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Antimicrobial Activity of some Italian Honeys against Pathogenic Bacteria

Marco Fidaleo, Antonio Zuorro* and Roberto Lavecchia

Dipartimento di Ingegneria Chimica, Materiali e Ambiente – Università "La Sapienza"
Via Eudossiana 18, 00184 Roma (Italy)

Some Italian honeys of different floral origin (acacia, orange, chestnut, coriander, eucalyptus, lime, wildflower) and one honeydew honey were investigated for their potential as natural antimicrobials against pathogens commonly associated with wound or burn infections. *S. aureus*, *S. epidermidis*, *E. coli*, *P. aeruginosa* and *P. mirabilis* were used as test microorganisms. The honeys examined showed moderate to high antibacterial activity, with honeydew, eucalyptus and wildflower honeys being the most effective. MIC values obtained by the agar incorporation method were in the range of 5–20% (v/v). Gram-positive bacteria were more susceptible to the inhibitory action of honey, probably because of their larger outer-membrane permeability to exogenous substances. Overall, the results suggest that some of the honeys considered here might have excellent potential for use in antimicrobial formulations for topical applications.

1. Introduction

The use of honey to treat infections dates back to the time of ancient Egyptians and Greeks, but only recently has its ability to inhibit bacterial growth been scientifically proven. (Lubsy et al., 2005; Molan, 2006; Lofti, 2008). Current evidence suggests that several factors may contribute to the antimicrobial properties of honey, the most important being osmolarity, acidity, the enzymatic generation of hydrogen peroxide and the presence of various non-peroxide compounds derived from the pollen or the nectar of flowers (Bogdanov, 1997; Shahid, 2009). Studies have also shown that marked differences may exist in inhibitory activity depending on the geographical origin of the honey and its floral source (Lubsy et al., 2005; Bogdanov et al., 2008).
Motivated by the above observations and in continuation of our research into natural antimicrobials (Fidaleo et al., 2010), we decided to investigate the antibacterial potency of Italian honeys against some of the microorganisms most frequently associated with wound or burn infections. Treatment of these infections is becoming increasingly difficult, at present, because of the emergence of resistance to most first-line antimicrobial agents, such as penicillins, tetracycline, chloramphenicol and macrolides (Levy and Marshall, 2004). As a first step to the above end, we focused on domestic honeys of different floral origin and on a honeydew honey, a product rich in oligosaccharides and minerals obtained from the excretions of plant-sucking insects (Astwood et al., 1998). To assess their relevance in wound management and treatment we evaluated their antimicrobial properties against both Gram-positive and Gram-negative bacteria, including a methicillin-resistant *Staphylococcus epidermidis* (MRSE)

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strain isolated from a clinical specimen. The activity of the honeys tested was also compared with that of a medical-grade honey derived from *Leptospermum scoparium*, a plant indigenous to New Zealand locally known as the "manuka" tree (Stephens et al., 2005). Manuka honey is one of the most utilized curative honeys in the world because of its documented efficacy in the treatment of infections caused by both antibiotic-susceptible and antibiotic-resistant pathogens (Molan, 2009; Cooper et al., 2010).

2. Experimental

2.1 Materials

Seven nectar honeys (acacia, orange, chestnut, coriander, eucalyptus, lime and wildflower) and one honeydew honey from different locations in Italy were obtained directly from the producers (Table 1). Three medical-grade Manuka honeys of varying strength (UMF 15+, 20+ and 25+) were purchased from Honey NZ International (Parnell, Auckland, NZ). All honeys were stored in the dark at 4 °C until use.

Mueller-Hinton Agar 2 and Mueller-Hinton broth were from Sigma-Aldrich (Milano, Italy). Glucose, fructose, maltose, sucrose and all other chemicals were of analytical grade and used without further purification.

2.2 Artificial honey

An "artificial honey" was used in control experiments. It was prepared by dissolving 20 g fructose, 15 g glucose, 4 g maltose and 1 g sucrose in 10 mL sterile deionized water, so as to obtain the following composition (wt%): 40% fructose, 30% glucose, 8% maltose and 2% sucrose.

2.3 Bacterial strains

Escherichia coli (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 10145) and *Proteus mirabilis* (ATCC 25933) were obtained from KairoSafe (Duino Aurisina, Italy). *Staphylococcus epidermidis* was isolated at the Department of Cardiac Surgery ("Tor Vergata" University, Rome, Italy) from the wound site of a patient who developed infection after heart surgery.

2.4 Antimicrobial activity assay

Susceptibility tests were made according to NCCLS (National Commitee for Clinical and Laboratory Standards) guidelines by the agar-well diffusion method and the agar incorporation technique. All assays were done at least in duplicate.

For the agar-diffusion assay, bacterial cells from an exponential-phase culture obtained from a single colony were spread on the surface of agar plates using a sterile swab soaked in the bacterial suspension. 9-mm wells were then cut in the agar and filled with 150 µL of honey. After overnight incubation at 37 °C, the plates were examined and the diameters of the inhibition zones measured.

Agar incorporation tests were carried out by including honey, from 2.5 to 20% (v/v) in 2.5% increments, in agar plates. After solidification at room temperature followed by 20 min drying at 40 °C, the plates were inoculated using sterile pipette tips, incubated overnight at 37 °C and then inspected for the presence of colonies.

Table 1: Honey samples tested and associated codes

Honey	Code	Honey	Code
Acacia (Momigno, I)	Ac1	Eucalyptus (Ostia, I)	Eu3
Acacia (Caserta, I)	Ac2	Wildflower (Teramo, I)	Wi1
Orange (Vibo Valentia, I)	Or	Wildflower (Teramo, I)	Wi2
Chestnut (Arezzo, I)	Ch1	Honeydew (Pistoia, I)	HD
Chestnut (Capri, I)	Ch2	Manuka (Auckland, NZ)	M15+
Coriander (Acqualagna, I)	Co	Manuka (Auckland, NZ)	M20+
Eucalyptus (Asiago, I)	Eu1	Manuka (Auckland, NZ)	M25+
Eucalyptus (Maremma, I)	Eu2		

3. Results and Discussion

Preliminary testing of bacterial susceptibility to common antibiotics indicated that the strain isolated from the patient's wound was a methicillin-resistant *S. epidermidis* (MRSE). An example of the results of agar-diffusion tests is shown in Figure 1. As can be observed, antimicrobially active honeys produced a growth inhibition halo around the well containing the honey sample. To quantify this activity we calculated the area of the inhibition zone, obtaining the values reported in Table 2.

Inspection of the data reveals that the five pathogens tested were susceptible to all of the domestic honeys examined, with the exception of *E. coli*, which showed resistance to one type of acacia honey (Ac1). Overall, their susceptibility to honeys increased in the order: *P. aeruginosa* < *E. coli* < *P. mirabilis* < *S. epidermidis* < *S. aureus*. A similar trend was observed for Manuka honey. The degree of inhibition by this honey increased with the declared UMF (Unique Manuka Factor) value, which is an indicator of the strength of the honey. Remarkably, Gram-positive bacteria (*S. aureus* and *S. epidermidis*) were more sensitive than Gram-negatives (*E. coli*, *P. aeruginosa* and *P. mirabilis*). This difference can be attributed to the lower outer-membrane permeability of Gram-negatives, which limits the entry of antimicrobial agents into the cells and the subsequent interaction with target sites (Nikaido, 2003).

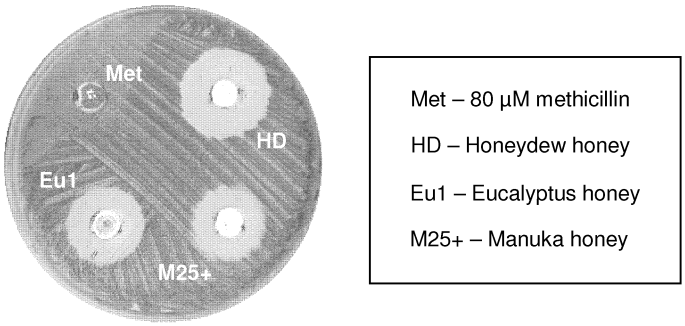

Figure 1: Susceptibility of S. epidermidis to methicillin and some honeys

Table 2: Antimicrobial activity of honeys against the five pathogens tested. Data are expressed as mean inhibition zone area (mm^2) ± SD

Honey	*S. aureus*	*S. epidermidis*	*E. coli*	*P. aeruginosa*	*P. mirabilis*
Ac1	125.1 ± 5.7	137.4 ± 0.0	0.0 ± 0.0	7.3 ± 0.0	14.9 ± 0.0
Ac2	250.5 ± 0.0	219.9 ± 0.0	49.5 ± 0.0	31.4 ± 0.0	69.1 ± 0.0
Or	235.0 ± 8.5	190.9 ± 0.0	40.3 ± 2.5	31.4 ± 0.0	69.1 ± 0.0
Ch1	235.0 ± 8.5	235.0 ± 8.5	40.3 ± 2.5	31.4 ± 0.0	69.1 ± 0.0
Ch2	266.4 ± 9.2	235.0 ± 8.5	59.1 ± 3.3	14.9 ± 0.0	69.1 ± 0.0
Co	163.4 ± 0.0	125.1 ± 5,7	23.0 ± 1.5	7.3 ± 0.0	40.3 ± 2.5
Eu1	282.7 ± 0.0	282.7 ± 0.0	59.1 ± 3.3	59.1 ± 3.3	113.1 ± 0.0
Eu2	176.9 ± 7.1	101.5 ± 5.0	31.4 ± 0.0	11.0 ± 0.4	69.1 ± 0.0
Eu3	282.7 ± 0.0	250.5 ± 0.0	59.1 ± 3.3	79.5 ± 4.2	101.5 ± 5.0
Wi1	266.4 ± 9.2	334.0 ± 10.5	59.1 ± 3.3	40.3 ± 2.5	49.5 ± 0.0
Wi2	250.5 ± 0.0	190.9 ± 0.0	40.3 ± 2.5	23.0 ± 1.5	69.1 ± 0.0
HD	467.3 ± 0.0	407.8 ± 11.8	101.5 ± 5.0	69.1 ± 0.0	101.5 ± 5.0
M15+	299.4 ± 9.8	219.9 ± 0.0	137.4 ± 0.0	7.3 ± 0.0	90.3 ± 9.1
M20+	316.5 ± 0.0	219.9 ± 0.0	163.4 ± 0.0	14.9 ± 0.0	205.2 ± 7.8
M25+	388.8 ± 0.0	334.0 ± 10.5	235.0 ± 8.5	31.4 ± 0.0	235.0 ± 8.5

Among domestic honeys, honeydew honey was the most effective, with an average activity against the five pathogens comparable to that of the strongest Manuka honey (M25+). Very high activities were also observed for eucalyptus (Eu1, Eu2), wildflower (Wi1) and chestnut (Ch1, Ch2) honeys. The strong inhibitory effect of honeydew honey (HD) and some blossom honeys (namely, Wi1, Eu1, Eu3, Ch1, Ch3, Ac2) against the MRSE clinical isolate is of great practical importance. Recently, in fact, *S. epidermidis* has evolved from a normal human commensal to an aggressive opportunistic pathogen through the acquisition of the ability to form biofilms and produce toxins (Vuong and Otto, 2002). In regard to HD, it is interesting to note that the high antimicrobial potency of this type of honey has already been highlighted (Vorlova et al., 2005) and could, at least in part, be ascribed to some of the substances excreted by aphids or other insects (Bogdanov et al., 2008). Agar incorporation experiments allowed estimation of the MICs (minimum inhibitory concentrations), which were determined as the lowest concentration of honey in water solutions giving complete inhibition of bacterial growth. A representative example of the results is shown in Figure 2. MIC values were in the range 5-20% (v/v) and most between 12.5 and 17.5%. *S. aureus* was the most susceptible, its growth being completely inhibited at honey concentrations between 5 and 7.5%. No significant differences were found between domestic and Manuka honeys (Table 3). Finally, no inhibitory activity was detected when honeys were replaced by the artificial honey, suggesting that osmolarity does not play an important role in growth inhibition. Thus, we can speculate that the observed activity is mainly due to the presence of antimicrobially active phytochemicals in the honey and/or the generation of hydrogen peroxide by the bee-derived enzyme glucose oxidase, according to the reaction: $C_6H_{12}O_6 + O_2 + H_2O \rightarrow C_6H_{12}O_7 + H_2O_2$ (Bogdanov, 1997).

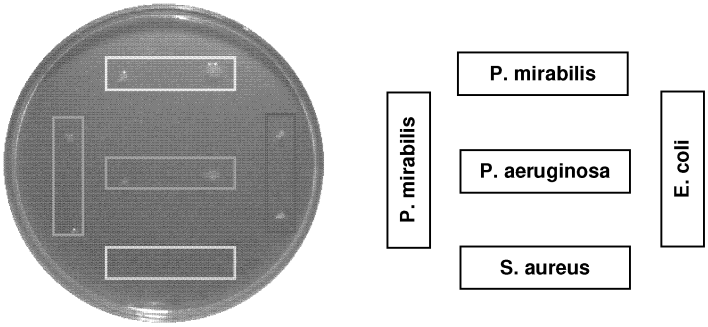

Figure 2: Effect of 12.5% (v/v) eucalyptus honey (Eu3) on the growth of the five pathogens

Table 3: MIC values (%v/v) for some honeys against the five pathogens tested

Honey	*S. aureus*	*S. epidermidis*	*E. coli*	*P. aeruginosa*	*P. mirabilis*
Ch2	5.0	17.5	12.5	10.0	15.0
Eu3	7.5	17.5	15.0	15.0	15.0
HD	5.0	17.5	15.0	15.0	15.0
M25+	5.0	17.5	15.0	15.0	15.0

However, until the precise nature of the factors responsible for the observed effects is established, it is difficult to assess which of the honeys examined here are more suited for use as antimicrobials. Recently, strong evidence has been provided that methylglyoxal, a phytochemical found at high levels in Manuka honey, could be the main responsible for its activity (Mavric et al., 2008, Adams et al., 2009). So, it is not unlikely that similar but as yet unidentified compounds are present in at least some of the Italian honeys studied, providing them with the ability to fight infections.

4. Conclusions

This is the first study to provide direct evidence for the efficacy of some Italian honeys against wound pathogens, including MRSE, whose increasing prevalence in nosocomial infections, particularly among immunocompromised patients, is a source of great concern. Although further research is needed to elucidate the mechanisms involved, the capacity of these honeys to inhibit the growth of both Gram-positive and Gram-negative bacteria make them promising candidates for use in topical treatment of infected wounds or burns.

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