



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 log₁₀ 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.

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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; MS², 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 H₂O₂ 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, H₂O₂, 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 H₂O₂. 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, DeBataista, & 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°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°C. Generally

plates with between 30 and 300 cfu were used for colony counting. The effect of the control sugar-matched honey was also investigated and control growth cultures in equivalent TSB broth for each bacterial isolate were carried out. Ethical approval for this project was granted by the Division of Health Sciences Ethics Committee, Queen Margaret University.

2.4. Detection of hydrogen peroxide in honey

Hydrogen peroxide was measured using a modification of previously described method (Kwakman et al., 2011). In brief, honeys were diluted to 40% (w/v) in PBS and incubated in an orbital incubator at 37 °C at 100 RPM for 2.5 h. The assay was performed in 96 well plates, 40 µL of sample was added and 135 µL of reagent containing 50 µg/mL *o*-dianisidine (Sigma) and 20 µg/mL horseradish peroxidase type I in 10 mM phosphate buffer pH 6.5 added. Absorbance was read at 430 nm at time zero and after 2.5 and 5 h. Results were calculated as the mean of triplicate readings taken at 2.5 and 5 h and were described in µg/mL using a hydrogen peroxide standard curve. The limit of detection was 1.4 µg/mL.

2.5. Estimation of sugar content using refractometry

The sugar content of the tested honeys was determined using a pocket refractometer (Bellingham and Stanley Limited, Basingstoke, UK) according to the manufacturer's guidelines as described previously (Schneider et al., 2012).

2.6. Estimation of pH

The pH was measured using a standard laboratory pH electrode (Hanna Instruments, UK Model H18519N pH meter) which was calibrated before use with buffers at pH 7.0 and 4.0.

2.7. Honey colour

Colour was determined using a previously described method (Kaskoniene et al., 2009). Honey samples were diluted to 50% (w/v) in distilled water and heated to 40 °C to dissolve all sugar crystals. The absorbance of the sample was read at 635 nm and converted to Pfund scale values. These values were classified into colour grades using the United States Standards for Grades of Extracted Honey (US Agricultural Marketing Service Fruit and Vegetable Division Processed Products Branch 1985). All honeys were also photographed.

2.8. Extraction and estimation of total phenol content

A previous method (Biesaga & Pyrzynska, 2012) was adapted. 10 mL of 50% (v/v) methanol in distilled water containing 0.2% (v/v) formic acid was added to triplicate 1 g samples of honey in centrifuge tubes. After vortex mixing to dissolve the honey, the samples were extracted using a blood tube rotator for 30 min at 4 °C. After centrifugation (10 min at 2500 × g, 5 °C), the supernatant was retained.

The total phenol content (TPC) of honey samples was estimated using an Folin-Ciocalteu method (Deighton, Brennan, Finn, & Davies, 2000). Briefly, 100 µL of extract was pipetted into triplicate 1.5 mL Eppendorf tubes and 150 µL of distilled water was added. 250 µL of Folin-Ciocalteu reagent was added and the sample was left for 3 min. Saturated sodium carbonate (500 µL) was added and blue colour developed for 1 h. Blank reactions containing only distilled water were used to provide a zero value. Samples were transferred to cuvettes and their absorbance read at 750 nm in a UV/visible spectrophotometer (Amersham Biosciences, model

Ultrospec 2100 Pro). The absorbances were compared against a phloroglucinol standard curve and TPCs were expressed in µg phloroglucinol equivalents (PGE)/mL.

2.9. Solid phase extraction of honey

Highland honey (TH; 10 g) was diluted in 40 mL of 0.1% (v/v) formic acid in ultrapure water and vortexed to ensure dissolution. This solution was subjected to solid phase extraction as described previously (McDougall, Kulkarni, & Stewart, 2009). Briefly, the sample was applied to a solid phase extraction (SPE) unit (Strata C18-E, GIGA SPE units, 10 g capacity; Phenomenex Ltd., Macclesfield, UK) pre-washed in 0.1% (v/v) formic acid in acetonitrile then pre-equilibrated in 0.1% (v/v) formic acid in ultra-pure water. The unbound material, which was enriched in the sugars, was collected. The SPE units were washed with a unit volume of 0.1% (v/v) formic acid then with 2 vol of ultra-pure water. The bound extracts were eluted with 80% (v/v) acetonitrile and aliquots were evaporated to dryness in a SpeedVac (Thermo Scientific, Waltham, MA, USA).

2.10. Characterization of phytochemical profiles by LC-MS

The honey extracts were concentrated X 10 fold using a SpeedVac evaporator. Honey extracts and the SPE bound sample were re-suspended in 5% (v/v) acetonitrile containing 0.1% (v/v) formic acid. Samples were analyzed using an LCQ-DECA system, comprising a Surveyor auto-sampler, pump and photo diode array detector (PDAD) and a ThermoScientific mass spectrometer ion-trap. The gradient was comprised of Solvent A (ultra-pure water containing 0.1% (v/v) formic acid) and solvent B (acetonitrile containing 0.1% (v/v) formic acid). 20 µL samples were eluted on a gradient of $t = 0-5$ mins, 2% B; 5–35 mins, a gradient of 2–40% B. A C18 column (Synergi HydroC18 with polar end capping, 2 mm × 150 mm, Phenomenex Ltd, UK) was used at a flow rate of 200 µL/min and three discrete channels were scanned by the PDAD (280, 365 and 520 nm). An electrospray ionisation (ESI) interface was used and the samples were analyzed in positive and negative mode. There were 2 scan events; full scan analysis, followed by data dependent MS² of the most intense ions using collision energies (source voltage) of 35% in wideband activation mode. Deionized water was used as a control for comparison. Vanillic acid and cyanidin-3-*O*-glucoside were used to tune the MS detector for negative and for positive mode respectively. Certain samples were also analyzed using near identical conditions but using a ThermoScientific Orbitrap MS capable of exact mass determination. All samples were run in triplicate.

2.11. Statistical analysis

All readings were made in triplicate, and experiments were conducted on two or three separate occasions. Data was recorded as a mean with standard error (SEM) and was analyzed in SPSS 19.0 and Microsoft Excel 2010. Experimental data was compared to corresponding controls using a two-tailed independent Student *t*-test. A *p* value of ≤0.05 was accepted as significant.

3. Results

3.1. Antimicrobial activity of honeys

The growth of all bacteria was inhibited by all honey samples at 75% (w/v) including the control sugar-matched honey (Fig. 1). The most effective antimicrobial honeys were Manuka Medihoney® (MH), Highland honey (TH) and Portobello 2011 (PB-11), which all

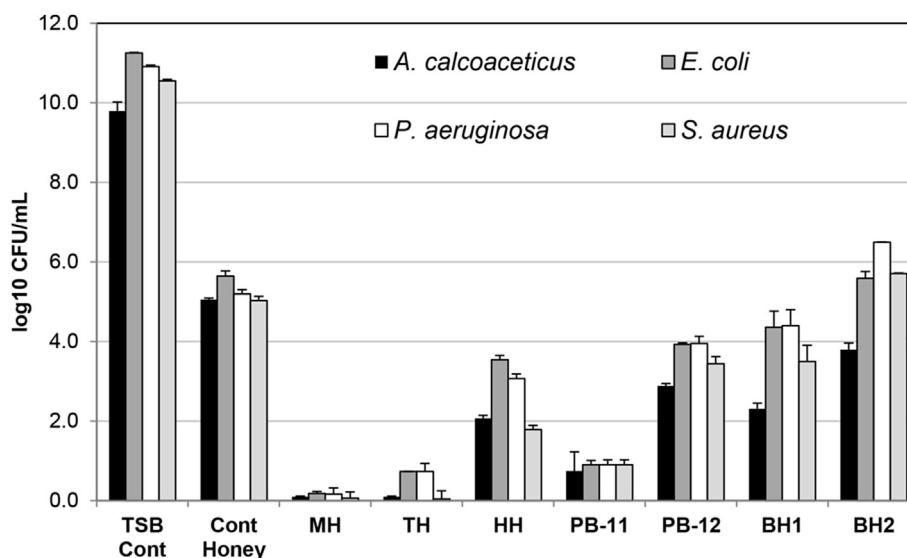


Fig. 1. Inhibition of growth of bacteria by 75% honey in TSB.

Results are expressed as the mean of triplicates \pm SE of log₁₀ cfu/ml after incubation at 37 °C for 24 h. TSB Cont = growth of bacteria in TSB without honey; Cont Honey = growth of bacteria in sugar-matched control honey.

reduced bacterial growth from ~ 10 log₁₀ cfu/mL in the respective TSB controls to ≤ 1 log₁₀ cfu/mL ($p \leq 0.001$). Manuka honey effectively prevented the growth of all bacteria, whereas TH and PB-11 were almost as effective but permitted limited growth. Heather honey (HH), Blossom honey 1 (BH1) and Portobello 2012 (PB-12) honey were less effective and reduced growth to between 2 and 4 log₁₀ cfu/mL depending on the bacteria but this was still significantly more effective than the sugar-matched control honey ($p \leq 0.001$). Within this less active group, even the least active honey Blossom honey 2 (BH2), which was not significantly different from the control sugar-matched honey for most bacteria, significantly reduced the growth of *Acinetobacter calcoaceticus* ($p \leq 0.05$). Indeed, there was a trend that suggested that *Acinetobacter* was generally more susceptible to inhibition by honey treatment than the other bacteria.

3.2. Comparative composition of honeys

Hydrogen peroxide activity was not detected in any honey sample at a limit of detection of 1.4 μ g/mL. All of the honeys had high sugar content ($>75\%$; Table 1) but no honey was significantly different from any other. All honey samples had acidic pH (between 4 and 5) and there were considerable differences in colour (see

Supplementary data for photographs). The darkest were the amber coloured Manuka, Heather and Highland honeys, both Blossom honeys were light amber and both Portobello honeys were classified as white.

The total phenol content (TPC) of the honeys could be described in two different groups: MH, HH and TH had relatively high TPCs (920–1248 μ g PGE/g FW) while BH1, BH2 and both Portobello honeys had lower concentrations (<300 μ g PGE/g FW). The honey samples with the highest TPC also had the highest antioxidant capacity (measured as FRAP) and there was a correlation between TPC and FRAP ($R^2 = 0.8475$; results not shown). Correlations between FRAP and TPC within similar types of samples have been noted before (Deighton et al., 2000). The darkest honeys had the greatest polyphenol concentration and antioxidant activity. These values fit within reported ranges. For example, Alzahrani et al. (2012) reported that their Manuka honey contained 899 μ g/g FW gallic acid equivalents (GAE), Islam et al. (2012) reported values for their mixed source Bangladeshi honeys ranging from 152 to 688 μ g GAE/g FW and Moniruzzaman, Khalil, Sulaiman, and Gan (2013) found that the lowest polyphenol content for their honey samples was 187 μ g GAE/g.

Table 1
Compositional data for honey samples.

Sample	pH	Sugar Content (%)	Total Phenol Content (μ g GAE/g FW)	Antioxidant capacity (FRAP) (Fe^{2+} mM/kg)	Colour
Manuka (MH)	4.25	78.7 \pm 0.4	1248 \pm 82 ^a	4.55 \pm 0.08 ^a	amber
Blossom Honey 1 (BH1)	4.5	77.4 \pm 0.2	243 \pm 9	2.73 \pm 0.08	light
Blossom Honey 2 (BH2)	5.0	79.9 \pm 0.3	292 \pm 3	2.69 \pm 0.04	light
Heather Honey (HH)	5.0	76.9 \pm 0.3	921 \pm 18 ^b	3.16 \pm 0.10 ^b	amber
Highland Honey (TH)	4.0	79.3 \pm 0.4	975 \pm 38 ^b	3.53 \pm 0.09 ^b	amber
Portobello 2011 (PB-11)	4.5	78.4 \pm 0.2	183 \pm 6	1.91 \pm 0.02	white
Portobello 2012 (PB-12)	4.5	79.3 \pm 0.2	286 \pm 7	2.49 \pm 0.03	white

The sugar content, the total phenolic content and antioxidant capacity are reported as means \pm SE ($n = 3$). The pH of honey is reported as median value of the range, $n = 9$. ND = not detected.

^a Significantly different than all other values.

^b Significantly different than all other values not marked with this symbol.

3.3. Compositional analysis by liquid chromatography mass spectrometry (LC-MS)

LC-MS analysis provided a unique and distinctive profile of phenolic and non-phenolic compounds in each honey sample (Fig. 2). Comparison of the composition of most active honey samples (i.e. MH, TH and HH honeys) was used to select components that may be associated with anti-microbial activity. Peaks

which were more abundant in the most effective honey samples but were absent or present at lower levels in less effective honey samples were highlighted (see Fig. 2). Only the traces after 5 mins are shown because the high sugar content of the samples eluted between 1 and 4 min. The Manuka honey contained characteristic derivatives including methyl syringate (Stephens et al., 2010; peak MH5). Phenyllactic and methoxyphenyllactic acid were also present as reported before in Manuka honeys (Oelschlaegel et al.,

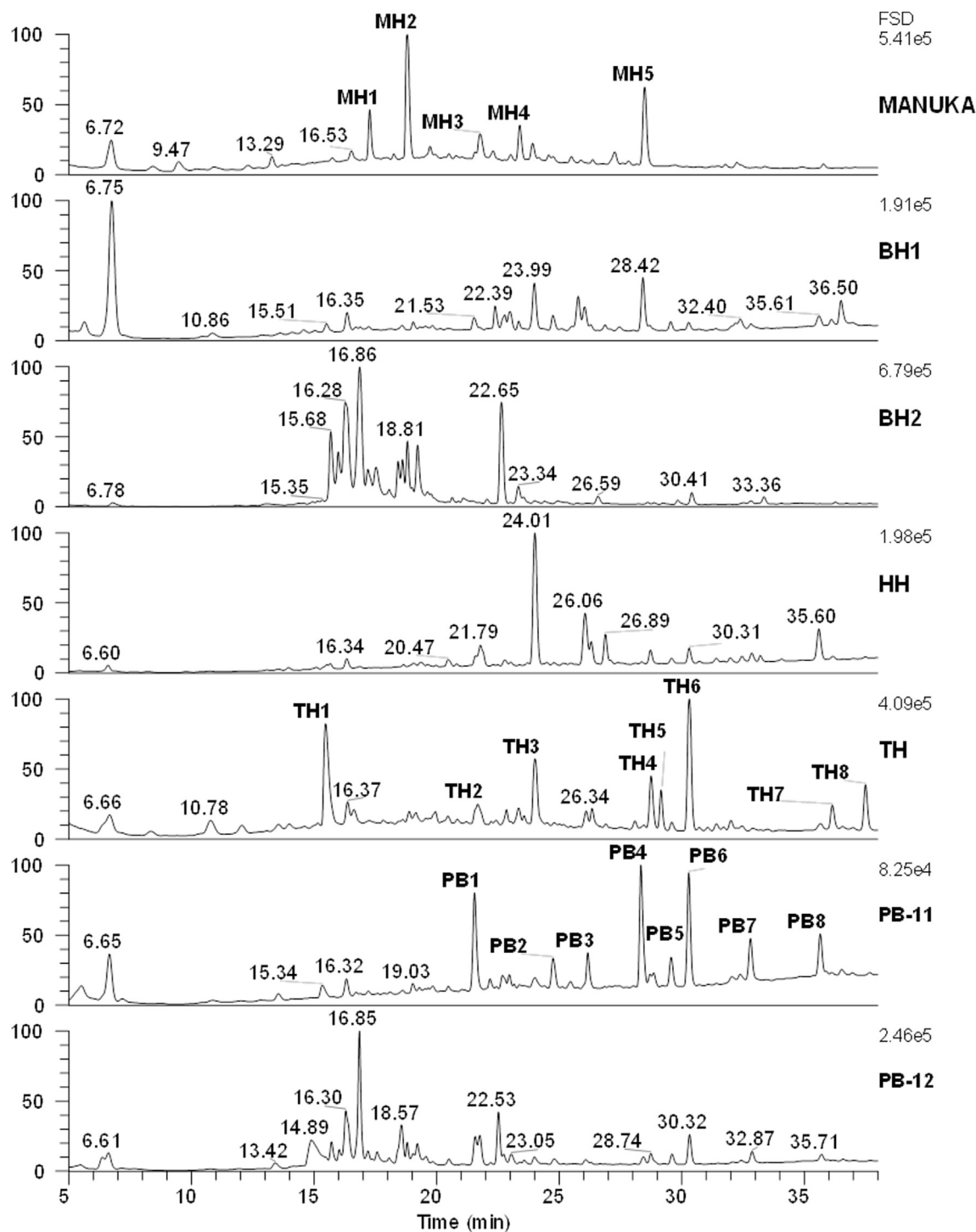


Fig. 2. UV traces of honey samples.

All traces are shown at 280 nm. The figures in the total right corners represent the full scale deflection of the PDAD. Selected peaks are labeled and are discussed in Table 2 and in the text.

2012) and peak MH2 may be a maltosyl-methyl syringate derivative as previously identified by Fearnley et al. (2012).

The TH honey also contained phenyllactic acid (peak TH2) and contained *cis*- and *trans*-isomers of abscisic acid (ABA), which have been identified in honeys from a range of floral sources (e.g. Oelschlaegel et al., 2012). PB-11 honey also contained a clear peak of *cis*-ABA (peak PB6) and of pinobanksin (PB8), a common component of heather honeys (Ferreres, Andrade, & Tomás-Barberán, 1996). In addition, both TH and PB honeys contained peaks with MS properties suggestive of hexoses of *m/z* 199 and 201 (*m/z* 361 and 363; peaks TH3, PB2 and PB3) and peak PB5 had *m/z* of 199 and may be a non-glycosylated form. To examine the nature of these components more closely, a sub-sample of TH honey was fractionated using solid phase extraction on C18 units and re-examined using LC-MS on an Orbitrap MS capable of exact mass determination (Table 2). The MS data confirmed the presence of phenyllactic acid, methoxyphenyllactic acid, *trans*-ABA and pinobanksin and provided predicted structural formulae at ~1 ppm error that matched these components in the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). In addition, the exact mass data for the *m/z* 363, 361, 201 and 199 peaks were consistent with hexoses of decenedioic and deacanedioic acids and the original fatty diacids respectively. The peak at *m/z* 201 also co-eluted with a standard of decanedioic acid and gave the same MS data (i.e. sebacic acid, Sigma Chem. Co. Ltd; results not shown). These fatty diacids have been identified in honeys from various sources (e.g. Schievano, Morelato, Facchin, & Mammi, 2013) and may arise from secretions from worker bees (Ramadan & Al-Ghamdi, 2012). However, to our knowledge, hexoside derivatives of these fatty diacids have not been previously identified in honeys.

4. Discussion

Our data on antimicrobial effectiveness generally agrees with previous work (Schneider et al., 2012) which showed that Scottish honeys were generally non-specific causing inhibition of both Gram-positive (*S. aureus*) and Gram-negative bacteria

(*P. aeruginosa*, *A. calcoaceticus* & *E. coli*). A wider screening which examined the activity of 29 honeys against common equine bacterial isolates found that although Manuka was effective, the best overall performing sample was a Scottish Heather Honey (Carnwarth, Graham, Reynolds & Pollock, 2014). Indeed, in this study, Highland (TH) and Portobello honey (PB-11) were almost as effective as the Manuka Medihoney® which suggests that these honeys could find use as antimicrobial treatments akin to Medihoney®. The honeys seemed particularly effective against the *Acinetobacter* isolate, which could be of interest considering the clinical issues surrounding antibiotic-resistant ABC infections. However further work examining the dose responses of the different bacteria is required to confirm this susceptibility.

A comparison of the general composition of the effective and non-effective honeys may reveal clues about the nature of the active antimicrobial components. Sugar content, H₂O₂ content or pH did not differ between the effective and non-effective honeys so these factors can be ruled out. Some of the most effective antimicrobial honeys, Manuka (MH), Highland (TH) and Heather honeys (HH) had higher polyphenol levels (and antioxidant activity) which suggests that antimicrobial action was related to the presence of these non-sugar components. Indeed, the least effective honey BH2, which had levels of inhibition that were similar to the sugar alone control “honey”, had low polyphenolic content. This trend agrees with work by Kaskoniene et al. (2009) who also described higher antimicrobial activity in honeys rich in polyphenols. However, the potential correlation between higher phenol content and antimicrobial activity does not hold across the sample set. For example, Portobello honey 2011 (PB-11) had the lowest TPC (183 µg GAE/g FW) but was almost as effective as the TH sample (TPC = 975 µg GAE/g FW) and was more effective than the heather honey (HH; TPC = 921 µg GAE/g FW). Indeed, there was no significant correlation between TPC and antimicrobial activity against any of the bacterial isolates (e.g. best correlation against *P. aeruginosa* had R² value < 0.45; see Supplementary data). FRAP, as a measure of antioxidant capacity, had a lower correlation against antimicrobial activity (R² values < 0.25; data not shown).

Table 2

The identification of selected components in MH, TH and PB-11 honeys.

Peak No.	T _R	PDA	<i>m/z</i>	MS ²	Putative Identity
MH1	17.27	275	372.9	326.7, <u>164.9</u>	Hexoside of <i>m/z</i> 165
MH2	18.80	260	580.9, 210.9	<u>534.7</u> , <u>322.9</u> , 210.9	Methyl syringate derivative ^a
MH3	21.78	275	165.0	<u>164.9</u> , 147.0	Phenyllactic acid
MH4	23.39	280	195.0	<u>176.9</u> , 148.9	Methoxyphenyllactic acid
MH5	28.49	260	210.9	195.9, 180.9, <u>166.9</u>	Methyl syringate
TH1	15.47	315	144.1	144.1	Unknown
TH2	21.68	275	165.0	<u>164.9</u> , 147.0	Phenyllactic acid
TH3	24.02	240	361.0	<u>300.9</u> , 270.8, <u>198.9</u>	Hexoside of <i>m/z</i> 199
TH4	28.75	260–290	308.9	262.9	ABA-formate adduct
			<u>262.9</u>	262.9, <u>219.0</u> , 201.0, 153.0	<i>trans</i> -abscisic acid (ABA)
TH5	29.12	275	455.0	<u>408.8</u> , 271.0, 247.0	Unknown
TH6	30.31	260–290	262.9	262.9, <u>219.0</u> , 201.0, 153.0	<i>cis</i> -abscisic acid
TH7	36.15	365	315.0	315.0, <u>301.0</u>	Isorhamnetin
TH8	37.50	270	No <i>m/z</i>	—	Unknown
PB1	21.55	270	717.0	No MS ²	Unknown
PB2	24.75	240	361.0	<u>300.9</u> , 276.9, <u>198.9</u>	Hexoside of <i>m/z</i> 199
PB3	26.17a	350	363.0	<u>302.9</u> , 272.9, <u>201.0</u>	Hexoside of <i>m/z</i> 201
	b		<u>447.1</u> , 301.0	<u>301.0</u>	Quercetin rhamnose
PB4	28.34	350	<u>431.0</u> , 285.0	285.1	Kaempferol rhamnose
PB5	29.57	240	199.0	<u>198.9</u> , 181.0, <u>155.0</u> , 137.0	<i>m/z</i> 199
PB6	30.29	260–280	262.9	262.9, <u>219.0</u> , 201.0, 153.0	<i>cis</i> -abscisic acid ^b
PB7	32.81	280	No <i>m/z</i>	—	Unknown
PB8	35.65	290	271.0	<u>271.0</u> , 253.0, 225.0, 151.0	Pinobanksin

The underlined MS² ions were the most abundant.

a & b - peaks co-elute.

^a Possibly a formate adduct of the maltosyl-methyl syringate derivative identified by Fearnley et al. (2012).

^b *trans*-abscisic acid is also present in PB-11 but is not as abundant as in TH.

Table 3Exact mass MS and MS² data on selected peaks from TH using Orbitrap MS.

M-H exact mass	MS ²	Predicted formula (Δ ppm)	Putative Identity [PubChem No.]
165.0558	146.9803	C ₉ H ₉ O ₃ (1.169)	Phenylactic acid [3848 ^a]
195.0664	151.0327	C ₁₀ H ₁₁ O ₄ (1.255)	Methoxy-phenylactic acid
361.1503	301.0219, 270.0477, <u>199.0093</u> ^b	C ₁₆ H ₂₅ O ₉ (1.011)	Hexoside of decanedioic acid
263.1286	<u>219.1562</u> , 201.1708, 153.0306	C ₁₀ H ₁₅ O ₄ (loss of hexose)	<i>trans</i> -abscisic acid (ABA) [5280896]
363.1657	303.1046, 272.8793, <u>201.0931</u>	C ₁₅ H ₁₉ O ₄ (0.814)	
		C ₁₆ H ₂₇ O ₉ (0.751)	
199.0976	181.0976, <u>155.0861</u> , 137.0756	C ₁₀ H ₁₇ O ₄ (loss of hexose)	Decanedioic acid [6442613]
201.1131	183.1011, 139.1217	C ₁₀ H ₁₅ O ₄ (1.095)	Decanedioic acid [5192]
271.0609	252.9962, <u>225.1189</u> , 177.0338, <u>150.9814</u>	C ₁₀ H ₁₇ O ₄ (0.994) C ₁₅ H ₁₁ O ₅ (0.850)	Pinobanksin [73202]

Putative identifications supported by Schievano et al. (2013), Oelschlaegel et al. (2012), and Stephens et al. (2010).

^a PubChem database numbers are given in square parentheses.^b The underlined MS² ions were the most abundant.

Total polyphenol content may be an important criterion for antimicrobial activity, the composition of polyphenols present in honey may be the key factor. Specific polyphenols could function individually or act synergistically with other components to increase overall antimicrobial effectiveness. For example, methyl syringate is a major phenolic constituent of Maunka honeys (Oelschlaegel et al., 2012) and it is reported to be an effective antimicrobial agent (e.g. Russell, Molan, Wilkins, & Holland, 1990). Phenylactic acid and its methoxy derivative have been reported in Maunka honey (e.g. Stephens et al., 2010; Oelschlaegel et al., 2012). However, phenylactic acid hexoside (peak MH1, Table 3) has not been previously identified and its possible antimicrobial activities are not known. The presence of various flavonoids in honeys (e.g. flavonols; quercetin, kaempferol and isorhamnetin derivatives) is well documented (e.g. Gheldof & Engeseth, 2002) and seems to be dependent on the floral source of the honey. The flavanone, pinobanksin, found in PB honey, has also been identified in various honeys (Gheldof & Engeseth, 2002; Kečkeš et al., 2013).

The overall antimicrobial effectiveness of the honeys could also be influenced by synergies with other components. Other components proposed to be antimicrobial, such as methylglyoxal and bee defensin-1 (e.g. Kwakman & Zaat, 2012) were not examined this study and these may be important in certain samples. Abscisic acid was identified in TH and this is the first report of this plant hormone in a Scottish honey. It has been reported as a constituent in Manuka honey (Stephens et al., 2010; Oelschlaegel et al., 2012), Portuguese heather honey (Ferreeres et al., 1996) and Acacia honeys (Schievano et al., 2013). Although the antibacterial action of abscisic acid has not been fully investigated, it has been shown to have antimycobacterial activity (Clark, Ellsworth, Li, Johnson & Gray, 2013) and its various other bioactivities (e.g. Guri, Hontecillas & Bassaganya-Riera, 2011) suggest that it may contribute to the antimicrobial action of Highland honey.

The presence of fatty diacids in certain honeys, in particular decanedioic and decendioic acids, has been noted before (e.g. Tan, Holland, Wilkins, & Molan, 1988). These diacids may arise from royal jelly and have been suggested to have antimicrobial effects (Isidorov, Czyżewska, Jankowska, & Bakier, 2011) so they may contribute to overall antimicrobial effectiveness. However, the putative glycosides of these diacids detected in both PB-11 and TH have not been previously detected in honeys and they may contribute to the antimicrobial effectiveness of these particular honeys. Although the antimicrobial potential of these novel components is unknown, initial analyses suggest that they were present at higher levels in the more effective honeys (results not shown) but further work is required to confirm their structure and potential activities.

Future work could involve classical sub-fractionation approaches to identify the active honey component(s) but this may nullify potential synergistic interactions different classes of components. Another interesting possibility is to extract polyphenol- and/or lipid-enriched fractions from active antimicrobial honeys then add them to a less active honey in an attempt to reconstitute activity. This would constitute a powerful method of determining the importance of various components to the antimicrobial activities of honey within a similar matrix.

5. Conclusions

This study examined the inhibitory activity of six Scottish honeys and Manuka honey against antibiotic-resistant *A. calcoaceticus*, *S. aureus*, *P. aeruginosa* and *E. coli* isolates. Two of the Scottish honeys (Highland honey (TH) and Portobello 2011) had similar effectiveness against these bacteria as Manuka. Antimicrobial activity of the honey samples appeared to be influenced but not explained by their total phenol content. LC-MS analysis of the honeys uncovered characteristic polyphenol components in the different honeys but also identified novel glycoside derivatives of fatty diacids, which may contribute to their antimicrobial activities.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.lwt.2017.01.023>.

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