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Antimicrobial activity of methylglyoxal, a phytochemical found in some types of honey, against pathogenic bacteria

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ABSTRACT

Recent evidence suggests that methylglyoxal (MG), a highly reactive ketoaldehyde, is the main responsible for the unusually high antimicrobial activity of some New Zealand honeys. To provide further support to this hypothesis and assess the potential of MG as a new natural antimicrobial agent, we performed comparative in-vitro activity tests on some of the microorganisms most frequently associated with human infections, including a methicillin-resistant *Staphylococcus epidermidis* (MRSE) strain from a clinical isolate. Very similar activity profiles were observed by using MG or a medical-grade (UMF 25+) Manuka honey as antimicrobial agents, with the following susceptibility order: MRSE, *S. aureus* > *E. coli*, *P. mirabilis* > *P. aeruginosa*. MG exhibited bacteriostatic and bactericidal activity against all the microorganisms tested, with MIC and MBC values ranging from 1.05 to 4.22 mM and 2.11 to 4.22 mM, respectively. Experiments made by adding 70 mM MG to an artificial honey and an equimolar glucose–fructose mixture showed that the activity of Manuka honey arises primarily from the presence of high levels of MG. The remarkable antibacterial potency of MG makes it an attractive candidate for the development of pharmaceutical compositions for the treatment of microbial infections.

KEYWORDS

Antibacterial activity;
Honey;
Methylglyoxal;
MRSE;
Pathogenic bacteria.

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[1-3]. 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[4,5]. In some honeys, the hydrogen peroxide formed from the oxidation of glucose by the enzyme glucose oxidase is the predominant antimicrobial agent. Other honeys display a pronounced non-peroxide activity which is thought to be, at least partly, related to as yet unidentified

compounds. This is the case of the honey derived from *Leptospermum scoparium*, a plant indigenous to New Zealand which is locally known as the "manuka" tree. Manuka honey has a long and traditional history of use in the treatment of infections and its efficacy is well documented[6,7]. However, despite the efforts made to characterize the compounds responsible for the unusually high non-peroxide activity, the chemistry behind the antiseptic properties of this honey has not yet been completely elucidated. Recently, Mavric et al.[8] have provided strong evidence that methylglyoxal (MG), a phytochemical found at high levels in Manuka honey, could be the main responsible for the observed non-peroxide activity. According to Adams et al.[9], this compound would originate from the non-enzymatic conversion of dihydroxyacetone, a reducing sugar which is present in the nectar of Manuka flowers.

MG, also known as pyruvaldehyde or 2-oxopropanal, is a highly reactive α-ketoaldehyde, i.e., a compound characterized by the presence of both an aldehyde and a ketone group in the molecule (Figure 1). MG occurs in appreciable amounts in dairy products, roasted coffee and fermented beverages[10]. In addition, it can be formed during cooking or prolonged storage of foods as a result of carbohydrate degradation[10]. In living organisms, MG is produced by enzymatic and nonenzymatic pathways, including protein glycation by glucose, lipid peroxidation and the metabolism of acetone and threonine[11,12].

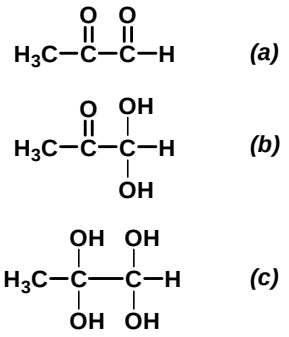

Figure 1 : Chemical structure of methylglyoxal (a) and its monohydrated (b) and dihydrated (c) forms.

Most of the research on MG has been focused on its accumulation in cells and on intracellular generation of stable Maillard reaction intermediates, the so-called AGEs (Advanced Glycation End-products), because of their possible implication in diabetic complications and neurodegenerative disorders[13,14]. In contrast, scant attention has been paid to assessing the effects of exogenous MG on bacteria, especially those that appear to be mostly susceptible to honey. There is, therefore, a need for more in-depth assessment of the antimicrobial properties of MG and its role in honey.

The main goals of this study were to provide further support to the hypothesis that MG is the main responsible for the antimicrobial efficacy of Manuka honey and to assess its potential as a new natural antimicrobial agent. To this end, we focused our attention on some of the microorganisms most frequently associated with human infections, including a methicillin-resistant strain of *Staphylococcus epidermidis* which is responsible for a growing number of nosocomial infections all over the world.

MATERIALS AND METHODS

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). Methicillin-resistant *Staphylococcus epidermidis* (MRSE) was isolated from a wound exudate at the Department of Cardiac Surgery ("Tor Vergata" University, Rome, Italy).

Antimicrobial agents and chemicals

MG (2-oxopropanal) and methicillin (2,6-dimethoxyphenylpenicillin) sodium salt were obtained from Sigma-Aldrich (Milano, Italy). MG was in the form of a 40% (w/v) aqueous solution. Active Manuka honey (UMF 25+) was purchased from Honey NZ International (Parnell, Auckland, NZ). UMF (Unique Manuka Factor) is an indicator of the antimicrobial potency of the honey and, according to the producer, UMF 25+ denotes a honey for therapeutic usage with very high activity levels.

An inverted sugar syrup, supplied by FPP (Nizza Monferrato, Italy), was used as an artificial honey. Its composition and other properties are reported in TABLE 1. Mueller–Hinton broth, Mueller–Hinton Agar 2, D-(+)-glucose (>99.5%) and D-(–)-fructose (>99%) were from Sigma-Aldrich (Milano, Italy). The equimolar glucose–fructose mixture was prepared by solubilizing

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40 g glucose and 40 g fructose in 20 mL of sterile deionized water.

All other chemicals were of analytical grade and used without further purification.

TABLE 1 : Composition and properties of the artificial honey.

Components / Properties	Value
Glucose	40 wt %
Fructose	35 wt %
Sucrose	5 wt %
Water	20 wt %
Phosphates (as PO_2O_5)	< 0.5 wt %
Sulphides (as SO_2)	< 0.1 wt %
Nitrogen (as NH_4)	< 0.5 wt %
Density at 20 °C	1.42 g/mL
pH	4.0 – 4.5

Antimicrobial activity assay

MG susceptibility tests were performed by conventional agar dilution and agar-well diffusion methods, and by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Susceptibilities to Manuka honey, artificial honey and the equimolar glucose–fructose mixture were assessed by the agar-well diffusion assay. Diluted honey samples were obtained by dissolving the appropriate amount of honey in sterile deionized water. All tests were run at least in triplicate and the results were averaged.

Agar dilution method

Mueller–Hinton agar plates were prepared by pouring 25 mL of the medium containing from 1.05 mM to 16.9 mM MG into each plate. After solidification at room temperature, followed by 20 min drying at 40 °C, the plates were inoculated using a sterile loop and incubated overnight at 37 °C.

Agar diffusion method

Bacterial strains 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 an aqueous solution at the appropriate MG (or honey) concentration. After overnight incubation at 37 °C, the plates were examined and the diameters of the inhibition zones were measured.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC values were determined in Mueller–Hinton broth using a two-fold serial dilution technique. Bacterial strains from an exponential-phase culture were grown overnight at 37 °C, using an inoculum of approximately 1.5×10^6 CFU/mL, in the presence of different concentrations of MG. Subcultures were then streaked on Mueller–Hinton agar plates, which were incubated at 37 °C for 18 h. After this time, the number of colonies formed on each plate was counted. MG concentrations were varied from 0.53 to 135 mM. The MIC was defined as the lowest concentration of MG giving complete inhibition of bacterial growth, and the MBC as the lowest concentration killing 99.9% of the original inoculum.

RESULTS

Susceptibility of bacterial strains to MG

Figure 2 shows the results of experiments performed by inoculation of bacteria onto agar plates containing increasing concentrations of MG. The five strains were all sensitive to the antimicrobial agent, but to varying degrees. *S. epidermidis* stopped growing at an MG concentration of 2.11 mM, followed by *E. coli*, *S. aureus*, *P. mirabilis* (4.22 mM) and *P. aeruginosa* (6.33 mM).

Agar-well diffusion tests gave the results in Figure 3, where the mean diameter of the inhibition zones is plotted against the concentration of MG. For all strains, a dose-dependent response was observed. Susceptibilities of *S. epidermidis* and *S. aureus* to MG were very high and nearly identical. Slightly lower but still pronounced effects were observed for *P. mirabilis*, *E. coli* and *P. aeruginosa*.

MIC and MBC values are listed in TABLE 2. Overall, the MICs were in the range 1.05 – 4.22 mM and the MBCs were between 2.11 and 4.22 mM. *S. aureus* and *S. epidermidis* had identical MICs (1.05 mM) and MBCs (2.11 mM). The MICs and MBCs for *E. coli* and *P. mirabilis* were the same and equal to 2.11 mM. *P. aeruginosa* had a MIC and an MBC of 4.22 mM.

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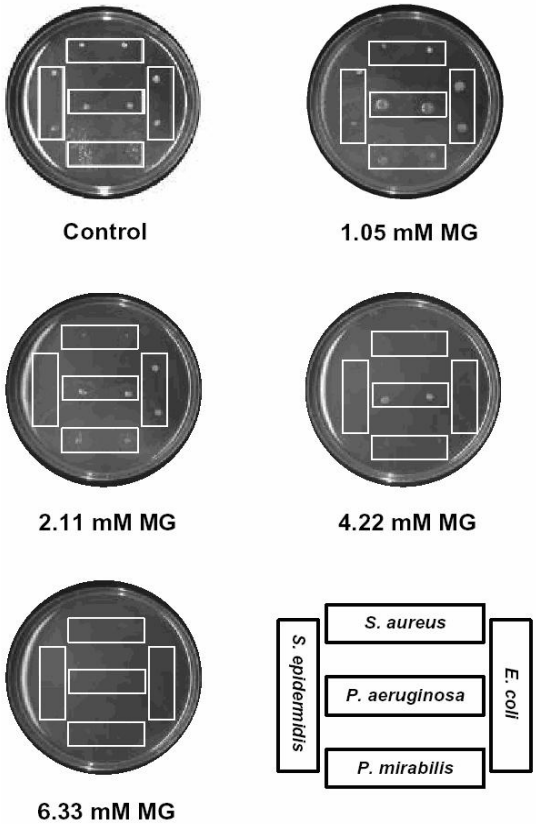

Figure 2 : Results of agar well diffusion assay showing the effect of increasing concentrations of MG on the growth of *E. coli, S. aureus, P. aeruginosa, P. mirabilis* and methicillin-resistant *S. epidermidis*.

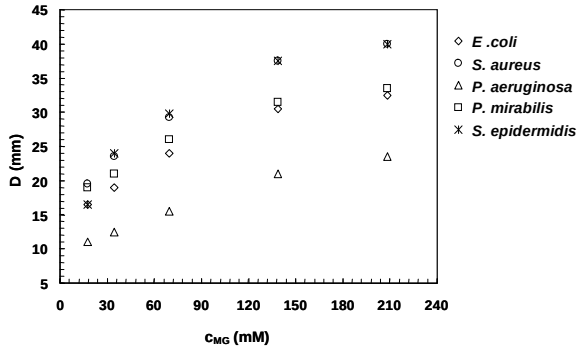

Figure 3 : Effect of MG concentration (c_{MG}) on the mean diameter of inhibition (D) for *E. coli, S. aureus, P. aeruginosa, P. mirabilis* and methicillin-resistant *S. epidermidis*.

TABLE 2 : Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MIC) values for MG against the microorganisms tested.

Microorganism	MIC [mM]	MBC [mM]	MBC/MIC
E. coli	2.11	2.11	1:1
S. aureus	1.05	2.11	2:1
P. aeruginosa	4.22	4.22	1:1
P. mirabilis	2.11	2.11	1:1
S. epidermidis	1.05	2.11	2:1

Susceptibility of bacterial strains to manuka honey

Figure 4 (panel A) shows the effects of increasing concentrations of Manuka honey on bacterial growth. At honey concentrations < 25% (v/v) little or no antibacterial activity was found. Above this value, dose-dependent effects were observed. The mean diameters of the inhibition zones were 12.8, 15.9, 17.7 and 20.1 mm for honey concentrations of 25, 50, 75 and 100%, respectively. The rank order of susceptibility was the same as that determined using methylglioxal, that is: *S. epidermidis*, *S. aureus* > *E. coli*, *P. mirabilis* > *P. aeruginosa*. A representative example of the effects of Manuka honey (75% v/v) and MG (35 and 25 mM) on *S. epidermidis* derived from the clinical isolate is presented in Figure 5.

Susceptibility of bacterial strains to artificial honey

The results of experiments using artificial honey or the glucose–fructose mixture are shown in Figure 4 (panel B). Both were applied as such or in combination with 70 mM MG. No activity was detected against the bacterial strains tested (*E. coli*, *S. aureus*, *P. aeruginosa* and *P. mirabilis*) when using the two materials as such. In contrast, high activity levels were

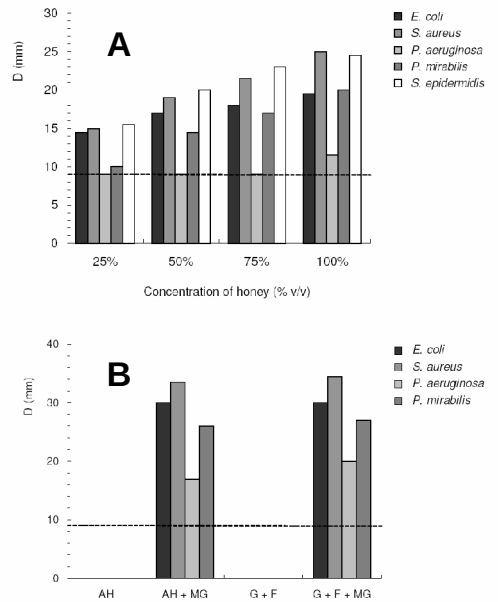

Figure 4 : Panel A: Effect of active manuka honey on the mean diameter of inhibition (D) for *E. coli, S. aureus, P. aeruginosa, P. mirabilis* and methicillin-resistant *S. epidermidis*. Panel B: Effect of the addition of 70 mM MG into artificial honey (AH) or the equimolar glucose–fructose mixture (G + F) on the growth of *E. coli, S. aureus, P. aeruginosa* and *P. mirabilis*. The dashed lines indicate the size of the agar well.

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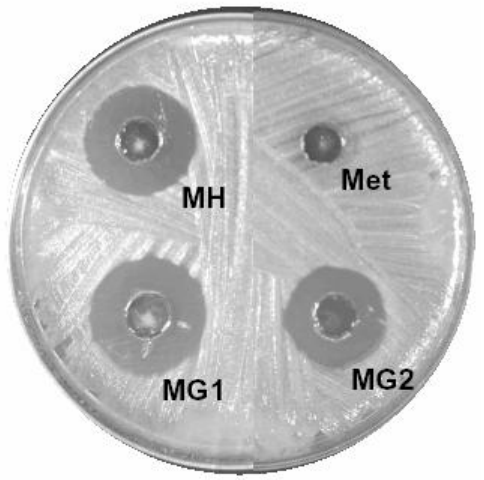

Figure 5 : Results of agar well diffusion assay showing the susceptibility of the clinical isolate of *S. epidermidis* to 70 µM methicillin (Met); 75% manuka honey (MH); 35 mM MG (MG1) and 25 mM MG (MG2).

observed on addition of MG to both of them. The resulting effects were very similar, with average inhibition zone diameters of 26.6 (artificial honey + MG) and 27.9 mm (glucose–fructose mixture + MG). Once again, the order of susceptibility was: *S. aureus* > *E. coli* > *P. mirabilis* > *P. aeruginosa*.

DISCUSSION

The microorganisms investigated in this study include three Gram-negative species (*E. coli*, *P. aeruginosa* and *P. mirabilis*) and two Gram-positives (*S. aureus* and *S. epidermidis*). They are among the bacterial pathogens most commonly associated with human infections[15,16]. Treatment of these infections is generally difficult because of increasing resistance against antibiotics and chemotherapeutic agents[17]. In particular, the prevalence of nosocomial and community-acquired infections caused by methicillin-resistant *Staphylococcus spp.* is growing worldwide, with important implications for patient health and therapy costs[18].

The results from the present study indicate that MG has significant in-vitro activity against all the microorganisms tested, including the methicillin-resistant isolate of *S. epidermidis*. According to susceptibility testing results, MG was not only inhibitory but also bactericidal against these pathogens. Concentrations higher than 2.11 mM (4.22 mM, for *P. aeruginosa*) were capable of killing more than 99.9% of the bacteria. *Staphylococcus spp.* showed higher sensitivity, while *P. aeruginosa* was the less susceptible, with an MIC two- to four-times smaller than those of other species.

Response variability to MG action suggests that there may be differences in the mechanistic pathways associated with the uptake and entry of MG into microbial cells. Gram-negative bacteria are known to be particularly resistant to many antimicrobial agents because of lower outer-membrane permeability, which prevents them from reaching target sites[19]. However, it is also known that porin channels in the outer membrane allow passive diffusion of small hydrophilic molecules (with a MW roughly less than 500-600 Da), as is indeed the case for MG (MW = 72.06 Da). This would explain the response of *E. coli*, *P. aeruginosa* and *P. mirabilis* to MG and suggest that their lower sensitivity, with respect to *S. aureus* and *S. epidermidis*, arises from factors related to other steps in the uptake process, such as adsorption to cell surface, diffusion into the periplasmic space and/or interaction with target sites[20]. Regarding the nature of these sites, it should be considered that MG, like other 2-oxoaldehydes, reacts readily with thiol groups of proteins as well as with the guanine bases of DNA, leading to inhibition of some enzyme activities and causing arrest of cell division[11]. It can, therefore, be speculated that its inhibitory and bactericidal activity is the result of an overall cellular damage caused by random multiple detrimental effects on cytoplasmic constituents, rather than interaction with specific target sites[21].

The ability of Manuka honey to inhibit the growth of the microorganisms investigated is in agreement with the available body of observations[6,22-27] and adds further support to the therapeutic potential of this type of honey for the treatment of infections. Although the precise mechanisms by which honey inhibits bacteria remain elusive, osmolarity due to high sugar content, hydrogen peroxide generation and phytochemicals are considered to be the factors that contribute most to its activity[4,28]. In undiluted honey, the high sugar content (about 80% w/w) reduces the water activity, i.e., the amount of water available to the microorganisms, thus limiting their growth[29]. On dilution, honeys containing the bee-derived enzyme glucose oxidase produce hydrogen peroxide (according to the reaction: $C_6H_{12}O_6 + O_2 + H_2O \rightarrow C_6H_{12}O_7 + H_2O_2$), a powerful oxidizing agent

that causes protein and DNA damage via the formation of hydroxyl radicals[30]. In the case of Manuka honey, experiments performed by adding catalase, the enzyme that converts hydrogen peroxide to water and molecular oxygen, have unequivocally demonstrated that its activity can only partly be attributed to hydrogen peroxide[6,31,32]. According to our results, also osmolarity would seem to play a marginal role in inhibiting bacterial growth, at least under the conditions of this study. In fact, no activity was detected when using artificial honey or the equimolar glucose–fructose mixture. Interestingly, however, the addition of 70 mM MG to both materials strongly inhibited the growth of all the bacteria tested. The similarity of activity profiles points to a common causative factor and, hence, to the direct implication of MG in the antimicrobial properties of the honey.

A quantitative analysis of the results reveals that the efficacy of pure Manuka honey is equivalent to that of an aqueous solution with an MG concentration of about 30 mM (see Figure 3 and panel A in Figure 4). If MG were the only responsible for the activity of the honey assayed, an apparent MG concentration of approximately 1500 mg/kg would result (considering a honey density of 1.4 kg/L[33]). The available literature values for MG concentration in Manuka honeys are roughly in the range 100–1000 mg/kg[8,34]. Since the honey used in this study (UMF 25+) has the highest activity level among medical-grade Manuka honeys, its MG concentration can reasonably be expected to be close to 1000 mg/kg. This value compares fairly well with the estimate of 1500 mg/kg, further supporting the hypothesis that the unusually high antimicrobial activity of Manuka honey arises from the presence of MG.

The observed overestimation of MG levels could be due to the contribution of other honey constituents, such as polyphenols, organic acids or as yet unknown compounds[4,35]. Another possible explanation is that MG in honey is more active than in water. In this respect, it should be remembered that in aqueous media a chemical equilibrium exists between unhydrated and hydrated (mono- and dihydrated) MG forms[9,11] (Figure 1), and that the unhydrated keto-aldeyde is the most reactive[36]. Considering the low water content of honey and that most of the water molecules are hydrogen bonded to hydroxyl groups of sugars[37], it seems reasonable to assume that, in honey, the above equilibrium is shifted toward the more reactive unhydrated form. This would imply a more pronounced reactivity and a higher antimicrobial efficacy than in water.

In conclusion, the results of this study provide further evidence for the efficacy of medical-grade Manuka honey against common microbial pathogens and MRSE, which is becoming an increasingly frequent cause of nosocomial infections. Second, and perhaps more importantly, comparison of the responses of the microbial species to MG and Manuka honey seems to support the hypothesis that the former is the main responsible for the observed honey activity. Finally, MG was found to possess not only inhibitory but also bactericidal activity against all the microorganisms tested. These features and the fact that MG is considered to be potentially safe for human consumption[38,39] make this compound an attractive candidate for the development of new pharmaceutical compositions for the treatment of microbial infections. Inclusion of MG or MG-containing honeys in food products could also represent a valid strategy to limit the spread of food-borne pathogens.

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