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Total Phenols, Antioxidant Capacity and Antibacterial Activity of Manuka Honey Chemical Constituents

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Authors' contributions

This work was carried out in collaboration between all authors. Authors ROA and PSN designed the study and wrote the protocol. Author GK performed the study, managed the literature searches, performed the statistical analysis, and wrote the first draft of the manuscript in partial fulfilment of his MSc degree. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To compare the total phenol content, antioxidant capacity and antibacterial activity of methyl syringate (MSY), methylglyoxal (MGO) and phenyllactic acid (PLA) as major components from Manuka honey

Study Design: *In-vitro* study.

Place and Duration of Study: Nutrition Innovation Centre for Food and Health (NICHE), School of Biomedical Sciences, University of Ulster, Coleraine Campus, between June 2016 and September 2017.

Methodology: Total phenols content (TPC) was determined using the Folin-Ciocalteu assay. Antioxidant capacity was evaluated as 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical quenching activity or iron (III) reducing antioxidant capacity (IRAC). Antibacterial activity was measured using the disc diffusion assay with, *E.coli*, *Bacillus subtilis* or *Staphylococcus aureus*.

Results: The TPC for MSY was 60.8 % gallic acid equivalents (GAE) and significantly higher than

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1.6-3.2% GAE observed for MGO or PLA. The antioxidant capacity for MSY (128% to 270% trolox equivalents (TE)) was significantly higher compared with -6% to 4.4%TE for MGO or PLA. A disc diffusion assay for MGO and PLA showed antibacterial power but MSY had no antibacterial activity. **Conclusion:** Methylglyoxal and PLA from Manuka honey showed antibacterial activity but no detectable antioxidant and total phenol character. Methyl syringate, which shows high antioxidant capacity and TPC, had no detectable antibacterial activity. Total phenols content and antioxidant power of Manuka honey is unlikely to be related to its antibacterial activity.

Keywords: Manuka honey; honey components; antibacterial; antioxidant; methylglyoxal; methyl syringate; phenyllactic acid.

1. INTRODUCTION

Honey has a complex chemical makeup, which gives it unique biological properties varying from wound healing, antimicrobial power to anticancer effects [1-3]. Honeys vary greatly in composition, influenced by geographic location, floral source, storage conditions and other factors [2]. Manuka honey is characterized by a high non-peroxide antibacterial activity that remains after dilution and pre-treatment with catalase [3]. Manuka honey also exhibits high antioxidant capacity and high levels of polyphenols [4,5,6]. The total phenol content and methylglyoxal (MGO) content increase with the Unique Manuka Factor (UMF)TM rating [4,5]; (UMF)TM is a commercial rating that indicates an equivalent concentration of phenol solution which gives the same antibacterial activity as honey [4]. Specific chemical markers for Manuka honey quality include MGO [4,5], methyl syringate (MSY) [7] and syringic acid glycoside or leptosin [8].

Antibiotic resistance is becoming a growing problem. Currently, bacterial resistance towards honey has not been observed. The antibacterial activity of Manuka honey correlates with the total antioxidant capacity and UMF rating [1-3]. Recent research suggests that MGO is a major antibacterial compound in Manuka honey [9,10,11,12]. However, the contributions of MSY and phenyllactic acid (PLA) to antibacterial activity of Manuka honey is uncertain [7]. Meanwhile, PLA was identified from lactic acid bacteria fermentation as a broad-spectrum antibacterial agent [13]. UMF-rated honey possess high levels of MGO and polyphenols, but the relations of these indices to antibacterial activity is not wholly understood. The aims of this study were to, compare total phenol content, antioxidant capacity and antibacterial power for MSY, MGO and PLA from Manuka honey (Fig. 1). The results are discussed in terms the possible basis for honey antibacterial activity.

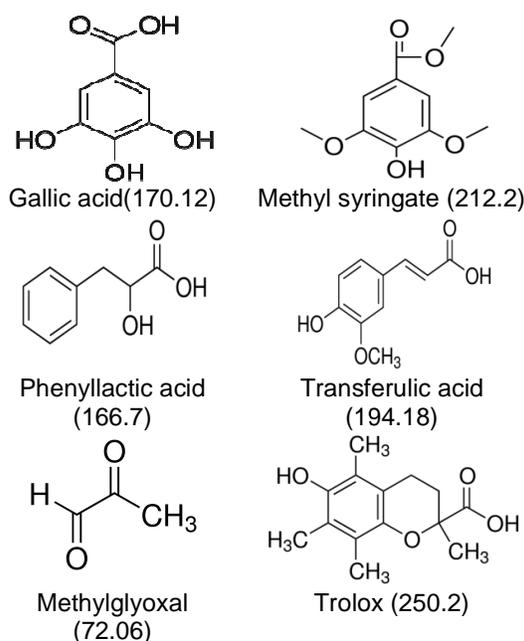


Fig. 1. Chemical structures some organic compounds in this report

Formula weights are shown in parenthesis

2. MATERIALS AND METHODS

2.1 Preparation of Samples

Manuka honey (MH) rated UMF18+ was from Comvita Ltd. (UK). Methyl syringate, MGO, PLA, TFA (1000 μ M) were dissolved in 20% methanol: water solvent as was trolox and gallic acid used as calibration standards. The reference antioxidants were diluted to 500 μ M, 250 μ M, 125 μ M, and 62.5 μ M before use.

2.2 Folin-Ciocalteu Total Phenol Assay

The Folin-Ciocalteu method [14] was used to determine the apparent TPC for honey MGO, PLA and MSY. This assay, which is dependent on tungsten-molybdate complexes is sensitive to

phenols and reducing agents. Gallic acid (50 µl; 0-1 mM) was added to microcentrifuge tubes, with 100 µl of Folin-Ciocalteu and 850 µl sodium carbonate. The microcentrifuge tubes were vortexed briefly and incubated at 37°C for 20 minutes. Thereafter, 200 µl of the sample was transferred to a 96-well microplate (x4 200 µl per sample), and absorbance was measured at 760 nm (A760) using a microplate reader (VersaMax model reader; Molecular devices, Sunnydale, California, USA).

2.3 Iron (III) Reducing Antioxidant Capacity (IRAC) Assay

The IRAC method is a modification of the Ferric Reducing Ability of Plasma (FRAP) assay [15], adapted for 96-well microplate format [16], and adjusted to function at pH 7 [17]. The IRAC reagent comprised 20 mg of ferrozine, dissolved with 18ml of Tris buffer (pH 7.0) and, mixed with 8mg of ferric (III) ammonium sulphate (8 mg) dissolved with 2 ml of deionized water. The microplate IRAC analyses involved adding 20 µl of sample (MSY, PLA, TFA or MGO at 0-1 mM) or reference antioxidant (trolox) to a flat-bottomed 96-well microplate followed by 280 µl of the IRAC solution. The reaction mixtures were incubated at 37°C for 30 minutes, and absorbance readings were recorded at 562 nm (A562) using a microplate reader (VersaMax model; Molecular devices, Sunnydale, California, USA). Each study was repeated on two or more occasions, with triplicate readings per experimental point. IRAC results are cited as, trolox equivalent antioxidant capacity (TEAC).

2.4 ABTS Assay for Antioxidant Capacity

The ABTS assay was performed as described previously [18] with adjustments made for 96-well microplate application. The microplate analyses were carried out by adding 20 µl of sample (MSY, PLA, TFA or MGO at 1 mM) or reference antioxidant (trolox) at concentrations stated to a flat-bottom 96-well microplate followed by 280 µl of the ABTS working solution. The reaction mixtures were then agitated briefly before being incubated at 37°C for in the dark 30 minutes, and absorbance readings were recorded at 734 nm (A734) using a microplate reader as above. The ABTS results are expressed as TEAC values.

2.5 Antimicrobial Determination

2.5.1 Bacterial strains and growth conditions

Bacterial strains were from the Centre of Molecular Biology (CMB), Ulster University. *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*) and *Bacillus subtilis* (*B. subtilis*) were routinely cultivated in either nutrient agar or broth medium at 37°C with shaking.

2.5.2 Determination of antimicrobial activity - disk diffusion

Disc diffusion antibacterial assays were performed as described previously [19]. A 24-hour bacteria liquid culture (200µl) was spread evenly over nutrient agar plates using a sterile spreader and allowed to dry. Six blank paper disks were added to each agar plate and 20µl of each test solution (and controls) were added. To ensure that paper discs were loaded with comparable amounts of tests compounds it was assumed that the maximum occurrence MSY, PLA or MGO from Manuka honey would not exceed 1500 ppm (1.5 mg/ml) [4], corresponding to PLA (9 mM), MSY (7 mM) and MGO (20 mM). Manuka honey (UMF 18+) tested as 25% w/v solution (20µl) loads ~7 µg per disc for each component. Therefore, we prepared MGO (2mg/ml), PLA (2mg/ml) or MSY (1.1mg/ml) using 20% sterile methanol: water solvent and loaded 20 µl (30-40 µg) per paper disc. Tests also involved 20% methanol: water solvent as negative control. Pen-Strep (5000 U/ml penicillin & 5000 µg/ml streptomycin; Gibco ThermoFisher Scientific) was used as a positive control for antibacterial assays (~16 µg per paper disc). Agar plates were incubated at 37°C for 24 hours and the diameter of the zones of inhibition was measured. We used a laminar flow cabinet to ensure sterility. Results are an average of duplicate observations.

2.6 Statistical Analysis

Antioxidant assays were conducted in triplicates on two separate occasions. Microplate data were exported to excel for graphing and calculations. Calibration graphs were produced by plotting absorbances minus reagent blanks on the y-axis, against the concentration of the specific standard; Gallic acid or trolox (in the assay) on the x-axis. Molar absorptivity was determined by taking the gradient of the linear regression trend line where $y=mx$. The

regression coefficient (R^2) was maximized by deletion of high points on the graph.

The disk diffusion assay for antibacterial activity was conducted in duplicate on two different occasions. The averages of the results are displayed in the figures and tables shown with errors given as standard deviation (SD) or standard error of the mean (SEM) where specified. To test for significance we used one-way Anova (IBM SPSS (V24)). An alpha value of $P < 0.05$ was used to determine significance.

3. RESULTS AND DISCUSSION

3.1 Calibration Characteristics of Folin, IRAC and ABTS Assays

Calibration graphs for antioxidant assays were linear ($R^2 > 0.98$) with an upper limit of 1.25-25 μM corresponding to a prepared concentrations of ≤ 0.5 mM. The equations of the line in Table 1 were employed for determining the total phenol (Folin assay) or antioxidant capacity (ABTS & IRAC) for PLA, MSY and MGO.

3.2 Total Phenols Content or Character

Manuka honey possesses high total phenol content, high antioxidant capacity, and high concentrations of MGO which increase with the UMF rating [2,3,4,5]. The pure constituents (MSY, MGO and PLA) investigated in this paper occur in high concentrations in Manuka honey [4, 6, 7] with two (MGO and MSY) proposed as makers of quality [7,8]. Stephens and co-

workers analyzed phenolic acids from 8 samples of Manuka honey and found an average, maximum and minimum PLA level of 1513 mg/kg, 1920 mg/kg or 980 mg/kg, respectively [4]. Syringic acid and MSY combined were the second major components with an average, maximum and minimum levels of 145 mg/kg, 429 mg/kg, and 5.2 mg/kg. In a separate analysis, the same samples of Manuka honey contained an average, maximum and minimum MGO value of, 1085 mg/kg, 1541 mg/kg, or 651 mg/kg honey [4]. Another study of honey phenolics identified a major component as 2-hydroxy-3-phenylpropionate (synonym, (R)-3-phenyllactate PubChem ID 5461132) with an average, minimum and maximum level of, 1769 mg/kg, 733 mg/kg and 4474 mg/kg [6]. In this study, we included trans-ferulic acid (TFA) because its chemical structure bears some similarity to PLA [20] except TFA has an OH group on its benzene ring making it a phenolic compound (Fig. 1).

The total phenol responses for different organic constituents from Manuka honey converted to mg-GAE/100g is shown in Fig. 2. To check the validity of this analysis, the total phenol character for gallic acid was determined to be 97684 (± 3254) mg/100g, which is ~98% GAE. The total phenol response for MSY was 60.8% GAE whilst TFA yielded 63.6% GAE (Fig. 2). The results for MGO and PLA indicated an apparent total phenol response of 3.2% GAE and 1.6% GAE, respectively. The high GAE value for MSY and TFA should be as expected as these are phenolic compounds with an OH group on their benzene ring (Fig. 1).

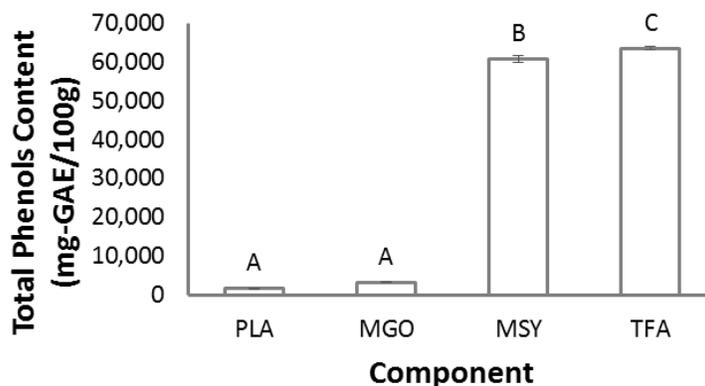


Fig. 2. Total phenols character for some components from honey
Phenylactic acid (PLA), methylglyoxal (MGO), methyl syringate (MSY), Trans-ferulic acid (TFA) is a phenolic comparator for PLA. Lettered data labels show significantly different groups ($P < 0.05$)

Table 1. Calibration parameters for folin and antioxidant capacity assays

Assay	Reference standard	Equation	R ²
Folin	Gallic acid	$\Delta A_{760} = 13447x$	0.9961
ABTS	Trolox	$\Delta A_{734} = 17603x$	0.9962
IRAC	Trolox	$\Delta A_{562} = 39968x$	0.9805

Calibration data for Folin-Ciocalteu, ABTS or Iron (III) Reducing Antioxidant Capacity (IRAC) assay respectively.

3.3 Total Antioxidant Capacity

Analysis using the ABTS assay showed that MSY had high radical quenching capacity, corresponding to 270% TEAC or 27000mg TE per 100g of MSY. TFA had a radical quenching capacity of 170% TEAC. As a check on the validity of this approach, ABTS analysis for trolox produced a TEAC value of 114370(±6511) mg/100g in other words 114± 6.5% TE. However, the radical quenching capacity was 4.2% TE for MGO and 1.7% TE for PLA and the values were not significantly different from each other (Fig. 2). In the preceding analysis, the results of ABTS assay were presented as TEAC units [18] and not as % inhibitory concentrations. Using values for TEAC allows a comparison of antioxidant (ABTS & IRAC) assay results (see below).

According to the IRAC assay, pure MSY showed antioxidant power equal to 128% TE, whilst TFA had antioxidant capacity equal to 89% TE. By comparison, Trolox used a reference for the IRAC assay showed antioxidant power of 107016 (± 5242) mg TE/100g or 107± 5.2% TE. When MGO and PLA were analyzed by the IRAC assay the antioxidant capacity was minus 6.6% TE, which is effectively, zero (Fig. 3).

The antioxidant capacity for MSY, TFA, PLA, and MGO are broadly consistent with their chemical structures (Fig. 1). The number of OH (O-) groups attached to benzene ring is a determinant for antioxidant power for phenols. The present results suggest that MSY is a major contributor to the antioxidant capacity for Manuka honey. By contrast, PLA and MGO, which occur in similar or greater quantities [4], do not contribute enormously to the antioxidant capacity for Manuka honey.

3.4 Antimicrobial Activity

3.4.1 Disk Diffusion

It is thought that MGO accounts for a large part of the non-peroxide antibacterial activity from Manuka honey [9,10,11,12] but the data for MSY is conflicting [1,7]. PLA is difficult to observe by HPLC analysis and is not characterized for antibacterial activity in Manuka honey [4]. The disc diffusion assay (Table 2) showed that MGO, PLA and unfractionated Manuka honey possess antibacterial activity. Apparently, MSY had no antibacterial activity against, *E. coli*, *S. aureus*, or *B. subtilis* strains, at the concentration tested.

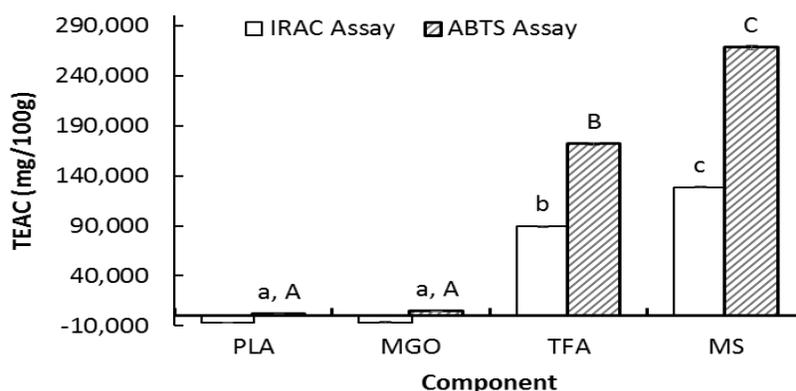


Fig. 3. Antioxidant capacity for honey compounds using the ABTS and IRAC assays

PLA = phenyllactic acid, MGO = methylglyoxal, TFA = tetra ferulic acid. Results are expressed as Trolox equivalent antioxidant capacity (TEAC). Within each assay, bars with different labels show significantly different groups ($P < 0.05$)

Table 2. Zone of inhibition from disc diffusion assay for MH components*

	MGO	MSY	PLA	MH	Pen-Strep	Meth
<i>E. coli</i>	30.5 ^D ± 0.5	6.5 ^A ± 0	12.5 ^B ± 0.5	23 ^C ± 0	32 ^D ± 0	6.5 ^A ± 0
<i>S. aureus</i>	30 ^D ± 0	6.5 ^A ± 0	13 ^B ± 0	23 ^C ± 1	29.5 ^D ± 0.5	6.5 ^A ± 0
<i>B. subtilis</i>	20 ^C ± 1	6.5 ^A ± 0	13 ^B ± 0	17.5 ^C ± 0.5	21.5 ^C ± 1.5	6.5 ^A ± 0

*Average diameter of the Zones of Inhibition from two replicates ± Standard error of the mean (SEM). The measurements include the size of the paper disk (6.5mm). Loadings per disc was 20 µl for Methylglyoxal (MGO; 2mg/ml), methyl syringate (MSY; 1.1 mg/ml), phenyllactic acid (PLA, 2mg/ml), Manuka honey (MH; 25% w/v), Pen-Strep (~8mg/ml) is a positive control; and Meth=methanol: water mixture (20%v/v) negative control, Treatment time was 24 hours before measurement

4. CONCLUSION

Manuka honey components (MSY, PLA and MGO) exhibit different levels of total phenol character, antioxidant capacity and antibacterial power. Analysis using the Folin-Ciocalteu method showed that PLA and MGO had virtually no phenols character. The phenols response for MSY was 60.8% GAE consistent with the chemical structure (Fig. 1). The antioxidant capacity of MSY was high (128-270% TEAC) whilst antioxidant capacity of MGO and PLA were virtually zero. Assays for antibacterial activity were positive for MGO and PLA but MSY had no activity. Therefore, the total phenol and antioxidant components from Manuka honey may contribute poorly to the antibacterial activity. By contrast, MGO and PLA with demonstrated antibacterial activity had low antioxidant or total phenol character.

Interestingly, MGO and polyphenol content are "competing" quality indicators for Manuka honey [4,5]. Obviously, MGO and PLA appear to be better measures for the antibacterial power for Manuka honey. In contrast, the antioxidant activity from Manuka honey is attributable to MSY and related polyphenols. It is noteworthy that *high* concentrations of MGO, PLA, and total polyphenols seem to co-exist for UMF-rated honey [4,5,6,16,21] and so the possibility exists for synergistic or destructive interactions between these components. The current results are interesting but more research, using a wider range of bacteria pathogens and assay conditions, will be needed to yield findings that are more definitive. We are currently seeking resources and collaborative opportunities from Public, Private and Industry sources to enable additional studies on the medicinal properties of honey.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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