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Compositional analysis of Scottish honeys with antimicrobial activity against antibiotic-resistant bacteria reveals novel antimicrobial components

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ABSTRACT

Antibiotic-resistant bacteria are a major health concern and honey may provide an alternative to antibiotic use under certain conditions. The antimicrobial action of six Scottish honeys and Manuka Medihoney® was compared against antibiotic-resistant *Acinetobacter calcoaceticus, Staphylococcus aureus, Pseudomonas aeruginosa and Escherichia coli*. Certain Scottish honeys, such as Highland and Portobello honey 2011, were comparable in effectiveness to the established antimicrobial Medihoney®, inhibiting growth to <1 compared to 10 $\log_{10}$ CFU/ml in the control. Heather honey was the next most active while Blossom honeys were less active. Bacteria were inhibited by a sugar-matched control, but to a lesser extent, indicating that antimicrobial activity was associated with non-sugar components, such as polyphenols. However, total phenol content or antioxidant capacity did not correlate with antimicrobial activity. Liquid chromatography-mass spectrometric analysis revealed that the composition of polyphenol and non-polyphenol components differed between honeys. In addition, candidate components that could be associated with antimicrobial activity were noted including novel fatty diacid glycoside derivatives not previously identified in honeys.

1. Introduction

Antibiotic resistant bacteria are a major world-wide health concern and the prevalence of hospital associated "superbugs" such as MRSA (methicillin resistant *Staphylococcus aureus*) has entered these terms into common parlance. The importance of the issue led the World Health Organization to focus their 2011 World Health day on the global problem of antimicrobial resistance (http://www.who.int/bulletin/volumes/89/5/11-088435/en/). The continuing importance and global reach of this issue was also highlighted in the publication of the UK government/Wellcome Trust Review on Antimicrobial Resistance (http://amr-review.org/sites/default/files/160525_Final%20paper_with%20cover.pdf) and in the recent United Nations declaration on antimicrobial resistance (http://www.un.org/pga/71/wp-content/uploads/sites/40/2016/09/DGACM_GAEAD_ESCAB-AMR-Draft-Political-Declaration-1616108E.pdf).

One group of bacteria which are increasingly recognized as an important antibiotic-resistant source of infection is the *Acinetobacter baumannii-Acinetobacter calcoaceticus* complex (ABC). The ABC contains four closely related groups of bacteria which are not readily phenotypically distinguishable (Gerner-Smidt, 1992) and includes opportunistic pathogens recently designated as "red alert" because of their propensity for multidrug resistance (Cerqueira & Peleg, 2011). As many as 74% of infections in some intensive care units and surgical wards have been attributed to ABC and isolates from centres in Australia, Austria, China, Pakistan, Venezuela, South

Abbreviations: ABC, *Acinetobacter baumannii-Acinetobacter calcoaceticus* complex; ANOVA, Analysis of Variance; cfu, Colony forming units; GAE, Gallic acid equivalents; LC-MS, Liquid Chromatography-Mass Spectrometry; *m/z*, Mass to charge ratio; MRSA, Methicillin resistant *Staphylococcus aureus*; MS, Mass Spectrometry; MS^2, Mass Fragmentation Spectra; NCTC, The National Collection of Type Cultures; RT, Retention time; SE, Standard Error; SPE, Solid phase extraction.

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Africa, South Korea, Taiwan, the USA and Great Britain (e.g. Higgins, Dammhayn, Hackel, & Seifert, 2010) confirm that multidrug resistant ABC has become a worldwide problem.

There is a clear need for alternatives to antibiotic use in the treatment of bacterial infections caused by such antibiotic-resistant bacteria and one possible alternative is honey. Honey has been shown to be an effective dressing for wounds (Gethin & Cowman, 2008) which can speed up healing time and reduce infection. As a result, honey is currently recommended in NHS wound management formularies of both England and Scotland (e.g. http://www.staffordshireandstokeontrent.nhs.uk/Wound%20Care%20Formulary%202012.pdf & http://www.ljf.scot.nhs.uk/LothianJointFormularies/Adult/Wound%20Section/Pages/default.aspx) specifically honey derived from Manuka (Robson, Dodd, & Thomas, 2009; *Leptospermum scoparium*) Medihoney®, MH). However, several varieties of honey have been shown to inhibit ABC bacteria *in vitro* (Alqurashi, Masoud, & Alamin, 2013; Blair, Cokcetin, Harry, & Carter, 2009) including clinically-relevant isolates of *A. baumannii* and *A. calcoaceticus* (Blair et al., 2009; Hannan, Barkaat, Usman, Gilani, & Sami, 2009). The studies on ABC support the role of honey as an alternative to antibiotics for treating wounds but they also highlight the variability of antimicrobial effectiveness between different honeys. Medihoney® and "Black seed honey" had minimum inhibitory concentrations of ~7% (w/w) (Blair et al., 2009; George & Cutting, 2007; Hannan et al., 2009) whereas Clover, Citrus and Nigella honeys failed to show inhibitory action until 40% (w/w) (Hassanein, Gebreel, & Hassan, 2010) and so the floral source of honey appears to be an important factor in the selection of effective antimicrobial wound dressings.

The quantity and presence of established antimicrobial factors in honey varies widely, which may influence overall effectiveness. For example, Manuka honey contained 44 times more of the bactericidal component methylglyoxyl than "Revamil® Source" medical honey (Kwakman, Velde, de Boer, Vandenbrouke-Grauls, & Zaat, 2011), but Manuka honeys lacked other antimicrobial factors such as H_2O_2 and bee defensin-1. Also the mean methylglyoxyl concentrations in Manuka honey from various regions of New Zealand varied by as much as 5 fold (Oelschlaegel et al., 2012). Understanding which are the active antimicrobial components in different honeys and their combined mechanisms of action will allow for the selection of more potent honeys for use against multidrug-resistant bacteria such as ABC. Several groups have begun to identify the antimicrobial factors in honey. By eliminating various factors one at a time, the antimicrobial activity of Revamil® Source honey against *B. subtilis, E. coli, P. aeruginosa and S. aureus* was attributed to osmotic effect, pH, H_2O_2, methylglyoxyl and bee defensin-1 content (Kwakman et al., 2010). However this approach precludes the examination of possible synergies between active components and a further study suggested that found that the action of Manuka honey could not be accounted for by these factors alone (Kwakman et al., 2011). Therefore there may be additional antimicrobial substances, at least in some honeys.

One possible source of antimicrobial activity in honeys is polyphenols. Polyphenols are known to exhibit antimicrobial activity against a range of bacteria but their action in honey has been suggested to be partly dependent on H_2O_2. This would explain why Kwakman et al. (2010) who studied components in isolation failed to attribute activity to polyphenols. Importantly different polyphenols have differential effects, some can kill certain bacteria which others fail to inhibit, and other apparently ineffective polyphenols may act synergistically to enhance effectiveness of bactericidal polyphenols (Alivarez, DeBatista, & Pappa, 2006). This highlights the importance of understanding the polyphenol composition of honeys intended for medical use. Indeed, it has been suggested that the floral source provides a polyphenol signature which can be identified in honeys (e.g. Ceksteryte, Klazlauskas, & Racys, 2006).

In previous work, "Portobello" honey from an apple orchard apiary in Edinburgh was shown to have similar antimicrobial effectiveness as Manuka Medihoney® against wound-infecting *E. coli*, *P. aeruginosa* and *S. aureus* (Schneider, Coyle, Warnock, Gow, & Fyfe, 2012). This study is a continuation of this work and examines the antimicrobial effectiveness of Portobello honeys and four other Scottish honeys compared with Manuka honey and a sugar only control "honey". However, in this study, we also include tests against *A. calcoaceticus,* one of the multidrug resistant ABC bacteria which challenge the medical community today.

The antimicrobial activities of the honeys was compared with known antimicrobial factors such as sugar content, pH, hydrogen peroxide content, total phenolic content and antioxidant capacity. The phytochemical composition of the honeys was also examined by liquid chromatography-mass spectrometry (LC-MS) techniques to uncover candidate antimicrobial components.

2. Materials and methods

2.1. Bacterial strains

Acinetobacter calcoaceticus NCTC10290 (a strain isolated from a skin abscess), *Staphylococcus aureus* NCTC 10655, *Pseudomonas aeruginosa* NCTC 10782 and *E. coli* NCTC 10418 (all of which were isolated from infected wounds) were supplied by the National Collection Type Culture, Porton Down, Salisbury UK. All strains were resistant to penicillin.

2.2. Honey samples

Six Scottish honeys were compared against Comvita Manuka Medihoney® (MH; Derma Sciences Ltd, Maidenhead, UK), a honey derived mostly from *Leptospermum* spp, including *Leptospermum scoparium* (Manuka). The Scottish honeys were mainly obtained in 2012 and were two Blossom honeys (BH1 from The Oaks Apiary, Falkirk and BH2 from the Heather Hills Apiary, Bridge of Cally, Perthshire), Heather honey (HH from an apiary in Nairn, Morayshire), Highland honey (TH from an apiary in Torridon, Wester Ross), and Portobello Orchard honey from two different years (PB-11 & PB-12 from an apiary in Portobello, East Lothian). PB-11 was previously examined by Schneider et al. (2012).

A sugar "control honey" (CH) was designed to match the sugar composition of Revamil Source honey as recently determined by Kwakman et al. (2010). The control honey was used as a negative control in the anti-bacterial assays and consisted of 38.5% fructose, 33.3% glucose, 6.2% maltose and 7.3% sucrose in distilled water (Okoro, 2013; Okoro, Coyle, & Fyfe, 2015).

2.3. Comparison of the antimicrobial activity of honeys in vitro using a broth culture assay

A broth culture assay was used to determine the inhibitory activity of honey against *A. calcoaceticus, S. aureus, P. aeruginosa* and *E. coli.* Previous work (Schneider et al., 2012) found that both 50% and 75% honey in tryptic soy broth (TSB at 3% (w/v); (Sigma Chem Co. Ltd) effectively reduced the number of colony forming units (cfu). Therefore, for comparative purposes cultures were carried out by inoculating 10 mL of 75% honey broths [i.e. 7.5 g honey made up to 10 mL TSB with 100 μL of starting culture from an overnight incubation of each bacterium in TSB. Inoculated broths were incubated for 24 h at 37^{O}C, sampled and then serially diluted using phosphate buffered saline (PBS) before being spread onto tryptic soy agar (TSA) plates and then incubated for 24 h at 37 ^{O}C. Generally