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ORIGINAL ARTICLE

Key role of hydrogen peroxide in antimicrobial activity of spring, *Honeydew maquis* and chestnut grove Corsican honeys on *Pseudomonas aeruginosa* DNA

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Significance and Impact of the Study: We started to determine the antibacterial efficiency of Corsican chestnut grove and honeydew maquis honeys on *Pseudomonas aeruginosa*. No morphological alteration of the bacterial surface was observed. Antimicrobial action seems to be related to the synergy between hydrogen peroxide and phenolic compounds. The exerted pro-oxidant activity leads to a degradation of *P. aeruginosa* plasmidic DNA. This is the first study that investigate the primary antibacterial mechanism of Corsican honeys.

Keywords
antimicrobials, honey, mechanism of action, oxidation, pseudomonas.

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Abstract

In honeys, several molecules have been known for their antibacterial or wound healing properties. Corsican honeys just began to be tested for their antimicrobial activity with promising results on *Pseudomonas aeruginosa*. So, identification of active molecules and their mode of action was determined. Hydrogen peroxide concentrations were evaluated and, in parallel, the minimal inhibitory concentrations (MIC) values were performed with and without catalase. More, the quantity of phenolic compounds and ORAC assay were measured. Observation of antibacterial action was done using scanning electron microscopy (SEM) followed by plasmidic DNA extraction. MIC values of chestnut grove and honeydew maquis honeys vary between 7 and 8%, showing a strong antimicrobial capacity, associated with a plasmidic DNA degradation. When catalase is added, MIC values significatively increase (25%) without damaging DNA, proving the importance of H_2O_2. This hypothesis is confirmed by SEM micrographies which did not show any morphological damages but a depletion in bacterial population. Although, such low concentrations of H_2O_2 (between 23 μmol l^{-1} and 54 μmol l^{-1}) cannot explain antimicrobial activity and might be correlated with phenolic compounds concentration. Thus, Corsican honeys seem to induce DNA damage when H_2O_2 and phenolic compounds act in synergy by a putative pro-oxidant effect.

Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium found in soil, coastal marine habitats and marshes. It is known to be multidrug-resistant, notably due to its ability to acquire new resistance to antibacterial agents (Roberts *et al.* 2012). Its spontaneous mutation rate can be up to a thousand times higher than usual values (10^8/10^9) (Maciá *et al.* 2005). Its ability to form biofilms on inert surfaces such as rock or medical apparatus further enhances its resistance (Stover *et al.* 2000; Campeau *et al.* 2014). *Pseudomonas aeruginosa* is a very common cause of nosocomial diseases, from 11 to 13·8% worldwide, and more in intensive care units, where infection rates range

from 13·2 to 22·6%. It is the second most frequent cause of hospital-acquired, ventilator- and healthcare-associated pneumonia, after *Staphylococcus aureus* infections (Driscoll *et al.* 2012). Researchers are currently seeking naturally occurring substances to produce new antibiotics or agents able to restore antibiotic action towards this bacterium.

The antibacterial activity of honeys can be attributed to various factors. In most samples, it relies greatly on levels of hydrogen peroxide (H_2O_2) (Brudzynski 2006), which is, along with gluconic acid, a by-product of glucose oxidation by the bee enzyme glucose oxidase (FAD-oxidoreductase, EC 1.1.3.4). Water plays a key role in the reaction, facilitating the enzyme's access to its substrate. Hydrogen peroxide production in diluted honeys is known to produce a long-lasting antiseptic effect (Manzoori *et al.* 2006). However, some New Zealand Manuka honeys have been shown to maintain their growth inhibitory levels even after removal of hydrogen peroxide (Molan and Russell 1988). Antibacterial action of those honeys was later related to methylglyoxal (Molan 1992). Nonperoxide activity is mostly due to acidity, osmolarity (Wahdan 1998; Molan 1992) or phenolic compounds (Aljadi and Yusoff 2003). Phenolic compounds are widespread in plants, being their most abundant secondary metabolites (over 8000 phenolic structures already discovered). They are known to be involved in defence against aggression by pathogens (Dai and Mumper 2010). Antibacterial activity can also be expressed by the action of antimicrobial peptides such as bee-defensin-I (Kwakman *et al.* 2010).

In a previous study, we tested the antibacterial activity of some protected designation of origin 'Miel de Corse – Mele di Corsica' Corsican honey samples on several strains (Poli *et al.* 2018). We showed that *P. aeruginosa* was very sensitive to some Corsican honeys. So, the work reported here focus on this particular strain: we set out to make a more thorough study of the entities involved and their mechanisms of action on this bacterium at the cell level.

Results and discussion

Antibacterial activity of 'spring', 'chestnut grove' and 'honeydew maquis' honeys on *P. aeruginosa*

Minimal inhibitory concentration (MIC_{90}) values of 'spring' (SPR), 'chestnut grove' (CG) and 'honeydew maquis' (HDM) samples for *P. aeruginosa* are given in Table 1: MIC_{90} is the concentration of diluted honey that inhibits 90% of the bacterial growth.

Corsican honeys might offer a way to help fight the highly resistant strain *P. aeruginosa*. Indeed, both CG and HDM Corsican honeys were particularly active on this Gram-negative bacterium: measured MIC_{90} values (7 or 8%) were below those obtained with different Manuka honeys (12·5 or 14%) (Alzahrani *et al.* 2012; Kuś *et al.* 2016). Their activity was also higher than in acacia, lavender or wild carrot honeys (respectively 13, 21 and 12%) (Alzahrani *et al.* 2012), and in Gelam, Kelulut, pineapple and Tualang Malaysian honeys (respectively 10, 20, 25 and 12·5%) (Zainol *et al.* 2013). Despite a lower antimicrobial activity with an MIC value of 13%, SPR can still be considered as moderately active according to the literature. These results are supported by scanning electron microscopy (SEM) observations of CG 2 (Fig. 1).

Compared with the homogenous and very dense flora of the control (Fig. 1a), bacterial cells observed in the presence of CG 2 at its MIC_{90} were dispersed throughout the support (Fig. 1b). However, no morphological alteration or structural damage appeared on micrographs. Furthermore, when honey concentration reached 2 MIC_{90}, *P. aeruginosa* cells were no longer able to survive (Fig. 1c). Similar results were observed in SEM micrographs of CG 1, HDM 1, HDM 2 and SPR (data not shown). As previously described in the literature (Brudzynski *et al.* 2012a), we investigated the putative link between the cell death observed and DNA damage.

P. aeruginosa plasmidic DNA degradation

As shown in Fig. 2, after incubating *P. aeruginosa* with active honeys diluted to their MIC_{90} values, we observed plasmid DNA degradation.

The control shows a visible fragment of DNA corresponding to *c.* 23 kb of undamaged plasmid (Raja and Selvam 2009). Moreover, this DNA was digested by EcoRI restriction enzyme showing three visible strips and no sign of smear. In the presence of MIC_{90} diluted honeys, a smear of lower intensity was identified under the control fragment. These phenomena were mostly visible for CG 2 and HDM 1, which had similar amounts of hydrogen peroxide (Table 1). The appearance of a smudge of small DNA fragments is known to indicate that its degradation has involved irreparable double-strand cuts, which are lethal for the cell (Brudzynski *et al.* 2012a). Also, incubating *P. aeruginosa* at twice the MIC_{90} values showed no visible strip (data not shown).

In honeys, key factors in DNA damage are reactions of phenolic auto-oxidation and production of radical oxygen species (ROS) involving hydrogen peroxide as a central entity (Brudzynski *et al.* 2012b): MIC_{90} values rose to 25% (Table 1) when H_2O_2 was removed by catalase in

Table 1 MIC_{90} values and hydrogen peroxide concentrations obtained before and after catalase treatment

Honeys	MIC_{90} *P. aeruginosa* (%)	MIC_{90} *P. aeruginosa* after catalase treatment (%)	Hydrogen peroxide (μmol l^{-1})	Hydrogen peroxide after catalase treatment (μmol l^{-1})
Moderately active honey				
SPR	13	25	$23^{c} \pm 1{\cdot}15$	$1{\cdot}09 \times 10^{-2\ a} \pm 0{\cdot}02$
Active honeys				
CG 1	7	25	$47{\cdot}6^{b} \pm 2.38$	$1{\cdot}99 \times 10^{-2\ a} \pm 0{\cdot}002$
CG 2	8	25	$52{\cdot}4^{a} \pm 2.62$	$2{\cdot}96 \times 10^{-2\ a} \pm 0{\cdot}01$
HDM 1	7	25	$54^{a} \pm 2.7$	$5{\cdot}84 \times 10^{-2\ a} \pm 0{\cdot}01$
HDM 2	8	25	$50^{ab} \pm 2.5$	$4{\cdot}13 \times 10^{-2\ a} \pm 0{\cdot}02$

CG 1, chestnut grove 1; CG 2, chestnut grove 2, SPR, spring; HDM 1, honeydew maquis 1; HDM 2, honeydew maquis 2.
Lowercase letters indicate significant differences between experimental conditions for hydrogen peroxide (concentration) according to a Kruskal–Wallis test at the 95% confidence level.

the five tested honeys, neutralizing their antibacterial efficiency. This data demonstrate the importance of hydrogen peroxide in antimicrobial activity. As shown in Fig. 2, the addition of catalase to the tested honeys showed a strip with the same size as the control, demonstrating that removing hydrogen peroxide abolished the antibacterial action. Measured concentrations of H_2O_2 (Table 1) provide further evidence: the hydrogen peroxide content did not differ substantially in the active CG and HDM honeys (from 47·6 μmol l^{-1} to 54 μmol l^{-1}), but was significantly lower in the moderately active SPR honey (23 μmol l^{-1}). Similar concentrations were found in moderately active *Brassica napus* and *Castanea sativa* honeys with H_2O_2 concentration close to 40 μmol l^{-1} (MIC_{90} values: 20%; Bucekova *et al.* 2014). These levels were still far from the H_2O_2 concentration in *Abies alba* active honeys, ranging around 200 μmol l^{-1} for a 10% MIC_{90} value.

Metal-catalysed H_2O_2 degradation by ions such as Cu^{+} or Fe^{2+}, naturally present in honeys (Bogdanov *et al.* 2007), produces hydroxyl radicals. These radicals are generated via a Fenton reaction ($H_2O_2 + Cu^{+}$ or $Fe^{2+} \rightarrow Cu^{2+}$ or $Fe^{3+} + OH + {\cdot}OH^{-}$) and are, according to the literature (Storz and Imlay 1999), more often responsible for DNA damage than endogenous H_2O_2. Moreover, hydrogen peroxide hydrolysis also produces oxygen, which speeds up auto-oxidation processes of polyphenols with simple structures like coumarin identified in some honeys (Cowan 1999; Jerković *et al.* 2011). Those kind of molecules are known to exert antibacterial and fungicidal activities (Cowan 1999) because they tend to lose their antioxidant capacity and become pro-oxidant. Table 2 represents the concentration of phenolic compounds and oxygen radical absorbent capacity (ORAC) assay values in Corsican honeys.

In both cases, they were significantly higher in the active honeys (CG 1, CG 2, HDM 1 and HDM 2) than in the moderately active (SPR) honey: the total phenolic content ranged from 73·62 mg gallic acid equivalent (GAE)/100 g of honey in HDM 1, to 86·15 mg GAE/100 g of honey in HDM 2, whereas only 24·07 mg GAE/100 g of honey was detected in SPR. These results are in agreement with those of Ciappini and Stoppani (2014), although the overall range was greater (5–265 mg GAE/100 g of honey in various clover and multifloral honeys from Chile and Czech Republic). ORAC assay values lay between 5·89 μmol l^{-1} of trolox equivalents (TE)/g of honey for CG 1 and 8·6 μmol l^{-1} TE/g of honey in HDM 2. In SPR, ORAC values were significantly lower (2·76 μmol l^{-1} TE/g of honey). So, CG and HDM honeys have higher antioxidant capacity than SPR honey. The same range of ORAC values were found for monofloral honeys of clover (5·37 μmol l^{-1} TE/g of honey), blueberry (4·59 μmol l^{-1} TE/g of honey) or borage (1·99 μmol l^{-1} TE/g of honey) (Brudzynski *et al.* 2012b). ORAC assay was preferred to the hydroxyl radical generating activity. Indeed, Brudzynski *et al.* (2012a) showed that it isn't possible to conclusively identify ·OH radicals as the inducers of bacterial cell death.

According to Dai and Mumper (2010), under conditions that favour their auto-oxidation (high pH with high concentrations of transition metal ions and molecular oxygen) phenolic antioxidants behave like pro-oxidants. Despite a high antioxidant capacity (ORAC, Table 2), the high content of hydrogen peroxide in active honeys, which induces an elevated amount of ROS, explains the pro-oxidant behaviour of phenolic compounds. It seems that the presence of these two types of entity confers antibacterial activity on Corsican honeys via plasmid DNA degradation.

In conclusion, we showed that *P. aeruginosa* is sensitive to several Corsican honeys. Indeed, CG and HDM honeys are the most active (MIC_{90} value between 7 and

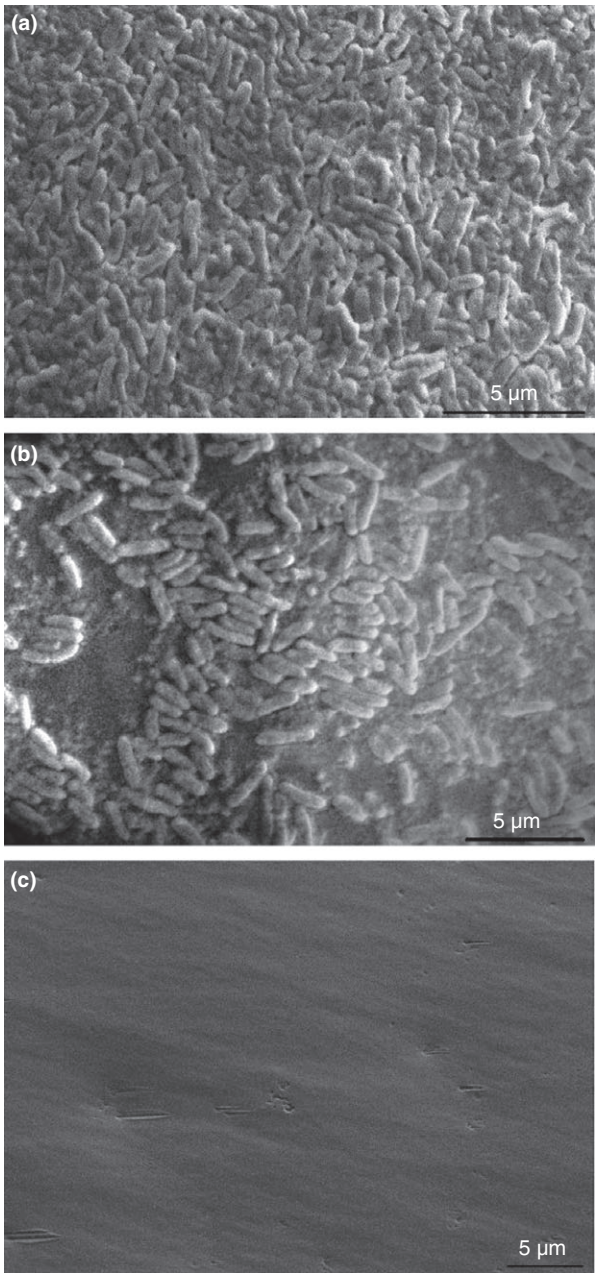

Figure 1 Scanning electron micrographies of *Pseudomonas aeruginosa* growth in absence (control: a) or in presence of CG 2 honey at MIC90 (b) and twice MIC90 (c). Scale bar = 5 μm.

8%), while SPR honey can be considered as a moderately active honey (MIC_{90} value equal to 13%). Their antibacterial efficiency seems to arise from an interaction between hydrogen peroxide and phenolic compounds. However, H_2O_2 is a key factor in phenolic compound auto-oxidation and in Fenton's reaction, which lead to reactive oxygen species formation. These ROS may be responsible for the *P. aeruginosa* plasmid degradation we highlight in this work. Since SEM micrographs showed no morphological degradation, this mechanism of action could be involved in Corsican honeys antibacterial activity. An in-depth study is now required to establish the complete phenolic profile for all the honeys and so identify active entities.

Materials and methods

Honey samples

Five honey samples from the year 2014, one 'spring' (SPR), two 'chestnut grove' (CG 1 and CG 1) and two HDM 1 and HDM 2, were purchased commercially from local apiarists and stored in darkness at 4°C. Sensory analyses were performed to check good conservation.

Bacterial strains and growth conditions

The *in vitro* antibacterial activity of Corsican honeys was tested against a laboratory-controlled strain of *P. aeruginosa* (CIP A22) routinely grown at 37°C on Mueller-Hinton 2 Agar (BioMérieux, Marcy l'Etoile, France).

Antibacterial assay

The antibacterial activity was expressed as the minimum inhibitory concentration (MIC) of honey that reduced bacterial growth spectrophotometrically by 90%. The test was adapted from Zainol *et al.* (2013) with slight modifications. Working bacteria culture was adjusted to be equal to 0·5 McFarland standard (10^8 CFU per ml), and further diluted 200-fold in Muëller–Hinton broth (MHB, oxoid) to reach $0{\cdot}5 \times 10^6$ CFU per ml. Honey samples were diluted (from 50 to 1%) in MHB and then filtered through 0·2 μmol l^{-1} filters (Sartorius AG, Göttingen, Germany). A volume of 190 μl of each honey dilution was aseptically transferred into 96-well microplates (Nunc™, Roskilde, Denmark) with eight replicates per dilution. The first two wells of each honey dilution served as dilution sterility controls (supplemented with another 10 μl of the respective honey dilution); six others were the test wells, in which 10 μl of bacteria culture was mixed. The last two rows were reserved for batch sterility (200 μl of MHB) and growth (10 μl of bacterial culture in 190 μl of MHB) controls. Plates were incubated in a shaker incubator (Stuart, Stone, UK) at 120 rev min^{-1}, at 37°C for 18–24 h. The absorbance of the wells was read at 620 nm using a microtitre plate reader (Sunrise, Tecan™) after incubation. Percentage bacterial growth inhibition was calculated using the following formula:

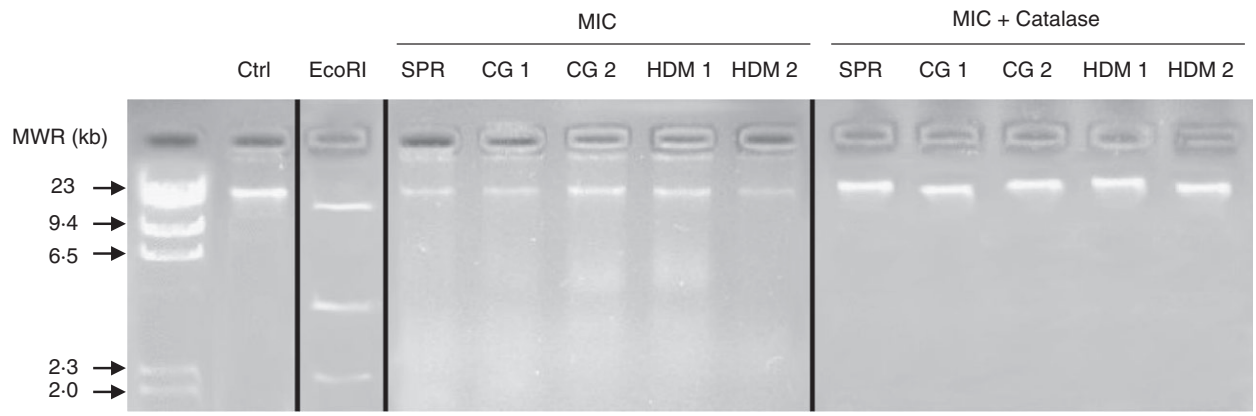

Figure 2 Action of 'Chestnut grove' (CG1 and CG2), 'Honeydew maquis' (HDM1 and HDM2) and 'Spring' (SPR) Corsican honeys on *Pseudomonas aeruginosa* plasmidic DNA (Ctrl: control; EcoRI: *P. aeruginosa* plamisdic DNA digested by EcoRI restriction enzyme).

Table 2 Total phenolic content and radical scavenging activity of Corsican honeys

Honeys	Total phenolic content (mg gallic acid equivalent/100 g of honey)	ORAC (μmol l^{-1}ol TE/g of honey)
Moderately active honey		
SPR	$24{\cdot}07^{c} \pm 1{\cdot}2$	$2{\cdot}76^{c} \pm 0{\cdot}28$
Active honeys		
CG 1	$76{\cdot}01^{b} \pm 3{\cdot}8$	$5{\cdot}89^{b} \pm 1{\cdot}03$
CG 2	$78{\cdot}41^{b} \pm 3{\cdot}92$	$6{\cdot}98^{b} \pm 1{\cdot}47$
HDM 1	$73{\cdot}62^{b} \pm 3{\cdot}68$	$7{\cdot}2^{ab} \pm 0$
HDM 2	$86{\cdot}15^{a} \pm 4{\cdot}31$	$8{\cdot}6^{a} \pm 0$

CG 1, chestnut grove 1; CG 2, chestnut grove 2, SPR, spring; HDM 1, honeydew maquis 1; HDM 2, honeydew maquis 2.
Lowercase letters indicate significant differences between experimental conditions for total phenolic content (concentration) or ORAC (concentration) assays according to a Kruskal–Wallis test at the 95% confidence level.

Throughput (HTP) Assay Innovotech Inc., Edmonton, AB, Canada) was fitted. These pegs allow bacterial development in a biofilm. The microplates were shaken at 37°C for 36 h. The pegs were immersed in 2·5% glutaraldehyde (Electron Microscopy Science™, Hatfield, PA, USA) fixing solution in sodium cacodylate (Sigma-Aldrich™, Saint-Louis, MO, USA) buffer 0·1 mol l^{-1} (pH 7·2) for 2–3 h at 4°C; they were washed in two successive 10 min baths of sodium cacodylate buffer, dried out for 120 h, and then metallized using a gold/palladium mixture. The pegs were then examined under a scanning electron microscope (Hitachi™ s-3400n, Tokyo, Japan) with an accelerating voltage of 5 or 10 kV. Each experiment was performed in triplicate. Scanning electron micrographies are a representation of the homogenous observation of the entire pegs surface.

Plasmid DNA extraction

Bacteria cultures adjusted to 10^7 CFU per ml were incubated overnight, and shaken at 37°C in MHB with most active honeys used at concentrations of MIC and 2 × MIC. Plasmid DNA was isolated using Quantum® prep plasmid miniprep kit (Bio-Rad™, Hercules, CA, USA) following the manufacturer's instructions.

Agarose gel electrophoresis

Aliquots of plasmid DNA (30 μl) were analysed by agarose gel (1%) electrophoresis with ethidium bromide in Tris base, acetic acid and EDTA 1X. Migration was

$$\left(1-\frac{(\text{absorbance of test well}-\text{absorbance of corresponding control well})}{(\text{absorbance of assay growth control}-\text{absorbance of sterility control})}\right)\times 100$$

Each experiment was performed in triplicate.

Scanning electron microscopy

Scanning electron microscopy allowed the behaviour of bacteria to be observed in solution with honey samples. For this purpose, 7·5 μl of each honey dilution was placed in a 96-well microplate containing 142·5 μl of a 0·5 McFarland standard diluted to reach $0{\cdot}5 \times 10^6$ CFU per ml. A plastic lid with 96 identical pegs protruding downward (MBEC™

carried out at 85 V for 40 min. Plates were examined under UV light. EcoRI restriction enzyme was used as a digestion control of untreated plasmid.

H_2O_2 assay and catalase treatment

Hydrogen peroxide in honey was measured with the Amplex® Red hydrogen peroxide/peroxidase assay kit (Molecular Probes, Invitrogen, Burlington, ON, Canada). The assay detects H_2O_2 using Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxasine), which reacts with hydrogen peroxide to produce the red fluorescent oxidation product resorufin. The assay was conducted according to the manufacturer's instructions. Fluorescence was measured with a spectrofluorimeter (FMP-850, Jasco™ Oklahoma City, OK, USA) at an emission wavelength of 590 nm using an excitation wavelength of 545 nm. The H_2O_2 standard curve was plotted using dilutions of a 100 μmol l^{-1} stock solution. Similarly, 40% honey dilutions were prepared extemporaneously (Bucekova *et al.* 2014), and were incubated with Amplex Red reagent for 30 min in the dark at room temperature. The blank was included in each plate and subtracted from the experimental measurements. Data were analysed with Jasco™ SpectraManager software. Statistical analyses were performed using R statistical software (www.R-project.org). Multiple mean comparisons were performed with the Kruskal–Wallis test at the 95% confidence level.

Honeys of known H_2O_2 concentration from Amplex Red assays were treated with catalase (Sigma-Aldrich™) at a ratio of 1000 U ml^{-1} for 2 h at room temperature. Catalase-treated honey samples were then used in the antibacterial assay to determine the MIC. Each experiment was performed in triplicate.

Total soluble phenolic content

To determine the total soluble phenolic content in honey samples, a phosphowolframate-phosphomolybdate complex was reduced to blue products by the action of phenolic compounds (Ciappini and Stoppani 2014). Distilled water was used to obtain 25 ml of diluted honey sample (4·0 ± 0·01 g), which was then filtered through Whatman No. 1 paper. To 1 ml of this solution,10 ml of distilled water with 1 ml of Folin-Ciocalteu reagent (Sigma-Aldrich) was added, and the mixture shaken gently. After 2 min, saturated sodium carbonate solution (2 ml) was added to the mixture, which was then adjusted to 25 ml with distilled water. The absorbance of the solution was read at 725 nm in a spectrophotometer (UV-1200, UVisco Jasco™) after incubation in the dark at room temperature for 2 h. The calibration curve was plotted with gallic acid as standard. The mean of three readings was used and expressed as milligrams of GAE per 100 g of honey. Statistical analyses were performed using R statistical software (ver. 2.13.1, www.R-project.org). Multiple mean comparisons were performed with the Kruskal–Wallis test at the 95% confidence level. Each experiment was performed in triplicate.

Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was carried out according to the method described by Dudonné *et al.* (2009). The reaction was run in 75 mmol l^{-1} phosphate buffer (pH 7·4) in black 96-well microplates (Greiner Bio-One, Kremsmünster, Austria) at 37°C on a Tecan Infinite® 200 PRO multimode reader (Tecan Group Ltd., Männedorf, Switzerland). Trolox (0–10 μmol l^{-1} final concentration) was used as standard; 25 μl of honey solutions (0·1–1 mg ml^{-1} final concentration) or trolox were mixed with 150 μl of fluorescein (70 nmol l^{-1} final concentrations) in the microplate and incubated for 20 min at 37°C; 75 μl of APPH solution (12 mmol l^{-1} final concentration) was then injected, and the fluorescence was measured every minute for 90 min at excitation and emission wavelengths 485 and 530 nm respectively. All samples were analysed in triplicate, and the ORAC values were expressed in μmol TE/g of honey. Statistical analyses were performed using R statistical software (www.R-project.org). Multiple mean comparisons were performed with the Kruskal–Wallis test at the 95% confidence level. Each experiment was performed in triplicate.

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Conflict of Interest

No conflict of interest declared.

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