133
16	isorhamnetin-3-*O*-neoh[a]	16.83		623	459, 315
17	Chlorogenic acid	18.40	298, 328	353	191, 179
18	isorhamnetin-*O*-pentoside	18.97	253, 346	447	315, 300
19	rosmarinic acid	20.23	294, 329	359	329, 286, 234
20	myricetin	20.30	255, 267 sh, 301 sh, 375	317	179, 151
21	tricetin	21.07	248, 267 sh, 302 sh, 351	301	151
22	methyl syringate	21.30	274	211	181
23	quercetin-3-*O*-rham[a]	21.63		447	301
24	*trans,trans*-abscisic acid	21.87	266	263	219, 201
25	*cis,trans*-abscisic acid	23.52	266	263	219, 201
26	quercetin	24.46	255, 370	301	179, 151, 121
27	naringenin 7-methyl ether	25.25	288, 320 sh	285	267, 252, 239
28	pinobanksin-5-methyl ether	25.31	286	285	267, 252, 239
29	quercetin 3-methyl ether	25.70	256, 355	315	300, 271, 255
30	*p*-coumaric acid isoprenil ester	26.42	294, 310	231	163, 119
31	pinobanksin	27.42	292	271	253, 225, 151
32	kaempferol	27.72	266, 370	285	161, 151, 135
33	isorhamnetin	28.37	253, 370	315	300, 151, 107
34	kaempferol methyl ether	28.79	265, 352	299	284
35	kaempferide	28.80	265, 364	299	284, 228, 212, 151, 132
36	quercetin 3,3-dimethyl ether	29.43	253, 355	329	314, 299, 285, 271
37	rhamnetin	30.94	256, 367	315	300, 165, 121
38	quercetin 3,7-dimethyl ether	32.00	256, 355	329	314, 299, 285
39	caffeic acid isoprenyl ester	32.83	298, 325	247	179, 135
40	caffeic acid benzyl ester	33.17	298, 325	269	178, 161, 134
41	chrysin	33.31	268, 314 sh	253	181, 151, 101
42	pinocembrin	33.57	289	255	213, 211, 151
43	galangin	34.03	265, 360	269	269, 241, 151
44	caffeic acid phenylethyl ester	34.24	295, 325	283	179, 135
45	6-methoxychrysin	35.08	265, 300 sh, 346 sh	283	268, 239, 211
46	galangin 5-methyl ether isomer	35.11	266, 302 sh, 360	283	268, 239
47	caffeic acid cinnamyl ester	36.05	295, 324	295	178, 134
48	pinobanksin-3-*O*-butyrate or isomer	39.28	292	341	271, 253
49	pinobanksin-3-*O*-pentenoate or isomer	41.43	292	353	271, 253

[a]hex (1 → 2) hex, hexosyl (1 → 2) hexoside; neoh, neohesperidoside; rham, rhamnoside.

samples: *a* = absorbance at time 0; *b* = absorbance after 120 min of incubation.

Antibacterial Activity. *Bacterial Strains, Drug Susceptibility, and Growth Conditions.* *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, and *Pseudomonas aeruginosa* as the main bacteria isolated from the oropharynx of patients suffering from oral mucositis (University Assistance Complex of León, Spain), as well as strains of these species from the Spanish Type Culture Collection (*S. aureus* CECT 86, *S. pyogenes* CECT 985, *E. coli* CECT 515 and *P. aeruginosa* CECT 110) were used. Clinical bacteria were identified using a MicroScan panel by Siemens (Camberley, U.K.).

The susceptibility of bacteria to different antibiotics was assessed by a plate microdilution method or a disk−plate diffusion method. Breakpoints were determined according to values defined by the Clinical and Laboratory Standards Institute.[18] Clinical strains, excluding *S. pyogenes*, exhibited resistance to several antibiotics tested. *S. aureus* was a methicillin-resistant strain (MRSA), *E. coli* was a

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Table 5. Phenolic Compounds and Abscisic Acid Contents (mg/100 g Honey) in the Different Honey Samples Studied[a]

compound name	honey sample															
	H1	H1a	H2	H2a	H3	H4	H4a	H5	H5a	H6	H6a	H7	H7a	H8	H8a	MkH
Cinnamic Acids and Derivates[b]																
UI 1			0.04	0.09				0.05	0.03	<LOQ	<LOQ	<LOQ	0.14	0.22	0.23	
UI 2			0.67	0.31				0.05	0.27	<LOQ	0.08	0.03		0.31	0.09	
kynurenic acid			1.16	0.40				0.27	0.36	<LOQ	0.10	0.12	0.12	0.45	0.15	
caffeic acid	0.18		<LOQ		0.11	<LOQ	0.04	0.03	0.03	<LOQ	<LOQ	0.03				
trans- cinnamic acid																0.07
UI 3			0.74	0.26				0.15	0.08			0.07		0.34		
chlorogenic acid												0.03				
rosmarinic acid						0.03										
p-coumaric acid isoprenyl ester					0.03											
caffeic acid isoprenyl ester			<LOQ	<LOQ	<LOQ	<LOQ	<LOQ					<LOQ				
caffeic acid benzyl ester												0.03			<LOQ	
caffeic acid phenylethyl ester			<LOQ	<LOQ	<LOQ	0.03	<LOQ	<LOQ	<LOQ						0.03	<LOQ
caffeic acid cinnamyl ester				<LOQ								<LOQ			<LOQ	<LOQ
Abscisic Acid[c]																
trans,trans-abscisic acid									<LOQ							
cis,trans-abscisic acid	1.62	0.97					<LOQ	<LOQ	0.05	1.27	2.09	0.13		2.77	5.12	
Other Phenolic Compounds[c]																
leptosperin																7.22
ellagic acid	0.63	<LOQ		1.00				0.17	0.45					0.27	0.18	
4-methoxyphenyllactic acid																1.95
methyl syringate																7.98
Total Phenolic Acids and Other Compounds																
	2.43^{cd}	0.97^{gh}	2.61^{bc}	2.06^{ef}	0.14^{lm}	0.06^{mn}	0.04^{n}	0.72^{hi}	1.27^{fg}	1.27^{fg}	2.27^{de}	0.44^{jk}	0.26^{kl}	4.36^{b}	5.80^{ab}	17.2^{a}
Flavonols and Glycosides[d,e]																
quercetin-3-*O*-hex (1→2) hex[h]										0.04	0.07					<LOQ
kaempferol-3-*O*-hex (1→2) hex[h]							0.03									
8-methoxykaempferol-3-*O*-neoh[h]								<LOQ	0.03							
quercetin-3-*O*-rutinoside		0.08				<LOQ	0.08									
kaempferol-3-*O*-neoh[h]		0.18				0.03							0.03			
isorhamnetin-*O*-pentoside															0.35	
myricetin										0.05	0.04					
tricetin										0.13	0.07					
quercetin	<LOQ	<LOQ	0.09	0.14	0.11	0.04	0.05	0.06	0.05	0.13	0.10	0.14	0.06	0.08	0.10	0.14
quercetin 3-methyl ether					0.05	<LOQ	0.04						<LOQ	0.06		
kaempferol	<LOQ	<LOQ	0.11	0.16	0.18	0.15	0.14	0.16	0.16	0.04	0.04	0.39	0.17	0.13	0.08	<LOQ
isorhamnetin	<LOQ	<LOQ	0.08	0.07	0.08	<LOQ	0.04	<LOQ	<LOQ		<LOQ		0.08	0.07	0.17	
kaempferol methyl ether			<LOQ		0.04	0.04	0.04	<LOQ	<LOQ			0.06	<LOQ	0.09		
kaempferide										<LOQ	<LOQ					
quercetin 3,3-dimethyl ether						<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.04	<LOQ	0.15	0.08	
rhamnetin	<LOQ	<LOQ	<LOQ	<LOQ	0.04	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.04	<LOQ	0.07	<LOQ	
quercetin 3,7-dimethyl ether	<LOQ	<LOQ	<LOQ	<LOQ	0.05	0.04	0.04	<LOQ	<LOQ	<LOQ	<LOQ	0.04	<LOQ	0.05	<LOQ	

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Table 5. continued

compound name	honey sample															
	H1	H1a	H2	H2a	H3	H4	H4a	H5	H5a	H6	H6a	H7	H7a	H8	H8a	MkH
Flavonols and Glycosides[d,e]																
galangin	<LOQ	<LOQ	<LOQ	0.04	0.09	0.07	0.07	0.05	0.06	0.06	0.08	0.15	0.04	0.10	0.05	<LOQ
galangin 5-methyl ether isomer	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ			
Flavanonols[f]																
pinobanksin 5-methyl ether	0.55	0.50			0.35							0.41				0.93
pinobanksin	0.08	0.06	0.19	0.28	0.47	0.26	0.32	0.37	0.39	0.55	0.64	0.56	0.25	1.20	0.25	0.03
pinobanksin-3-*O*-butyrate or isomer					<LOQ	<LOQ						<LOQ				
pinobanksin-3-*O*-pentenoate or isomer					<LOQ									<LOQ		
Flavanones[f]																
naringenin 7-methyl ether						0.16	0.17									
pinocembrin	<LOQ	<LOQ	0.10	0.12	<LOQ	0.29	0.21	0.18	0.23	0.28	0.34	0.56	0.12	0.53	0.21	0.14
Flavones[g]																
chrysin	0.06	0.07	0.07	0.14	0.24	0.15	0.20	0.05	0.15	0.09	0.21	0.33	0.04	0.34	0.13	0.14
6-methoxychrysin			<LOQ	<LOQ	0.04	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ		0.03	<LOQ	0.03	<LOQ	<LOQ
Total Flavonoids																
	0.69^{lm}	0.89^{jk}	0.64^{m}	0.95^{ij}	1.74^{bc}	1.25^{h}	1.45^{de}	0.88^{jk}	1.08^{hi}	1.38^{fg}	1.60^{cd}	2.75^{ab}	0.80^{kl}	2.90^{a}	1.42^{ef}	1.38^{fg}
Total Phenolic Compounds																
	3.12^{de}	1.86^{jk}	3.25^{cd}	3.01^{fg}	1.88^{jk}	1.29^{mn}	1.47^{lm}	1.50^{kl}	2.35^{hi}	2.65^{gh}	3.87^{bc}	3.19^{ef}	1.06^{n}	7.26^{ab}	7.22^{ab}	18.6^{a}

[a]Different letters in the same line indicate significantly different values ($p < 0.05$). [b]Calculated using the calibration curve of caffeic acid at λ 320 nm. [c]Calculated using the calibration curve of gallic acid at λ 280 nm. [d]Flavonols were calculated using the calibration curve of quercetin at λ 360 nm. [e]Flavonol glycosides were calculated using the calibration curve of rutin at λ 340 nm. [f]Calculated using the calibration curve of naringenin at λ 280 nm. [g]Calculated using the calibration curve of chrysin at λ 340 nm. [h]hex (1→2) hex, hexosyl (1→2) hexoide; neoh, neohesperidoside; rham, rhamnoside.

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producer of β-lactamases, and *P. aeruginosa* showed resistance against 9 of 14 antibiotics tested (Supporting Information).

All bacteria were grown in Mueller Hinton broth (MH; Sigma-Aldrich, St. Louis, MO, USA) at 37 °C with shaking (180 rpm) until the exponential growth phase (JP Selecta, Barcelona, Spain). Prior to experiments, bacteria were subcultured twice in MH agar to ensure the purity of cultures.

Honey Susceptibility. The minimal inhibitory concentration (MIC) was determined according to the M07-A9 protocol.[18] Honey concentrations between 400 and 6.25 mg/mL were tested. MIC values were defined after 24 and 48 h of incubation. MIC was the lowest concentration that prevented any discernible growth.

The minimal lethal concentration (MLC) was also determined by inoculating on MH agar plates 20 μL of each concentration tested from 96-well microtiter plates in which no growth was observed. MLC was defined as the lowest concentration that prevented any bacterial growth and reduced the viability of the initial inoculum by at least 99.9%. The tests were carried out in triplicate.

Statistical Analyses. Statistical analysis was performed using different packages (car, HH, agricolae, psych) of the open source statistical program R (version 3.3.3).[19] All variables were tested for the assumptions of normality and homoscedasticity. A Kruskal–Wallis test applying Bonferroni correction was utilized to compare the results between samples. $p < 0.05$ was considered to be significant. In addition, Spearman's correlation coefficient in bivariate linear correlations was used to study the relationship between bioactive compounds and bioactivity.

RESULTS AND DISCUSSION

Bioactive Compound Quantification (Vitamin C, TPC and TFC). The main bioactive compounds were quantified in the different honey samples. Vitamin C in honey comes essentially from nectar or honeydew and pollen, whereas phenolic compounds come from propolis in addition to nectar and pollen.[20] Thus, depending on honey botanical and geographical sources, bioactive compounds content might fluctuate considerably, as hereby reported (Table 3).

Vitamin C was detected in all honey samples. However, contents deeply differed among them, ranging from 0.34 to 75.9 mg/100 g of honey ($p < 0.001$). Sample H7a, a thyme honey, registered significantly higher values of AA, which corroborates that described in previous studies for this variety of honey.[21] On the other hand, two eucalyptus (H6 and H6a) and two polyfloral honey samples (H4a and H7) showed the lowest contents.

Similarly, the amounts of TPC and TFC varied considerably among samples ($p < 0.001$). TPC ranged between 23.1 and 158 mg/100 g of honey and TFC between 1.65 and 5.93 mg/100 g of honey. H1, a polyfloral honey, presented the highest values for both TPC and TFC, but in not all cases were the two parameters correlated. The lowest values of TPC and TFC were found in eucalyptus honey (H6 and H6a).

In addition, it is important to take into account that although the Folin–Ciocalteu assay is widely used to determine TPC in food extracts, it is not specific for phenolic quantification, considering that other types of compounds present in honey such as reducing sugars and amino acids can also reduce the Folin–Ciocalteu reagent.[22] In the present study a correction factor for interfering substances in the determination of TPC was not used because sugars, as principal interaction components in honey, present low solubility in methanol.[23] Nevertheless, it is necessary to consider that TPC determined may have values higher than the real ones. Similar circumstances occur with TFC; results may show an overestimation as some nonflavonoid compounds can exhibit absorbance at 510 nm.[22] However, despite the limitations posed, these methods allow a rapid and estimated evaluation of the availability of these compounds and their potential antioxidant activity.[24]

Identification and Quantification of Individual Polyphenols. Characterization of phenolic compounds and other bioactive components in honey intended for medical uses is essential, since these minor substances might be responsible for many of their health protective effects.[3]

The HPLC-ESI/MS analysis of honey extracts permitted identifcation of 49 different phenolic compounds on the basis of their UV and mass spectra and their MS/MS fragmentations (Table 4). However, only 46 of these were quantified due to some compounds coeluting under a single chromatographic peak with the same retention time (Table 5).

Cinnamic acids and their derivatives were the main phenolic acids found. Three compounds (UI 1, UI 2, and UI 3) were considered unknown but were tentatively identified. UI 3 (UV spectrum 319 sh, 309, 298 sh nm; MS m/z 144; MS^2 m/z 133) was previously described by Tomás-Barberán, Martos, Ferreres, Radovic, and Anklam[25] as marker of chestnut honey. UI 1 (UV spectrum 328 sh, 316, 306 sh nm; MS m/z 188; MS^2 m/z 144) and UI 2 (UV spectrum 330, 318 sh nm; MS m/z 188; MS^2 m/z 144) compounds are probably kynurenic acid derivatives in view of the similarities among the UV spectra and MS fragmentations of the three compounds (Table 4). Interestingly, the samples in which *Castanea sativa* was the predominant or secondary pollen (samples H2, H2a, H5, H5a, H8, and H8a), presented higher amounts of UI 1, UI 2, and UI 3, as well as kynurenic acid, which suggests the relationship between these compounds and a chestnut source. Furthermore, both isomers of abscisic acid previously described in other varieties of honey[26] were detected but only *cis,trans*-abscisic acid could be quantified in some samples. Other phenolic compounds, characteristic of MkH, as well as ellagic acid were identified.

Concerning flavonoids, four subclasses of compounds were discriminated: flavonols, flavanonols, flavanones, and flavones, as well as some flavonol glycosides mainly from quercetin, kaempferol, isorhamnetin, and 8-methoxykaempferol, which were previously described in different types of honey.[15] Moreover, specific floral markers were found in monofloral samples: myricetin and tricetin in eucalyptus honey,[15] kaempferol and derivatives in rosemary honey,[25] kynurenic acid in chestnut honey,[27] and leptosperin, 4-methoxyphenyllactic acid, and methyl syringate in MkH.[28]

The wide variability of honey samples was reflected in the phenolic profiles (Table 5). MkH was very different from the rest, and among other honey samples only eight compounds (quercetin, kaempferol, rhamnetin, quercetin 3,7-dimethyl ether, galangin, pinobanksin, pinocembrin, and chrysin) were common to all of them, as could be expected from their propolis origin and presence in beeswax. Furthermore, results evidenced three types of honey samples: those characterized by profiles dominated by phenolic acids (H1, H2, H2a, H8, H8a, and MkH in which phenolic acids represent between 60.1 and 92.6% of total phenolic compounds quantified), others in which flavonoids prevailed (H3, H4, H4a, H7, and H7a, in which flavonoids represent between 67.6 and 97.3% of total phenolic compounds quantified), and finally, those in which none of these compounds stood out (H1a, H5, H5a, H6, and H6a, in which phenolic acids and flavonoids represent around 50% of total phenolic compounds quantified).

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Table 6. Minimal Inhibitory Concentrations (MIC) and Minimal Lethal Concentrations (MLC) (g/mL) of Honey Samples against Reference and Clinical Strains of Bacteria[a]

	Gram-positive bacteria								Gram-negative bacteria										
	SA CECT86		MRSA		SP CECT985		SP clinical		EC CECT515		EC clinical		PA CECT110		PA clinical		statistical analysis[b]		
	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	R/C	G^+/G^-	[M]
H1	0.10	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.25	0.25	0.20	0.20	0.20	0.25	0.20	0.20	NS	NS	NS
H1a	0.05	0.05	0.10	0.10	0.20	0.20	0.20	0.20	0.20	0.20	0.10	0.10	0.20	0.20	0.20	0.20	NS	NS	NS
H2	0.05	0.05	0.10	0.10	0.20	0.20	0.20	0.20	0.20	0.20	0.10	0.10	0.20	0.20	0.10	0.10	NS	NS	NS
H2a	0.10	0.10	0.10	0.10	0.20	0.20	0.25	0.25	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	NS	NS	NS
H3	0.25	0.25	0.25	0.25	0.20	0.20	0.20	0.20	0.30	0.30	0.25	0.30	0.25	0.25	0.25	0.25	NS	*	NS
H4	0.25	0.30	0.20	0.20	0.25	0.25	0.20	0.30	0.30	0.30	0.25	0.25	0.30	0.35	0.25	0.25	NS	*	NS
H4a	0.20	0.20	0.20	0.25	0.20	0.25	0.20	0.25	0.30	0.30	0.25	0.30	0.30	0.30	0.25	0.25	NS	*	NS
H5	0.20	0.20	0.25	0.25	0.20	0.20	0.20	0.20	0.35	0.35	0.25	0.25	0.25	0.25	0.25	0.25	NS	**	NS
H5a	0.10	0.10	0.10	0.10	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	NS	*	NS
H6	0.10	0.10	0.10	0.10	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.30	0.20	0.20	NS	*	NS
H6a	0.10	0.20	0.10	0.10	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	NS	NS	NS
H7	0.10	0.10	0.25	0.25	0.20	0.20	0.20	0.20	0.20	0.20	0.25	0.25	0.20	0.20	0.25	0.25	NS	NS	NS
H7a	0.20	0.20	0.20	0.20	0.20	0.25	0.25	0.25	0.35	0.40	0.30	0.35	0.25	0.35	0.25	0.25	NS	**	NS
H8	0.10	0.10	0.10	0.10	0.20	0.20	0.20	0.25	0.20	0.20	0.10	0.20	0.20	0.20	0.20	0.20	NS	NS	NS
H8a	0.05	0.05	0.05	0.05	0.10	0.20	0.20	0.20	0.10	0.10	0.10	0.20	0.10	0.10	0.10	0.10	NS	NS	NS
MkH	0.10	0.10	0.10	0.10	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	NS	*	NS

[a]SA, *Staphylococcus aureus*; MRSA, methicillin resistant *Staphylococcus aureus*; SP, *Streptococcus pyogenes*; EC, *Escherichia coli*; PA, *Pseudomonas aeruginosa*. [b]R/C, reference strains vs clinical strains; G^+/G^-, Gram-positive bacteria vs Gram-negative bacteria; [M], MIC vs MLC; NS, no significant differences were observed; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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The content of total phenolic compounds ranged between 1.06 and 18.6 mg/100 g of honey in H7a and MkH, respectively. No correlation between total content of phenolic compounds quantified by HPLC and Folin−Ciocalteu assay was observed. This disparity might be explained because not all phenolic compounds present in honey samples were identified and/or quantified by HPLC and quantification of TPC through a Folin−Ciocalteu assay is only an estimation which was probably overvalued.[29]

In addition to their antioxidant and free radical scavenging abilities, polyphenols possess anti-inflammation, modulation of signal transduction, antimicrobial, and antiproliferation activities.[30] In addition to quantity, the specific phenolic profile may be a key factor, as particular polyphenols could function individually or act synergistically with other components to increase bioactive properties.[10] This standpoint highlights the importance of understanding the polyphenol composition of honey samples intended for medical uses.

Antioxidant Activity. Owing to the complex nature of matrix and involvement of multiple reaction characteristics and mechanisms, the antioxidant capacity of honey cannot be evaluated accurately by any single method. Therefore, a combination of assays will provide more information on the antioxidant properties.[31,32] In the current study three spectrophotometric methods were used.

Regarding the RSA assay, the sample H7a displayed the lowest concentration able to scavenger 50% of the free radicals (Table 3). The high concentration of vitamin C detected in this honey sample could explain the greater activity observed. AA has been described as a reducing agent capable of rapidly catching several reactive oxygen and nitrogen species (ROS and RNS).[30,33] However, no correlation between AA and RSA was observed. The absence of linear relations between variables does not exclude the presence of other nonlinear associations. Moreover, considering phenolic quantification by HPLC, H7a was the sample with the lowest concentration, which suggests that vitamin C is responsible for the antioxidant effects.

Similarly, phenolic compounds (TPC and TFC) may elucidate the results regarding the RP assay. On their behalf, phenolic compounds are capable of scavenging free radicals through electron and proton transfer mechanisms, as much as chelating metals,[30] which could explain the significant correlation observed between TPC and TFC with honey reducing capacity ($R = -0.80, -0.64$; $p < 0.01$, respectively). H1 was the sample that exhibited the highest values of TPC and TFC and likewise the best antioxidant activity in this assay.

Conversely, in the β-carotene inhibition bleaching assay no correlation was observed with bioactive compounds. The difficulties in finding relationships between data may be due to the lipid/water matrix used, especially because of the emulsifier introduced in the system against phase separation. The emulsifier may change the antioxidant distribution in the emulsified medium, and in turn the antioxidant activity, making it more difficult to interpret the results. Moreover, emulsifiers form micelles, which may trap antioxidants in these self-assembled structures and carry them to the water phase.[34]

In this assay, samples H2a, H5, and H5a presented similarly high antioxidant activities (more than 90% inhibition). In contrast, the H7a sample, which presented the best results in the RSA assay, acted as a pro-oxidant. This performance is apparently due to the high content of AA detected in this sample, which indeed exhibited a negative correlation with the inhibition of β-carotene bleaching ($R = -0.61$; $p < 0.05$). The pro-oxidant behavior of AA has been previously described[30,35] as a result of the formation of an ascorbyl radical during the oxidation reaction.[35]

A correlation between TPC and antioxidant activity was observed, suggesting that phenolic compounds are some of the main species responsible for the antioxidant capacity of honey.[33] However, for some samples, similar contents in TPC and TFC did not correspond to similar antioxidant capacities. This suggests that, although phenols remain the largest class of antioxidants found in nature, the overall antioxidant capacity of each sample results from the combined activity of other nonphenolic compounds.[32]

Among those compounds are proteins, amino acids, peptide inhibitors of oxidative enzymes, enzymes such as catalase or/and glucose oxidase, and organic acids such as gluconic, citric, and malic that could act by chelating metals and thus favor the action of other antioxidants such as polyphenols.[11,29] Moreover, the antioxidant properties of melanoidins (high-molecular-weight polymers formed in the final stage of the Maillard reaction)[36] have been described. Finally, because of the complex composition of honey, the interactions among the different compounds with antioxidant capacity and the possible synergies between them can also play an important role in the overall antioxidant capacity.[29,31,37]

Different assays provided different results, since each test assessed diverse action mechanisms in which a great variety of phytochemicals take part.

Antibacterial Activity. Honey antibacterial activity is associated with its physicochemical properties, as much as multiple compounds originating from the nectar of plants, pollen, propolis, and from the honeybee itself.[38] All honey samples exhibited antibacterial capacity against reference and clinical strains. However, effective concentrations ranged between 0.05 and 0.40 g/mL depending on honey variety and microorganism (Table 6).

Overall, *S. aureus* strains seemed to be the most sensitive bacteria, whereas *E. coli* strains were the most resistant. The outer membrane surrounding the peptidoglycan layer of Gram-negative bacteria offers a greater resistance to the entrance of antimicrobials.[31,39] However, in the current study, significant differences between Gram-positive and Gram-negative bacteria were not observed for all samples. Being a water-soluble substance, is feasible that honey was capable of accessing the periplasmic space of the bacteria through the porins, which act as hydrophilic conduits, as happens with other water-soluble molecules such as lactic acid.[39]

Significant differences between clinical and reference strains were not observed ($p > 0.05$), suggesting that honey samples were effective even against drug-resistant bacteria. New therapeutic options against emerging multi-drug-resistant pathogens are necessary, even more considering that some common infections have recently become extremely difficult or even impossible to treat.[40] Due to its peculiarities, honey might be a good option,[20,31] with little chance to resistance development by acting in a multifactorial way upon several bacteria target sites.[41] However, this natural substance remains underestimated in mainstream healthcare, in part due to the lack of comprehensive scientific evidence supporting its clinical use.[2]

Furthermore, honey samples exhibited not only bacteriostatic but also bactericidal effects. MLC values were similar or slightly higher than MIC values, and no significant differences between the concentrations were observed ($p > 0.05$).

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Honey antimicrobial activity has been related to physicochemical properties such as high osmolarity, low water activity, and acidity. Moreover, recent studies revealed that polyphenols are key components on antimicrobial effects of honey,[10,32] on their own or by reacting with H_2O_2. Thereby, benzoic acid can react with H_2O_2, resulting in peroxy acids, which are more stable and powerful than hydrogen peroxide and are capable of producing bacteria DNA degradation.[4,6] Conversely, in the present study no significant correlations between phenolic compounds and antibacterial activity were observed, as has been described in other studies.[42,43] Honey compounds interact among themselves, displaying an additive, synergistic, or antagonistic activity[7] which might not be explained by a simple linear relation.

To sum up, considering antioxidant activity, the honey samples with greatest potential were H1 and H2a, corresponding to a polyfloral and a chestnut honey, respectively. However, when the antibacterial capacity was analyzed, the best samples were H1a, H2, and H8a, corresponding to an avocado, a chestnut, and a polyfloral honey, respectively. Nevertheless, bioactivity needs to be understood as a combination of beneficial effects, and from this standpoint, H1a, H2, and H8a were the best samples; in addition to a greater antibacterial capacity, their antioxidant potential was appropriate. Although MkH bioactivity is well-known, in this study other varieties of honey were demonstrated to possess greater activity. Curiously, the phenolic profile seems to be a key factor, since honey samples with greater activity were not related with higher phenolic contents by HPLC, as occurred with H1a. No specific phenolic compounds have been described in avocado honey. Nevertheless, evidence encourages the study of possible markers characteristic of this variety, which could explain its higher bioactive functions.

Moreover, it is essential to underline that although polyfloral honey exhibited good bioactivity, its composition is even more variable than monofloral honeys due to the contribution, in different proportions, of several floral origins without any of them predominating. Not only the major but also a secondary floral source might considerably affect the composition and, consequently, bioactive properties.

Finally, considering that the potential therapeutic application of honey might result in dilution depending on the malady to treat, in vivo concentrations must be greater than those obtained as optimal in vitro, in order to maintain high levels of bioactive compounds in the lesion environment. For some drugs, cytotoxicity may then become a limitation, but this should not be an issue with honey, which could be used undiluted. Defining a correct posology for honey application will be essential for clinical success.

In conclusion, bioactive component contents and related bioactive activities among distinct varieties of honey were rather variable and depended primarily on their botanical origin, which confirms the initial hypothesis. The great variability observed reinforces the necessity to choose a proper type of honey for clinical application. Therefore, screening of a particular honey type composition, as well as its antioxidant and antimicrobial properties, is necessary prior to studies assessing in vivo the therapeutic potential of this natural product.

TPC and TFC provide a rapid and cheap estimation of phenolic compounds present in honey and their potential biological activity. However, these methods could overestimate phenolic content when other interference substances are present; therefore, other techniques that are more precise, such as HPLC-MS, are mandatory. In addition, knowing the phenolic profile is essential in order to identify the association between specific phenolic compounds and particular bioactivity properties.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b05436.

MIC values for different drugs against the bacteria tested (PDF)

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Notes

The authors declare no competing financial interest.

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