

# Antioxidant and antimicrobial effects of phenolic compounds extracts of Northeast Portugal honey

Letícia Estevinho \*, Ana Paula Pereira, Leandro Moreira, Luís G. Dias, Ermelinda Pereira

CIMO-Mountain Research Center, Escola Superior Agrária de Bragança, Instituto Politécnico de Bragança, Quinta de Santa Apolónia, Apartado 1172, 5301-855 Bragança, Portugal

Phenolic compounds of dark and clear honeys from Trás-os-Montes of Portugal were extracted with Amberlite XAD-2 and evaluated for their antioxidant and antimicrobial activities. The antioxidant effect was studied using the in vitro test capacity of scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical and of reducing power of iron (III)/ferricyanide complex. The antimicrobial activity was screened using three Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus lentus*) and three Gram-negative bacteria (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli*). The results obtained from the partial identification of honey phenolic compounds by high-performance liquid chromatography with a diode array detector showed that *p*-hydroxybenzoic acid, cinnamic acid, naringenin, pinocembrin and chrysin are the phenolic compounds present in most of the samples analyzed. Antioxidant potential was dependent of honey extract concentration and the results showed that dark honey phenolic compounds had higher activity than the obtained from clear honey. In the biological assays, results showed that *S. aureus* were the most sensitive microorganisms and *B. subtilis*, *S. lentus*, *K. pneumoniae* and *E. coli* were each moderately sensitive to the antimicrobial activity of honey extracts. Nevertheless, no antimicrobial activity was observed in the test with *P. aeruginosa*.

## 1. Introduction

Research indicates that honey have functional properties in human health promotion which depend largely on the floral source of the honey. These properties could be associated to honey high osmolarity and antibacterial properties (Effem, 1988). The major antibacterial properties are related to the level of hydrogen peroxide determined by relative levels of glucose oxidase and catalase (Weston, 2000). Honey differences in antimicrobial activity can be in part a reflection of these levels as well. The non-peroxide factors contributing to honey antimicrobial and antioxidant activity are the lysozyme, phenolic acids and flavonoids (Snowdon and Cliver, 1996). Taken as a whole, these factors give honey unique properties as a wound dressing: it leads to rapid clearance of infections, rapid debridement of wounds, rapid suppression of inflammation, minimization of scarring, and stimulation of angiogenesis as well as tissue granulation and epithelium growth (Molan, 2002).

Although the honey therapeutic action has been taken some attention by researchers, studies only have been done on screening the raw honey samples on antimicrobial activity (Taormina et al., 2001; Basualdo et al., 2007) and on antioxidant capacity (Rauha et al., 2000; Frankel et al., 1998). Others studies have shown that

individual phenolic compounds have growth inhibition on a wide range of Gram-positive and Gram-negative bacteria (Davidson et al., 2005). Phenolic compounds are one of the most important groups of compounds occurring in plants, comprising at least 8000 different known structures (Bravo, 1998). These compounds are reported to exhibit anticarcinogenic, anti-inflammatory, anti-atherogenic, antithrombotic, immune modulating and analgesic activities, among others and exert these functions as antioxidants (Vinson et al., 1998).

The honey phenolic compounds under study are the phenolic acids and flavonoids, which are considered potential markers of the honey botanical origin. The phenolic acids are divided in two subclasses: the substituted benzoic acids and cinnamic acids. The flavonoids present in honey are divided in three classes with similar structure: flavonols, flavones and flavanones. These are important due to their contribution to honey colour, taste and flavour and also due to their beneficial effects on health. Moreover, honey phenolic compounds composition and consequently antioxidant capacity depends on their floral sources used to collect honey which predominance are dependent of seasonal and environmental factors (Al-Mamary et al., 2002; Yao et al., 2003). So, different honey properties were expected since the composition of active compounds in honey from different locations should be different.

In this study, phenolic compounds of dark and clear honeys were extracted and analyzed. A screening of honey extracts

antibacterial activities against Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus* and *Staphylococcus lentus*) and Gram-negative bacteria (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli*) was also performed. The microorganisms *S. aureus*, *S. lentus*, *P. aeruginosa*, *K. pneumoniae* and *E. coli* are the isolated microorganisms from human skin wounds most representative in the Portugal Northeast Hospital Center. Also, the *B. subtilis* microorganism was used since it has a high sensibility to drug substances. Moreover, antioxidant properties of honeys extracts was also studied by testing their reducing power and scavenging effect on DPPH radicals activities.

## 2. Materials and methods

### 2.1. Reagents

#### 2.1.1. Reagents and standards for chemical analysis

All chemicals and reagents used were either analytical or HPLC grade. The water was treated in a Milli-Q water purification system (Milipore, Bedford, MA, USA) before use. The Amberlite XAD-2 was purchased to Supelco. The phenolic compounds used for the identification and quantification of phenolic acids and flavonoids in honey were purchased from Sigma-Aldrich and they are: benzoic acid, caffeic acid, cinnamic acid, *m*-coumaric acid, *p*-coumaric acid, ellagic acid, ferulic acid, gallic acid, gentisic acid, *p*-hydroxybenzoic acid, 2,4-dihydroxybenzoic acid, protocatechuic acid, salicylic acid, syringic acid, vanillic acid, galangin, kaempferol, quercetin, myricetin, apigenin, luteolin, chrysin, hesperetin, genkwanin, pinocembrin, naringenin and pinobanksin.

#### 2.1.2. Standards and reagents for microbiological assays

Ampicillin (analytical standard compounds) was of the highest available quality and purchased from Merck. Water was treated in a water purification system (TGI Pure water System, USA).

### 2.2. Honey samples

From Trás-os-Montes Beekeepers Association of Montesinho Natural Park of Bragança's District in Portugal, 20 honey samples were collected randomly with different colours, from lighter to darker honey for the phenolic profile evaluation. Dark and clear honey samples (two kinds of colour usually found in this region), with a typical phenolic compounds profile, were selected for phenolic compounds extraction for antibacterial and antioxidant properties study. In fact, Trás-os-Montes's honey and other regions of Portugal's in general is produced from nectar of *Lavandula*, *Echium* and *Erica* plants (Dias et al., 2008). Clear honey is obtained mainly from the nectar of *Lavandula* plants, ambar honey due to *Echium* as main plants and dark honey, generally a multi-floral honey, with *Erica* predominant nectar.

### 2.3. Honey extracts

Two honey phenolic compounds extraction procedures were carried out according to the extract amount needed for the subsequent analysis.

#### 2.3.1. Phenolic compounds extraction procedure for antimicrobial and antioxidant assays

The phenolic compounds (flavonoids and phenolic acids) extraction from the two honey samples was carried out as reported previously (Andrade et al., 1997a; Yao et al., 2003). The honey samples, about 300 g, were mixed with 1.5 L of HCl  $1 \times 10^{-2}$  mol/dm<sup>3</sup> and, after dissolution, filtered with a cotton filter for solid particles removal. Amberlite XAD-2 was added to the filtrate and afterwards, the mixture was stirred with magnetic stirrer for about 3 h at room temperature. The Amberlite particles were packed in a column, washed with HCl  $1 \times 10^{-2}$  mol/dm<sup>3</sup> (300 mL) and with desionized water (300 mL) for sugar and other honey compounds removal. The adsorbed phenolic compounds were extracted from the Amberlite by elution with 500 mL of methanol, which was evaporated by reduced pressure (Rotavapor Buchi RE 111 with a Buchi 461 Waterbath, 2002). The residue, dissolved in a little volume of water, was extracted for the phenolic compounds, three times with 30 mL of diethyl ether. The combined extracts suffered evaporation and, after measuring the weight extract, part of the residue was re-dissolved either with DMSO (dimethyl sulfoxide) for antimicrobial activity assays or with methanol for antioxidant tests.

#### 2.3.2. Phenolic compounds extraction for HPLC analysis

The honey sample, about 10 g, was mixed with hesperetin and 60 mL of HCl  $1 \times 10^{-2}$  mol/dm<sup>3</sup>. The Amberlite XAD-2 particles were packed in a glass chromatographic column, washed with 100 mL of methanol, 200 mL of desionized water and 50 mL of HCl  $1 \times 10^{-2}$  mol/dm<sup>3</sup> (this procedure was repeated for each sample). The honey mixture was filtered, with a cotton filter for solid particles removal, directly

to the column of Amberlite XAD-2. The eluate was added again to the column and then washed with HCl  $1 \times 10^{-2}$  mol/dm<sup>3</sup> (50 mL) and with desionized water (200 mL) for sugar and other honey compounds removal. The adsorbed phenolic compounds were extracted from the column by elution with 125 mL of methanol that was evaporated by reduced pressure (Rotavapor Buchi RE 111 with a Buchi 461 Waterbath, 2002). The residue dissolved in a little volume of water, was extracted, for the phenolic compounds, three times with 15 mL of diethyl ether. The combined extracts suffered evaporation and the final residue was re-dissolved with 1.00 mL of methanol. This solution was filtered with 0.2 µm Whatman nylon filter and analyzed by HPLC (Andrade et al., 1997b).

### 2.4. HPLC analysis

The honeys phenolic compounds analysis was performed in liquid chromatography equipped with Varian Pump model 9010, Rheodyne injector, Dyode-Array detector Varian ProStar model 330, column Heater Jones Chromatography model 7981 and using LC WorkStation Software. The analyses were carried out with the internal standard calibration method. The sample was injected in the HPLC system with a C18 column (Merck LichroCart 125-3), thermostated at 35.2 °C. The mobile phase consisted of phosphoric acid at pH 2.5 (eluent A) and methanol (eluent B). The injection volume for all samples was 10 µL. The elution was with flow rate 0.5 mL/min and the gradient program was as follows: 95–83% A (10 min), 83–74% A (10 min), 74–42% A (20 min), 42–5% A (10 min) and 5–95% A (10 min). The phenolic acids and flavonoids analysis were done at 280 nm and identification was carried out using the peak retention time and spectrum compared with those from commercial standards (Yao et al., 2003).

### 2.5. Antimicrobial tests

#### 2.5.1. Microorganisms and culture conditions

To determine the antimicrobial efficacy of the honey extracts, five bacterial strains *B. subtilis* CECT 498, *S. aureus* ESA 40, *E. coli* ESA 31, *P. aeruginosa* ESA 25, *K. pneumoniae* ESA 8 and *S. lentus* ESA 10 were selected. The microorganisms CECT were obtained from the Valencia University of their Spanish-type culture collection (CECT), while the microorganisms ESA were strains clinically isolated by Centro Hospitalar do Nordeste and identified in the Microbiology Laboratory of Escola Superior Agrária de Bragança. Microorganisms were cultured aerobically at 37 °C (Scientific 222 oven model, 2003) in bacteria nutrient agar medium for 24 h, without agitation. For experimental use, the microorganisms were sub cultured in liquid media, incubated for 24 h at 37 °C, with agitation (orbital shaker incubator: Stuart Scientific SI50 model, 2001) and used as the source of inoculums for each experiment.

#### 2.5.2. Test assays for antimicrobial activity

A screening of antibacterial activities with three Gram-negative bacteria (*E. coli*, *P. aeruginosa* and *K. pneumoniae*) and three Gram-positive bacteria (*B. subtilis*, *S. aureus* and *S. lentus*) was performed by measuring the minimal inhibitory concentrations (MICs in mg of extract/mL of DMSO) which were determined by an adaptation of the agar streak dilution method based on radial diffusion (Hawkey and Lewis, 1994; Ferreira et al., 2004). The MIC (minimal inhibitory concentration) considered to be the lowest concentration of the tested sample capable to inhibit growth of bacteria, after 24 h. Each inhibition zone diameter was measured three times (using a ruler) and the average was taken; standard deviations were less than 5%. Positive control using only inoculation and negative control using only DMSO in the hole were also carried out. Suspensions of the microorganisms were prepared to contain approximately  $10^8$  cfu/mL and then 100 µL of these suspensions were inoculated in plates containing agar medium. A 50 µL volume of each honey phenolic compounds extract (the range of phenolic compounds solutions concentration, mg of extract per mL of DMSO, used on these assays varies between 40 and 0.4 mg/mL) was pipetted into a hole (depth 3 mm, diameter 4 mm) made in the centre of the agar. Different concentrated solutions of ampicillin in DMSO were used as standard, under the same experimental conditions. All these assays were carried out in quadruplicate.

### 2.6. Antioxidant activity tests

#### 2.6.1. Reagents

BHA (2-*tert*-butyl-4-methoxyphenol) and  $\alpha$ -tocopherol were purchased from Sigma (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, USA). Methanol pro analysis was obtained from Pronalab (Lisboa, Portugal). Water was treated in a water purification system (TGI Pure Water Systems, USA).

#### 2.6.2. Reducing power assay

The reducing power was carried out according to the procedure described by Oyaizu (1988). A volume of 2.5 mL of solution of honey extracts with different concentrations (solutions with 12.5, 25 and 50 mg of extract/mL of methanol) were mixed with 2.5 mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and 2.5 mL

of 1% potassium ferricyanide. After incubation of this mixture at 50 °C for 20 min, a volume of 2.5 mL of 10% trichloroacetic acid (w/v) was added and the mixture was centrifuged at 1000 rpm in a refrigerated centrifuge at −4 °C (Centorion K24OR-2003), for 87 min. The following step consisted in the mixture of 5 mL of the upper layer with 5 mL of desionized water and 1 mL of 0.1% of ferric chloride. After mixture homogenization, the solution absorbance was measured spectrophotometrically at 700 nm. All the assays were carried out in triplicate. The extract concentration providing 50% inhibition (EC50) was calculated from the graph of absorbance registered at 700 nm against the correspondent honey extract concentration. BHA and  $\alpha$ -tocopherol were used as reference compounds.

### 2.6.3. Scavenging effect assay

The capacity to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was monitored according to a method reported before (Hatano et al., 1988). Volumes of 0.3 mL of various concentrations of honey extracts (solutions with 12.5, 25 and 50 mg of extract/mL of methanol) were mixed with 2.7 mL of methanolic solution containing DPPH radicals ( $6 \times 10^{-5}$  mol/l). Each mixture was shaken vigorously and left to stand in the dark until a stable absorption value was obtained at 517 nm. DPPH scavenging effect was calculated as percentage of DPPH discoloration using the equation: scavenging effect (%) =  $[(ADPPH-AS)/ADPPH] \times 100$ , where AS is the absorbance of the solution at each level of the sample extract added and ADPPH is the absorbance of the DPPH solution. All the assays were carried out in triplicate. The extract concentration providing 50% inhibition (EC50) was calculated from the graph of scavenging effect percentage against honey extract concentration. BHA and  $\alpha$ -tocopherol were used as reference compounds.

## 3. Results and discussion

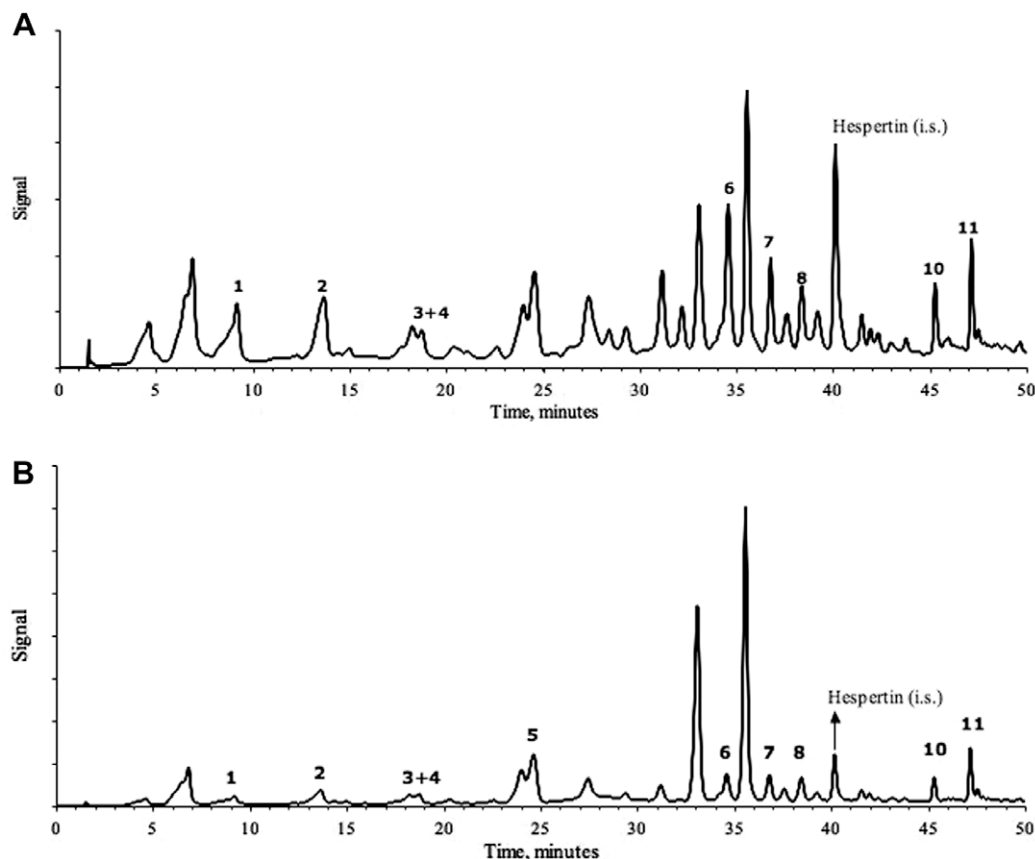
### 3.1. Phenolic compounds in honey

HPLC analysis of phenolic compounds in honey samples of Parque Natural de Montesinho Beekeepers Association of Trás-os-Montes region in Portugal showed that *p*-hydroxybenzoic acid, naringenin, pinocembrin and chrysin are the phenolic compounds

present in all honey samples analyzed. About 14 phenolic compounds have been identified (five flavonoids and nine phenolic acids) and the phenolic pattern of honey contains protocatequic acid, *p*-hydroxybenzoic acid, caffeic acid, chlorogenic acid, vanillic acid, *p*-coumaric acid, benzoic acid, ellagic acid, and cinnamic acid as well as the flavonoids naringenin, kaempferol, apigenin, pinocembrin and chrysin. Fig. 1 shows the typical HPLC chromatograms obtained for a clear and a dark honey samples. Of these compounds, phenolic acids are in higher concentrations than the flavonoids compounds. There were others compounds present in the chromatogram that had similar flavonoid and phenolic acid spectra and chromatographic behaviour but they could not be identified due to lack of availability of standard compounds. These identified phenolic compounds were already reported in honey (Andrade et al., 1997a; Yao et al., 2003; Aljadi and Yusoff, 2003). The total phenolic content extracted of the two honey samples used for the antibacterial and antioxidant properties study, measured by weight before the dissolution with DMSO, was in average 4.1 mg/100 g and 13.0 mg/100 g of honey for the clear and dark honeys, respectively. Dark honey samples have a significantly higher content of phenolic compounds extracted than clear honey samples ( $P < 0.01$ ).

### 3.2. Antimicrobial assays

The antimicrobial capacity of phenolic compounds, in a general way, is well known (Pereira et al., 2006; Rauha et al., 2000). As previously described, individual phenolic compounds present in honey extracts were identified and quantified, but we chose to submit the entire extracts to antimicrobial activity studies. In fact, total



**Fig. 1.** Typical HPLC chromatogram of phenolic compounds of a clear (A) and dark (B) honeys from Montesinho Natural Park. Phenolic acids are: (1) protocatequic acid, (2) *p*-hydroxybenzoic acid, (3) caffeic acid, (4) chlorogenic acid, (5) *p*-coumaric acid, (6) ellagic acid and (7) cinnamic acid. Flavonoids are: (8) naringenin, (9) kaempferol, (10) pinocembrin and (11) chrysin.

food extracts may be more beneficial than isolated constituents, since a bioactive individual component can change its properties in the presence of other compounds present in the extract (Borchers et al., 2004), corresponding to a synergistic effect. The minimal inhibitory concentration (MIC) values for bacteria (*B. subtilis*, *S. aureus*, *S. lentus*, *P. aeruginosa*, *E. coli*, and *K. pneumoniae*) were determined and results are presented in Table 1 (average of quadruplicate assays with percentage relative standard deviations less than 5%).

The results showed that honey phenolic compounds extracts obtained from the dark and clear honey have similar antimicrobial capacity inhibitory, with the exception of the *P. aeruginosa* microorganisms, but with different response degrees depending on the tested microorganism to honey extracts. The *S. aureus* was the most sensitive microorganism, with lower MIC (0.4 mg/mL). *Bacillus subtilis*, *S. lentus*, *K. pneumoniae* and *E. coli* were moderately sensitive to the antimicrobial activity of honey. In general, the Gram-positive bacteria were more sensitive to the honey phenolics compounds extracts than the Gram-negative bacteria. Our results were in agreement with the data observed by Cooper et al. (1999), Mundo et al. (2004) and Agbaje et al. (2006). These authors also observed that *S. aureus* was the most sensitive bacteria to manuka and pasture honeys. Results of the inhibitory activity of raw honey against pathogens have been presented by Taormina et al. (2001) and Basualdo et al. (2007) which are similar to the results obtained in this work, that have been carried out in different experimental conditions.

As expected, MICs ampicilline standards present lower MICs than honeys extracts. Usually, those pure active compounds reveal more activity than crude extracts (Pereira et al., 2006). In fact, concerning honey extracts activity, it is necessary to have phenolic compound concentrations 60 times higher than ampicilline in order to obtain similar antimicrobial activity for *S. aureus*, a more sensitive microorganism, and 600 times higher for *S. lentus*, *E. coli* and *K. pneumoniae*, more resistant microorganisms.

### 3.3. Antioxidant assays

Although several publications dealing with antioxidant activity of raw honey have appeared (Frankel et al., 1998; Aljadi and Kamaruddin, 2004; Al-mamary et al., 2002), no such information is available about antioxidant properties of the phenolic compounds extract of Trás-os-Montes honey of Portugal. In the present study, the antioxidant potential of phenolic compounds honey extracts (dark and clear honey) was measured by two different biochemical assays: reducing power and scavenging activity on DPPH radicals.

#### 3.3.1. Reducing power assay

Fig. 2 shows the reducing power of phenolic compounds extracts obtained from dark and clear honey as a function of their concentration. The presence of reducers (i.e. antioxidants) causes the reduction of the  $\text{Fe}^{3+}$ /ferricyanide complex to the ferrous (II) form, which can be measured by the formation of Perl's Prussian blue at 700 nm. The reducing power of the honey extracts in-

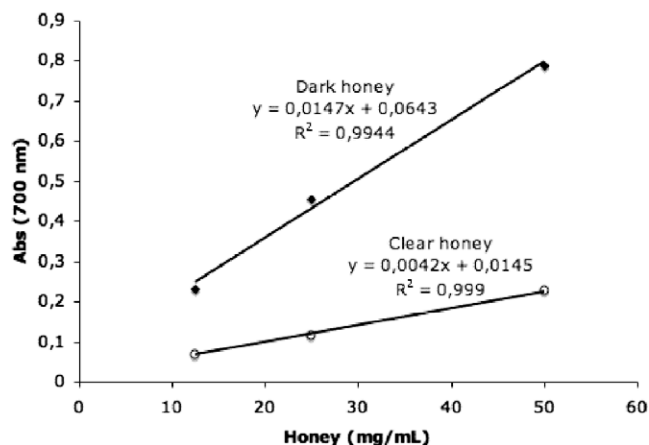


Fig. 2. Reducing power of methanolic extracts from honey.

creased with concentration: at 50 mg of extract/mL of DMSO the reducing power was 0.79 and 0.23, respectively for dark and clear honey; at 25 mg of extract/mL of DMSO, the reducing powers of the phenolic compounds extracts from honey were 0.45 and 0.12, and at 12.5 mg of extract/mL of DMSO were 0.23 and 0.07, respectively for dark and clear honey. Reducing power of BHA at 3.6 mg/mL and  $\alpha$ -tocopherol at 8.6 mg/mL was only 0.12 and 0.13, respectively. All these values are averages of the results obtained from triplicate assays and data variation was less than 5% (percentage relative standard deviation). The phenolic compounds extract from dark honey ( $\text{EC}_{50}$  29.6 mg/mL) proved to have a higher reducing power than the one obtained from the clear honey ( $\text{EC}_{50}$  value 115.6 mg/mL). These results showed that phenolic compounds extract from the dark honey sample has a higher reducing power than the one obtained from the clear honey sample.

#### 3.3.2. Radical scavenging effect assay

The radical scavenging effects of honey extracts were tested using methanolic solution of the "stable" DPPH free radical which exhibits a deep purple colour with maximum absorption at 517 nm. The DPPH free radical has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition (Amarowicz et al., 2004), unlike laboratory-generated free radicals such as the hydroxyl radical and superoxide anion. Antioxidant molecules can quench DPPH free radicals, resulting in discolouration of DPPH because of their conversion into a colourless/bleached product (i.e. 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine). Moreover, the more rapidly the absorbance decreases, the more potent the antioxidant activity of the extract. The scavenging effects of methanolic extracts from dark honey were examined and compared against clear honey (Fig. 3). Results are expressed as the ratio percentage of sample absorbance decrease relatively to the absorbance of DPPH solution in the absence of extract, at 517 nm. From the analysis of Fig. 3, we can conclude that the scavenging effects of honey extracts on DPPH

**Table 1**  
Antimicrobial activity phenolic compounds honey extracts against selected microorganisms

	MIC (mg/mL)					
	<i>Bacillus subtilis</i>	<i>Staphylococcus aureus</i>	<i>Staphylococcus lentus</i>	<i>Pseudomonas aeruginosa</i>	<i>Klebsiella pneumoniae</i>	<i>Escherichia coli</i>
Dark	4 (++++)	0.4 (++++)	40 (++++)	40 (–)	4 (++++)	40 (++++)
Clear	4 (++++)	0.4 (++++)	40 (++++)	40 (–)	40 (++++)	40 (++++)
Ampicillin	0.0125 (++++)	0.00625 (++++)	0.00625 (++++)	0.00625 (++++)	0.00625 (++++)	0.00625 (++++)

Note: Each value is expressed as mean ( $n=4$ ) and standard deviations were less than 5%. No antimicrobial activity (–) or inhibition (–), inhibition zone <1 mm. Slight antimicrobial activity (+), inhibition zone 2–3 mm. Moderate antimicrobial activity (++), inhibition zone 4–5 mm. High antimicrobial activity (+++), inhibition zone 6–9 mm. Strong antimicrobial activity (++++), inhibition zone >9 mm.



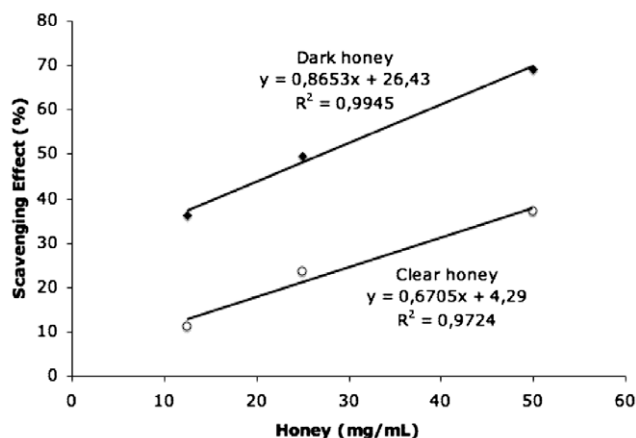


Fig. 3. Scavenging effect (%) on DPPH radicals of methanolic extracts from honey.

Table 2

EC<sub>50</sub> values<sup>a,b</sup> (mg/mL) of honey extracts in reducing power and DPPH scavenging assays

Samples	Reducing power (EC <sub>50</sub> <sup>a</sup> )	DPPH (EC <sub>50</sub> <sup>b</sup> )
Clear	115.6	68.17
Dark	29.64	27.24

<sup>a</sup> EC<sub>50</sub> (mg/mL): effective concentration at which the absorbance is 0.5.

<sup>b</sup> EC<sub>50</sub> (mg/mL): effective concentration at which 50% of DPPH radicals are scavenged.

radicals increased with the concentration increase and were high (69.2% at 50 mg of extract/mL of methanol) for dark honey. Extract from the clear honey presented moderate RSA (radical scavenging activity) values (37.0% at 50 mg/mL), respectively for the dark and clear honeys extracts. For other extract concentrations similar differences in magnitude were achieved: for 25 mg of extract per millilitre of methanol the RSA obtained were 49.4% and 23.5% and for 12.5 mg of extract per millilitre of methanol the RSA were 36.3% and 11.0%. However, the scavenging effects of BHA (3.6 mg/mL) and  $\alpha$ -tocopherol (8.6 mg/mL) were 96% and 95%, respectively. All these values are averages of the results obtained from triplicate assays and data variation was less than 5% (percentage relative standard deviation).

In Table 2, we present the EC<sub>50</sub> values for reducing power and DPPH scavenging effects obtained from each honey methanolic extract. Overall, dark honey revealed better antioxidant properties than clear honey (lower EC<sub>50</sub> values). The antioxidant activity of dark honey was higher than that of clear honey, which was due to the difference in their phenolic compounds contents and consequently their floral sources, as well verified by Aljadi and Kamaruddin (2004) and Al-Mamary et al. (2002) in their works with raw honey.

By comparing these findings with that reported by Frankel et al. (1998) and Chen et al. (2000), we noted that the free radical scavenging activities of both honeys were higher than the activities of Hawaiian Christmas berry, Tupelo, Sweet clover, Soy bean and Aca-cia honeys, but less than Gelam that of Buckwheat honey. However, ours results are similar to those obtained by Aljadi and Kamaruddin (2004) with Coconut honeys and by Baltrusaityte et al., 2007 ( ) with Lithuania honeys.

#### 4. Conclusions

The present study demonstrates that honey phenolic compounds are partially responsible for honey antibacterial and anti-

oxidant activity, corroborating the relevance of honey as a healthy alimentary product as a source of antioxidant and multi-resistant bacteria drug substances. We have shown that phenolic compounds extract obtained from the dark honey sample have stronger antioxidant activity (which can be attributed to their reductive capacity and scavengers of free radicals, and antimicrobial activity) than the clear honey sample. These results, also attributed to the differences in the phenolic compounds profile which are dependent of the honey geographic origin (flora predominance), are in accordance with other works that state that dark honey sample have phenolic compounds with higher microbiological inhibitors properties. Moreover, the extracted phenolic compounds from other natural products, for example, tables olives (Pereira et al., 2006), mushrooms (Barros et al., 2007), grape juice and wine (Mato et al., 2007), have lower antimicrobial effects comparatively to the ones obtained from phenolic compounds of honey extracts. These results show that honey is a natural product with therapeutic characteristics and that further studies should be carried out.

#### Conflict of interest statement

The authors declare that there are no conflicts of interest.

#### References

- Agbaje, E.O., Ogunsanya, T., Aiwerioba, O.I.R., 2006. Conventional use of honey as antibacterial agent. *Annals of African Medicine* 5, 79–81.
- Aljadi, A.M., Kamaruddin, M.Y., 2004. Evaluation of the phenolic contents and antioxidant capacities of two Malaysian floral honeys. *Food Chemistry* 85, 513–518.
- Aljadi, A.M., Yusoff, K.M., 2003. Isolation and identification of phenolic acids in Malaysian honey with antibacterial properties. *Turkish Journal of Medicine and Sciences* 33, 229–236.
- Al-Mamary, M., Al-Meer, A., Al-Habori, M., 2002. Antioxidant activities and total phenolic of different types of honey. *Nutrition Research* 22, 1041–1047.
- Amarowicz, R., Pegg, R.B., Rahimi-Moghaddam, P., Barl, B., Weil, J.A., 2004. Free-radical scavenging capacity of selected plant species from the Canadian prairies. *Food Chemistry* 84, 551–562.
- Andrade, P., Ferreres, F., Amaral, M.T., 1997a. Analyses of honey phenolic acids by HPLC, its application to honey botanical characterization. *Journal of Liquid Chromatography of Related Technologies* 20, 2281–2288.
- Andrade, P., Ferreres, F., Gil, M.I., Tomás-Barberán, F.A., 1997b. Determination of phenolic compounds in honeys with different floral origin by capillary zone electrophoresis. *Food Chemistry* 60 (1), 79–84.
- Baltrusaityte, V., Veuskutonis, P.R., Ceksteryte, V., 2007. Radical scavenging activity of different floral origin honey and beebread phenolic extracts. *Food Chemistry* 101, 502–514.
- Barros, L., Calhela, R.C., Vaz, J.A., Ferreira, I.C.F.R., Baptista, P., Estevinho, L.M., 2007. Antimicrobial activity and bioactive of Portuguese wild edible mushrooms methanolic extracts. *European Food Research and Technology* 225, 151–156.
- Basualdo, C., Sgro, V., Finola, M.S., Marioli, J.M., 2007. Comparison of the antibacterial activity of honey from different provenance against bacteria usually isolated from skin wounds. *Veterinary Microbiology* 124 (3–4), 375–381.
- Borchers, A.T., Keen, C.L., Gerstwin, M.E., 2004. Mushrooms, tumors, and immunity: an update. *Experimental Biology and Medicine* 229, 393–406.
- Bravo, L., 1998. Polyphenols: chemistry, dietary sources, metabolism and nutritional significance. *Nutrition Reviews* 56, 317–333.
- Chen, L., Mehta, A., Berenbaum, M., Zangerl, A.R., Enjeseth, N.F., 2000. Honeys from different floral sources as inhibitors of enzymatic browning in fruit and vegetables homogenates. *Journal of Agricultural and Food Chemistry* 48, 4997–5000.
- Cooper, R.A., Molan, P.C., Harding, K.G., 1999. Antibacterial activity of honey against strains of *Staphylococcus aureus* from infected wounds. *Journal of the Royal Society of Medicine* 92, 283–285.
- Davidson, P.M., Sofos, J.N., Brenem, A.L., 2005. *Antimicrobials in Foods*, third ed. Marcel Dekker Inc., New York. pp. 291–306.
- Dias, L.A., Peres, A.M., Vilas-Boas, M., Rocha, M.A., Estevinho, L., Machado, A.A.S.C., 2008. An electronic tongue for honey classification. *Microchimica Acta* 163, 97–102.
- Effem, S.E.E., 1988. Clinical observations on the wound healing properties of honey. *British Journal of Surgery* 75, 679–681.
- Ferreira, I.C.F.R., Calhela, R.C., Estevinho, L.M., Queiroz, M.J.R.P., 2004. Screening of antimicrobial activity of diarylamines in the 2,3,5-trimethylbenzo(b)thiophene series: a structure-activity evaluation study. *Bioorganic and Medicinal Chemistry Letters* 14, 5831–5833.

- Frankel, S., Robinson, G.E., Berenbaum, M.R., 1998. Antioxidant capacity and correlated characteristics of fourteen unifloral honeys. *Journal of Apicultural Research* 37, 27–31.
- Hatano, T., Kajawa, H., Yasuhara, T., Okuda, T., 1988. Two new flavonoids and other constituents in licorice root – their relative astringency and radical scavenging effects. *Pharmaceutical SOC Japan* 36, 2090–2097.
- Hawkey, P.M., Lewis, D.A., 1994. *Medical Bacteriology—A Practical Approach*. Oxford University, UK. pp. 181–194.
- Mato, I., Suárez-Luque, S., Huidobro, J.F., 2007. Simple determination of main organic-acids in grape juice and wine by using capillary zone electrophoresis with direct UV detection. *Food Chemistry* 102 (1), 104–112.
- Molan, P., 2002. Not all honeys are the same for wound healing. *Bulletin of the European Tissue Repair Society* 9, 5–6.
- Mundo, M.A., Padilla-Zakour, O.I., Worobo, R.W., 2004. Growth inhibition of food borne pathogens and foods spoilage organisms by select raw honeys. *International Journal of Food Microbiology* 97, 1–8.
- Oyaizu, M., 1988. Effects of amino-acids, organic-acids or sugar on antioxidative activity of browning products prepared from glucosamine. *Journal of the Japanese Society for Food Science and Technology* 35, 846–850.
- Pereira, J.A., Pereira, A.P.G., Ferreira, I.C.F.R., Valentão, P., Andrade, P.B., Seabra, R., Estevinho, L., Bento, A., 2006. Table olives from Portugal: compounds, antioxidant potential, and antimicrobial activity. *Journal Agricultural and Food Chemistry* 54, 8425–8431.
- Rauha, J.P., Remes, S., Heinonen, M., Hopia, A., Kähkönen, M., Kujala, T., Pihlaja, K., Vuorela, H., Vuorela, P., 2000. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *International Journal of Food Microbiology* 56, 3–12.
- Snowdon, J.A., Cliver, D.O., 1996. Microorganisms in honey. *International Journal of Food Microbiology* 31, 1–26.
- Taormina, P.J., Niemira, B.A., Beuchat, L.R., 2001. Inhibitory activity of honey against foodborne pathogens as influenced by the presence of hydrogen peroxide and level of antioxidant power. *International Journal of Food Microbiology* 69, 217–225.
- Vinson, J.A., Hao, Y., Su, X., Zubik, L., 1998. Phenol antioxidant quantity and quality in foods: vegetables. *Journal of Agricultural and Food Chemistry* 46, 3630–3634.
- Weston, R.J., 2000. The contribution of catalase and other natural products to the antibacterial activity of honey: a review. *Food Chemistry* 71, 235–239.
- Yao, L., Datta, N., Tomás-Barberán, F.A., Ferreres, F., Martos, I., Singanusong, R., 2003. Flavonoids, phenolic acids and abscisic acid in Australian and New Zealand *Leptospermum* honeys. *Food Chemistry* 81, 159–168.