Analysis of the flavonoid component of bioactive New Zealand mānuka (*Leptospermum scoparium*) honey and the isolation, characterisation and synthesis of an unusual pyrrole.

Running title: Flavonoids and an unusual pyrrole in New Zealand mānuka honey.

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ABSTRACT:

The flavonoid components of New Zealand mānuka (*Leptospermum scoparium*) honey have been quantified in a series of 31 honeys of varying non-peroxide antibacterial activity to clarify discrepancies between previous studies reported in the literature. Total flavonoid content was 1.16 mg/100 g honey. The principal flavonoids present were pinobanksin, pinocembrin, luteolin and chrysin and together these represented 61% of the total flavonoid content. **1**, 2-formyl-5-(2-methoxyphenyl)-pyrrole , which was weakly correlated with the non-peroxide antibacterial activity, was isolated from the flavonoid fraction and separately synthesised. **1** did not display inhibitory activity against *S. aureus* in vitro and thus the origin of the correlation, which is still unknown, is not a direct contribution.

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INTRODUCTION:

New Zealand mānuka (*Leptospermum scoparium*) honey is noted for its non-peroxide antibacterial activity (Allen, Molan & Reid, 1991). This activity has been attributed principally to the presence of methylglyoxal (Adams *et al*., 2008, Mavric, Wittman, Barth & Henle, 2008), which originates from dihydroxyacetone in the nectar of the flower (Adams *et al*., 2009), the reasons why some individuals of this species produce high levels of dihydroxyacetone are unknown. Because of its non-peroxide antibacterial activity, New Zealand mānuka honey commands a premium price world-wide and is thus subject to fraud and substitution; to establish authenticity, markers of floral origin are urgently needed. Flavonoid profiles have been used to determine the floral origin of honeys (Tomas-Barberan, Ferreres, Garcia-Viguera & Tomas-Lorente, 1992, Tomas-Barberan, Ferreres, Garcia-Viguera & Tomas-Lorente, 1993, Anklam, 1998); flavonoids thus represent a possible biomarker for mānuka honey. Flavonoids are known for their antibacterial and other bioactivities (Cushnie & Lamb, 2005, Havsteen, 2002, D'Arcy, 2005) and there exists a possibility that one or more components of the flavonoid fraction of mānuka honey contribute directly or indirectly (by involvement in the biosynthetic pathway generating high levels of dihydroxyacetone) to the non-peroxide antibacterial activity of the honey. Weston, Brocklebank and Lu (2000) found the principal flavonoids in mānuka honey to be pinobanksin with 4.3, 6.3, 5.0 μg/100g honey respectively for high (10 samples), low (3 samples) and zero (6 samples) antibacterial activity respectively , pinocembrin with 5.1, 7.3, 4.5.0 μg/100g honey for high, low and zero antibacterial activity respectively, chrysin with 2.2, 2.7, 2.2 μg/100g honey for high, low and zero antibacterial activity respectively and galangin with 2.2, 3.4, 1.9 μg/100g honey for high, low and zero antibacterial activity respectively. This is in contrast to the work of Yao, Datta, Tomas-Barberan, Ferreres, Martos and Singanusong (2003), who found the principal flavonoids in two samples of mānuka honey to be quercetin (430 μg/100g honey), isorhamnetin (400 μg/100g honey), an unknown flavonoid (390 μg/100g honey), chrysin (380 μg/100g honey) and luteolin (380 μg/100g honey) with a total flavonoid content of 3060 μg/100g honey. There is a very marked qualitative

and quantitative difference between these two sets of results and indeed Yao *et al*. (2003) recommended further analysis of New Zealand mānuka honeys using a large sample set and the HPLC detection methodology that they espoused, that is detection of flavonones at 290 nm and of flavones at 340 nm using diode array detection.

Pyrroles in honey can arise from Maillard reactions or from the pyrolysis of amino acids when honey is heated (Jerković, Mastelić, Marijanović, Klein & Jelić, 2007). 1*H*-Pyrrole has been found in *Robinia pseudoacacia L*., *Castanea sativa L*. and *Salvia officinalis* L (Jerković *et al*., 2007, Jerković, Mastelić & Marijanović , 2006). 1*H*-Pyrrole-2-carboxylic acid has been found in *Paliurus spina-christi* (Jerković, Tuberoso, Marijanović, Jelić & Kasum, 2009). 2-Acetylpyrrole has been found in *abbamele,* a honey-based Sardinian product (Jerković, Kasum, Marijanović & Tuberoso, 2011) and 1*H*-pyrrole-3,4-diacetic acid has been found in pine honey (*Pinus brutia* Ten) (Eraslan, Kanbur, Silici & Karabacak, 2010). Phenyl substituted pyrroles are also found in natural products, pyrrolnitrin is a tryptophan-derived, antifungal antibiotic isolated from *Pseudomonas pyrrocinia* (van Pée & Ligon, 2000). Both pentachloropseudilin and pentabromopseudilin, which are produced by an *Actinoplanes* sp. strain*,* are strongly active against Gram-positive bacteria. The latter is also known to inhibit a number of different enzyme systems and has high *in vitro* activity against leukemia and melanoma cell lines (van Pée & Ligon, 2000).

This study aimed to investigate the flavonoid profiles of a large sample set of New Zealand mānuka honeys using the detection methods suggested by Yao *et al.* (2003) and to establish if there was any link between this profile and the afore-mentioned non-peroxide antibacterial activity, which is a feature of these honeys. During the course of this investigation an unidentified peak in the HPLC chromatogram of the flavonoid fraction was shown to be weakly correlated to antibacterial activity. The compound, 2-formyl-5-(2-methoxyphenyl)-pyrrole , **1**, was subsequently isolated from the flavonoid fraction and fully characterised.

MATERIALS AND METHODS

Materials: Thirty one mānuka honey samples with non-peroxide antibacterial activities ranging from very low to 27.3 UMF™, were kindly supplied by Comvita New Zealand Ltd., Te Puke, New Zealand. A bulk sample of active mānuka honey (6 kg, Haddrell's, Cambridge, New Zealand) was used for the optimisation and testing of the extraction method. Mānuka honey (15 kg, Natures Country Gold, Hamilton, New Zealand) was used for the isolation of flavonoids.

3 was used as a model compound and **4** as an analogue for the characterisation of **1**. **3**, 5-(2-nitrophenyl)-2-furaldehyde, was obtained from Acros Organics. **4**, 2-Formyl-5-phenyl-pyrrole, was generously donated by Dr J. T. Reeves of the Department of Chemical Development, Boehringer Ingelheim Pharmaceuticals Inc., Ridgefield, CT 06877-0368, USA.

Methanol was redistilled from drum grade or HPLC grade (Scharlau), Milli-Q water was obtained from a Barnstead E-pure system (18.2 MΩcm). Dried and purified diethyl ether, hexanes, tetrahydrofuran and dichloromethane were obtained from a Pure Solvent Purification System (Model: PS-SD-5) as needed and used promptly. Dimethyl sulfoxide-d_6 (99.9 atom% D containing 0.03% v/v TMS) and chloroform-d_1 (99.8 atom% D) were obtained from Sigma-Aldrich Inc. Lithium diisopropylamide (LDA), *o*-methoxyacetophenone, oxazole-4-carboxaldehyde, diisobutylaluminium hydride (DIBALH) and triethylamine (Et_3N) were obtained from Sigma-Aldrich Inc., methane sulphonyl chloride (MsCl) was obtained from Riedel-de Haën. Caffeic acid (≥98%), (+)-catechin hydrate (98%), chlorogenic acid (predominantly *trans*) (≥ 95%), chrysin, *trans*-cinnamic acid (99+%), *p*-coumaric acid (≥ 98%), (-)-epicatechin (97%), galangin (no stated purity), gallic acid (97%), luteolin (≥ 98%), myricetin (approx 85%), pinocembrin (95%), quercetin dihydrate (98%), (+)-rutin hydrate (95%) and syringic acid (98%) were obtained from Sigma-Aldrich Inc., isorhamnetin (pure), kaempferol (pure) and naringin (pure) were obtained from Indofine Chemical Company Inc.

XAD-2 Amberlite resin (pore size 9 nm, particle size 0.3-1.2 mm) was obtained from Sigma-Aldrich Inc. Sephadex LH-20 resin was obtained from Amersham Pharmacia Biotech AB and silica gel was obtained from Merck.

General Methods: Evaporation of aqueous samples under reduced pressure was accomplished using a rotary evaporator with a water bath set to 35 ^{o}C, smaller samples were reduced in volume by evaporation under a stream of dry nitrogen (35 ^{o}C). UV-Visible spectra of isolated flavonoids were recorded on a Varian Cary 100 Scan UV-Visible Spectrophotometer, sodium methoxide, sodium acetate, sodium acetate/boric acid, aluminium chloride and aluminium chloride/hydrochloric acid were variously added to measure changes in the UV spectrum (Markham & Mabry, 1975, Jurd, 1962, Mabry, Markham & Thomas, 1970) for purposes of identification.

Isolation of Flavonoid Fraction: Phenolics were extracted from samples of honey using XAD-2 resin according to the method of Ferreres, Tomas-Barberan, Gil & Tomas-Lorente (1991). Phenolic acids and flavonoids were separated using Sephadex-LH20 according to the method of Bohm (1998).

Semi-preparative High Performance Liquid Chromatography (HPLC): Semi-preparative HPLC of bulk fractions of honey for isolation of flavonoids was carried out using two Waters 515 pumps, an Alltech Elite Degassing System, a Rheodyne 7725i injector fitted with a 500 µL sample loop, and a Waters 996 photodiode array detector. A reversed phase octadecylsilane Waters Xterra Prep column (PR_{18}, 10 µm, 7.8 mm x 300 mm) was used in this system. A binary mobile phase was used; this consisted of solvent A which was Milli-Q water acidified with acetic acid (0.075% v/v) and methanol (5% v/v) and solvent B which was methanol. The overall flow rate was 4 mL/min. The gradient used was held at 60% A for 5 min, decreasing to 50% A at 10 min and 20% A at 60 min and finally 0% A at 60.2 min.

*Analysis of flavonoids and isolation of **1** by High Performance Liquid Chromatography (HPLC):* Analysis of flavonoid extracts and isolation of **1** was carried out using two Waters 515 HPLC pumps with their flows combined through a static mixer (Grace Binary Large Volume Mixer SS Housing with 350 µL Mixer Cartridge). Both solvents were degassed prior to entering the pumps by passing through a Waters In-Line Degasser AF. Samples and standards were warmed (40 ^{o}C) and injected manually using a Rheodyne 7725i injector system fitted with a Rheodyne loop (5 µL for analyses, 50 µL for isolations). Separation was achieved using a Waters Symmetry Shield™ octadecylsilane HPLC column (RP18, 5 µm, 3.0 x 250 mm) with a Waters Universal Sentry™ Guard SymmetryShield™ Column. A binary gradient was used, solvent A was Milli-Q water and methanol (5% v/v), acidified with acetic acid (0.075% v/v), solvent B was methanol. The gradient was run at a constant combined flow rate of 0.3 mL/min. Detection was achieved using a Waters 996 Photodiode Array Detector (240-400 nm). Gradient method 1 (for analyses and initial isolation of **1**) consisted of 70% A for 15 min, decreasing to 40% A at 20 min and 0% A at 60 min and held for 10 min. Gradient method 2 (for final purification of **1**) consisted of 70% A for 5 min, decreasing to 55% A at 10 min, 20% A at 50 min and 0% A at 52 min and held for 8 min.

Gas Chromatography-Mass-Spectrometry (GC-MS): GC-MS was carried out on a HP 6890 series GC fitted with a Phenomenex ZB-5 5% phenyl-methylsiloxane) column (30 m x 0.25 mm x 0.025 µm) interfaced to a HP 5973 mass selective detector. Conditions used were 120 °C (0.75 min), 50 °C/min up to 200 °C, and 10 °C/min up to 295 °C (held 15 min). The mass spectrometer was operated in either total ion chromatogram (TIC) or a selected ion monitoring (SIM) mode using *m/z* 201 ($M^{+\cdot}$), 158 and 130 ions in the case of **1**.

High Resolution Mass Spectrometry (HRMS): Mass spectra were recorded in positive-ion mode on a Bruker MicrOTOF mass spectrometer with electrospray interface and MeOH as mobile phase. Assignments of major peaks were confirmed by comparison of the high-resolution isotope pattern of the ions with the theoretical pattern obtained using isotope ratios.

Nuclear Magnetic Resonance Spectroscopy (NMR): 1D- and 2D- ^{1}H and ^{13}C NMR spectra of samples were obtained using a Bruker Avance DRX-400 spectrometer. ^{1}H and ^{13}C experiments were carried out at 400.13 and 100.62 MHz respectively using a 5 mm inverse $^{13}C/^{1}H$ probe head except for the ^{13}C spectrum of **1,** which was acquired using a 5 mm dual $^{13}C/^{1}H$ probe head. Samples were dissolved in dimethylsulfoxide-d_6 or chloroform-d_1 for NMR analysis. Operation of the NMR spectrometer and processing of spectra were performed using Bruker Topspin 1.3 software.

Measurement of UMF™: Measurement of non-peroxide antibacterial activity was carried out using the method previously described by Allen *et al.* (1991) and isolated fractions were mixed with clover honey as an artificial matrix for testing (Adams *et al*., 2008).

*Measurement of Bioactivity of **1**:* Bioactivity testing was carried out at the ithree Institute, School of Medical and Molecular Sciences, University of Technology, Sydney, Australia. *Staphylococcus aureus* strain NCTC 8325 was used throughout this part of the study. Growth assays were set up in cation-adjusted Mueller Hinton II Broth (CaMHB, Becton Dickinson) whereas biofilm assays were performed in tryptic soya broth (TSB, Oxoid).

For a growth study, **1** was diluted in dimethyl sulfoxide (DMSO) and then further serial dilutions were made in DMSO. Each dilution was added in duplicate to a 96-well plate to a final DMSO concentration of 2%. An overnight culture was diluted to 1 x 108 cfu/mL (determined by back-titration on tryptic soy agar plates) and 150 µL was added to each well of the 96-well plates. Controls included a serial dilution of Lincomycin (to assess plate-to-plate variation), a positive control with bacteria alone in CaMHB with 2% DMSO and a negative (no bacteria) with 150 µl CaMHB containing 2% DMSO. Plates were incubated in a shaking incubator at 37 ^{o}C for 22 h and absorbance was measured at a wavelength of 595 nm using a plate reader (Biotek Synergy HT).

For a biofilm assay, plates were set up as for the growth assay except that bacteria were grown in TSB. Plates were then sealed with AeraSeal (Excel Scientific) and incubated at 37 ^{o}C in a humidified

incubator for 24 h. Plates were washed with PBS and dried at 60 ^{o}C for 1 h. Biofilm was stained by the addition of 200 µL of 0.2% crystal violet at room temperature for 1 h. Plates were washed 3 times with water and air-dried and the amount of biofilm biomass was quantified by destaining the biofilm with 250 µL of 30% acetic acid. Plates were incubated on a shaking platform at room temperature for 15 min and absorbance was then read at a wavelength of 635 nm.

For a disc diffusion test, an overnight culture (100 µL) was streaked out with a sterile cotton swab on a tryptic agar plate. Discs containing 10 µg of **1**, rifampicin (positive control) or DMSO (negative control) were placed on the lawn of bacteria. Plates were then incubated overnight at 37 ^{o}C and assessed for zones of bacterial inhibition.

*3-hydroxy-1-(2-methoxyphenyl)-3-(oxazol-4-yl) propan-1-one (**5**):* To THF (20 mL) at -84 ^{o}C was added LDA (2.5 mL, 5.5 mmol). The resultant solution of LDA was added dropwise to a solution of *o*-methoxyacetophenone (0.6 mL, 4.5 mmol). The reaction mixture was stirred at -84 ^{o}C for 30 minutes before a solution of oxazole-4-carboxaldehyde (0.5 g, 5.5 mmol) in a minimal amount of THF was added dropwise at -84 ^{o}C. The reaction mixture was stirred for 30 minutes at -84 ^{o}C, quenched with saturated aqueous NH_4Cl (20 mL) and allowed to warm to room temperature. The layers were allowed to separate, and the aqueous layer was extracted with EtOAc (2 x 20 mL). The combined organic layers were dried ($MgSO_4$), filtered and concentrated. The product was recrystallized from CH_2Cl_2/hexanes yielding **5** as pale yellow crystals (0.751 g, 68%), Mpt: 104 – 107 ^{o}C, HRMS: found: 270.0735, calculated for $C_{12}H_{12}NO_4Na$ $[M+Na]^+$: 270.0737, ^{1}H NMR (400.13 MHz, DMSO-d_6) δ ppm 3.87 (s, 2-OCH_3), 5.36 (d, *J* = 5.4 Hz, OH), 3.26 (dd *J* = 8.2, 16.2 Hz, H_a-2'), 3.37 (dd *J* = 4.6, 16.2 Hz, H_b-2'), 5.06 (m, H-3'), 7.89 (s, H- 5'), 8.26 (s, H-6'), 7.16 (d, *J* = 8.6 Hz, H-3), 7.54 (t, *J* = 7.5 Hz, H-4), 7.03 (t, *J* = 7.5 Hz, H-5), 7.52 (d, *J* = 8.6 Hz ,H- 6), ^{13}C NMR (100.62 MHz, DMSO-d_6 δ ppm 199.6 (C-1'), 50.4 (C-2'), 62.6 (C-3'), 143.3 (C-4'), 135.1 (C-5'), 151.5 (C-6'), 128.1 (C-1), 158.0 (C-2), 112.3 (C-3), 133.6 (C-4), 120.9 (C-5), 129.4 (C-6).

192 *2-formyl-5-(2-methoxyphenyl)-pyrrole* (**1**): A solution of **5** (0.23 g, 0.9 mmol) in THF (4 mL) was
193 treated at 0 °C with Et_3N (0.4 mL) followed by dropwise addition of MsCl (0.1 mL), the reaction
194 mixture was stirred at 0 °C for 1 h. Aqueous NaOH (2 M, 6 mL) was added and the reaction mixture
195 heated (70 °C, 72 h). After cooling to room temperature, the reaction mixture was diluted with
196 saturated aqueous $NaHCO_3$ solution, extracted with EtOAc (2 x 20 mL) and the organic layer dried
197 ($MgSO_4$), filtered and concentrated. The product was purified by chromatography on a silica gel
198 column (EtOAc – hexane, 1:9 → 1:0), the fraction containing **1** was concentrated and further
199 purified with preparative layer chromatography using a circular plate (220 mm diameter, 2 mm
200 silica layer: Merck PF245) installed on a Chromatotron (Harrison Research) using 50 mL portions of
201 hexane-diethyl ether mixtures (4:1, 2:3, 4:1) as eluent. This yielded **1** as a yellow solid (0.36 mg,
202 0.2%), HRMS: found: 224.0653, calculated for $C_{12}H_{11}NO_2Na$ $[M+Na]^+$: 224.0682, ^1H NMR (400.13
203 MHz) and ^{13}C NMR (100.62 MHz) see Table 4.

204 The major product formed during the synthesis of **1** was (*E*)-1-(2-methoxyphenyl)-3-(oxazol-4-yl)-
205 prop-2-en-1-one, **6**, and its (*Z*)-isomer (minor) eluting in GC-MS at 6.28 min and 5.60 min
206 respectively. Chromatography under the conditions used for **1** gave **6** as orange crystals (0.075g,
207 36%), Mpt: 75 – 80 °C, HRMS: found: 252.0644, calculated for $C_{13}H_{11}NO_3Na$ $[M+Na]^+$: 252.0631, ^1H
208 NMR (400.13 MHz, DMSO-d_6) δ ppm 3.86 (s, 2-OCH_3), 7.42 (dd, *J* = 15.5, 0.8 Hz, H-2'), 7.35 (d, *J* =
209 15.5 Hz, H-3'), 8.48 (t , *J* = 0.8 Hz, H-5'), 8.50 (s, H-6'), 7.19 (dd, *J* = 8.3, 1.0 Hz, H-3), 7.55 (td, *J* = 8.3,
210 1.8 Hz, H-4), 7.06 (td, *J* = 7.5, 1.0 Hz, H-5), 7.48 (dd, *J* = 7.5, 1.8 Hz, H-6), ^{13}C NMR (100.62 MHz),
211 DMSO-d_6 δ ppm 191.9 (C-1'), 131.1 (C-2'), 127.0 (C-3'), 136.3 (C-4'), 141.7 (C-5'), 153.1 (C-6'), 128.4
212 (C-1), 157.7 (C-2), 112.3 (C-3), 133.1 (C-4), 120.6 (C-5), 129.5 (C-6), 55.8 (2-O<u>C</u>H_3). The ^1H NMR
213 spectrum of the crude reaction mixture also showed signals at 6.85 ppm (*J* = 12.6 Hz) and 6.99 ppm
214 (*J* = 12.6 Hz) consistent with the presence of the olefinic protons of the corresponding (*Z*)-isomer.

215

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RESULTS AND DISCUSSION

Flavonoid profiles: The flavonoid profiles of 31 different mānuka honey samples were analysed. XAD-2 extraction was used according to the method of Ferreres *et al.* (1991) but modified to use 5 g rather than 50 g samples, which modification was made to allow flavonoid profiling of a much larger set of honey samples. To validate this modification multiple extractions of 50 g (3 replicates), 20 g (3 replicates) and 5 g (5 replicates) samples were performed and the total area of the chromatogram (340 nm) in the phenolic region was measured. The results, $(1.7 \pm 0.8) \times 10^8$, $(1.9 \pm 1.0) \times 10^8$ and $(2.1 \pm 1.0) \times 10^8$ for 50, 20 and 5 g respectively, indicate that any variation due to sample size is insignificant compared with the intra-sample size variation. Phenolic acids and flavonoids were separated using Sephadex-LH20 (Bohm, 1998). The HPLC chromatogram of the flavonoid fraction of a typical mānuka honey is shown in Fig.1. Flavonoids and phenolic acids were identified by HPLC co-elution with an authentic standard and for some confirmed by isolation and characterisation by NMR and UV spectroscopy (caffeic acid, *p*-coumaric acid, pinobanksin and 8-methoxykaempferol). Several compounds were identified as flavonoids by their characteristic UV spectra, Table 1, but isolation and identification has so far proven elusive. Unknown flavonoids 1, 2 and 5 showed only a Band II absorption for λ_{max} indicating that they are flavanones and/or dihydroflavonols while unknown flavonoids 3, 4 and 6 showed both Band I and Band II absorptions indicating that they are flavones and/or flavonols. Quantification of the flavonoids was achieved by comparing their absorbance in the HPLC chromatograms to four external standards. Pinocembrin at 290 nm was used to quantify all flavanones and dihydroflavonols, chrysin at 340 nm was used to quantify flavones and flavonols with unsubstituted B rings and kaempferol at 340 nm was used for those flavones and flavonols with a singly oxygenated B ring, while quercetin at 340 nm was used for all other flavones and flavonols, Table 2. Total flavonoid content was 1.16 mg/100 g honey with a 95% confidence interval of 0.16 mg/100 g honey and a range of 0.594-2.235 mg/100 g honey. This value is considerably higher than the 0.0147 mg/100 g total flavonoid content reported by Weston *et al.* (2000) and closer to that reported by Yao *et al*. (2003), that is 3.06 mg/100 g of honey

for two samples. Venugopal and Devarajan (2011) found 3.34 mg (chrysin equivalents)/100 g of honey in a single sample of mānuka honey using a chemical method.

The principal flavonoids present in the HPLC chromatograms were pinobanksin (PB, 0.27 ± 0.04 mg/100 g honey), pinocembrin (PC, 0.17 ± 0.02 mg/100 g honey), luteolin (L, 0.14 ± 0.02 mg/100 g honey) and chrysin (C, 0.13 ± 0.02 mg/100 g honey) and together these represented 61% of the total flavonoid content. Hitherto Oelschlagel, Gruner, Wang, Boettcher, Koelling-Speer and Speer (2012) have qualitatively reported the presence of luteolin in mānuka honey extracts analysed by UPLC while Daher and Gülaçar (2010) have observed the presence of pinocembrin in two mānuka honey samples examined by SPME followed by GC-MS.

In this study in addition to the four principal flavonoids five other known flavonoids quercetin (Q) , 8-methoxykaempferol (8MK), isorhamnetin (IR), kaempferol (K) and galangin (G) and six unknown flavonoids 1-6 (F1-6) were detected at lower levels. In addition to the well known propolis-derived flavonoids pinocembrin, pinobanksin, chrysin and galangin, as found for example in the studies of 19 mānuka honeys by Weston, Mitchell and Allen(1999) and Weston *et al*. (2000), we detected the presence of a further five known flavonoids by comparison with standards. The identification of chrysin and luteolin as major components of the flavonoid fraction of mānuka honey is consistent with the results of Yao *et al*. (2003) which showed quercetin (13.8%), isorhamnetin (12.9%), an unknown flavanone (12.7%), chrysin (12.6%) and luteolin (12.6%) as the main components of two mānuka honey samples; isorhamnetin and quercetin were however only found at low levels in the present study. The quercetin content of the 31 mānuka honeys in the present study was found to be highly variable with levels ranging from 0.000 to 0.115 mg/100 g honey. This high variability suggests that any quercetin found in mānuka honey probably originates from floral sources (introduced by nectar or pollen) other than mānuka trees. Pinobanksin, which was the main component of the mānuka honey flavonoids in the present study, was not observed in the two mānuka honey samples analysed by Yao *et al*. (*2003*). In the current study a significant level (an

268 average of 14% of the total flavonoid content assuming a detector response similar to quercetin)of
269 a non-flavonoid peak which eluted at 34.4 minutes, **1** (λ_{max} 342 nm), was identified in all of the
270 flavonoid extracts.

271 *Antibacterial activity of phenolic acid and flavonoid fractions:* Fractions obtained from the XAD-2
272 extraction were tested for antibacterial activity to determine the contribution of the phenolic acids
273 and flavonoids to the non-peroxide, antibacterial activity of mānuka honey. The aqueous fraction
274 of the XAD2 extraction contained the sugars and other polar compounds including methylglyoxal,
275 and the methanol fraction contained the phenolics including the flavonoids and phenolic acids. As
276 a comparison, the activity of the original mānuka honey and a sample of inactive clover honey
277 (used as an artificial matrix for the methanol fractions) were tested. Extraction and testing was
278 carried out in duplicate. The results of the activity testing are shown in Table 3. The phenolic
279 extract, at concentrations equivalent to natural honey, made such a small contribution to the non-
280 peroxide antibacterial activity it was not measurable by this assay, which concurred with the
281 findings of Weston *et al.* (2000) and the attribution of this activity to water-soluble methylglyoxal
282 (Adams *et al*., 2008, Mavric *et al*., 2008). At 25 times their natural concentration the phenolics did
283 display antibacterial activity, antibacterial activity has previously been attributed to such
284 compounds as well as other types of bioactivity (Cushnie & Lamb, 2005, Havsteen, 2002, D'Arcy,
285 2005). Although the flavonoid content of honey does not make a significant contribution directly to
286 the non-peroxide antibacterial activity of honey, it is possible that there is some indirect
287 contribution and to ascertain this, scatterplots were created of UMF™ versus peak area. The only
288 two substances from the flavonoid profile that showed activity correlations were the non-flavonoid
289 compound **1** (R^2 = 0.36) and luteolin (R^2 = 0.23), Fig. 2.

290 *Isolation and characterisation of **1**:* The observation that **1** showed a moderate R^2 = 0.36
291 correlation with UMF levels raised the possibility that it might be a minor contributor to the
292 bioactivity of manuka honeys and also serve as a marker compound for UMF activity, it was

293 therefore of interest to isolate this substance and to determine its structure and ascertain if it
294 exhibited any significant bioactivity. **1** was isolated from the flavonoid fraction by HPLC with
295 gradient method 1 and purified by HPLC with gradient method 2, Fig. 3. The ^{1}H NMR spectrum of **1**
296 in DMSO-d_6 gave a 3 proton singlet at 3.96 ppm typical of a -OCH_3 or -$COOCH_3$ group, 6 proton
297 signals in the region 6-8 ppm typical of aromatic or conjugated olefinic protons and a singlet signal
298 at 9.52 ppm suggestive of a -CHO group. A broad peak at 11.79 ppm, which disappeared upon pre-
299 saturation of the HOD peak, was attributed to an exchangeable proton. H,H-COSY revealed the
300 presence of two separate spin systems, a 2 proton spin system comprised of mutually coupled
301 signals (J = 3.9 Hz) centred at 6.77 and 7.04 ppm. The coupling constant exhibited by these
302 protons, while not typical of a *cis*-coupled aryl proton or olefinic proton in a 7- or 8-membered ring,
303 was typical of a *cis*-coupled proton in a 5-membered ring such as a furan, pyrrole or thiophene ring
304 (*27*). The second 4 proton system was comprised of signals centered at 7.02, 7.14, 7.33 and 7.77
305 ppm and showed couplings and correlations typical of an *ortho*-substituted benzene. The small
306 quantity of isolated sample meant that ^{13}C NMR showed only protonated signals even after
307 prolonged acquisition, namely OCH_3 (55.6 ppm), five or six aromatic or olefinic CH groups (114-
308 129.5 ppm) possibly including two unresolved signals at *circa* 120.7 ppm and a conjugated
309 aldehyde group (178.8 ppm). This was verified in an HSQC spectrum. An HMBC spectrum
310 determined with a 65 msec mixing time revealed the presence of 4 quaternary carbons at 156.2,
311 136.5, 132.5 and 119.2 ppm. A 1D selective NOESY experiment in which the aldehyde proton at
312 9.52 ppm was irradiated enhanced the signal at 7.04 ppm indicating that the aldehyde group was
313 spatially close to the *cis*-coupled proton spin system and distant from the aromatic protons. A
314 similar experiment irradiating the methoxyl protons caused enhancement of the aromatic proton
315 at 7.77 ppm indicating that the methoxyl group was a substituent on the aromatic ring. Thus it was
316 concluded that **1** consisted of an *ortho*-substituted aromatic ring to which a methoxyl group was
317 attached and a five-membered aromatic ring containing a heteroatom and to which an aldehyde
318 group was attached. Hosoya *et al*. (2003) have reported the ^{1}H NMR spectrum of 5-(2-

methoxyphenyl)-2-furaldehyde (**2**) in chloroform-d_1. The reported spectrum of **2** shows similarities to that of **1**, however the exchangeable proton at 11.79 ppm was not observed in **2**.

GCMS of **1** showed a base peak at *m/z* 201 and a much smaller peak at *m/z* 202. Although aldehydes typically show a large $[M-H]^+$, ion in this case it seemed likely that *m/z* 201 was the molecular ion and 202 was the corresponding isotope peak. The molecular mass of **2** is 202 and the substitution of N for O in the five-membered ring would yield a molecular mass of 201 and be consistent with the presence of an exchangeable proton in **1**.

3 was utilised as a model compound to assist in identification of **1**. The HMBC spectrum of **1** determined with a correlation (mixing) time of 65 msec did not include correlations which defined the point at which the proposed pyrrole ring was linked to the methoxyl substituted ring. A series of HMBC experiments were run using a specimen of **3** in which the mixing time was varied from 35 to 200 msec and the intensity of the correlation between H-6' and C-5 was determined, this showed a maximum at 50 msec. Using this optimised mixing time the HMBC spectrum of **1** revealed the presence of a ^3J correlation between the aromatic proton at 7.77 ppm (H-6') and a quaternary carbon (C-5) at 136.5 ppm in the five-membered ring moiety. Other structurally significant HMBC correlations observed for **1** are depicted in Fig. 4.

GC-MS analysis of **3,** under identical conditions used for **1,** afforded a peak which had a retention time of 11.98 min and showed a weak M^+ ion at *m/z* 217 together with a base peak fragment ion at *m/z* 188 attributable to a $(M - CHO)^+$ ion. The observation that the retention time of **3** was greater than that determined for **1** is consistent with the conclusion that **1** was a less polar, lower molecular weight variant of **3**. It was concluded that **1** was 2-formyl-5-(2-methoxyphenyl)-pyrrole. Reeves, Song, Tan, Lee, Yee & Senanayake (2007) have reported the synthesis of another analogue of **1**, 2-formyl-5-phenyl-pyrrole, **4**. The structure of **4** only differs from that proposed for **1** in that it lacks the aryl ring methoxy substituent. A sample of **4** was generously donated by Dr J.T. Reeves.

The ^{1}H and ^{13}C NMR assignments for 1 are given in Table 4 and compared with those which were determined for the specimen of **4** supplied by Dr Reeves. Notable points of similarity in the ^{1}H spectrum include the broad signal for the exchangeable proton attached to N, the chemical shift of the aldehyde proton and the coupling constant (3.9 Hz) of the *cis*-protons in the pyrrole ring. When **1** was subsequently synthesised (see below) the ^{1}H NMR spectrum was also recorded in chloroform-d_1 and the two pyrrole protons (H-3' and H-4') which were doublets in DMSO-d_6 were found to be a doublet of doublets in chloroform-d_1. Homonuclear decoupling irradiating the NH proton signal reduced H-3' and H-4' to doublets with J = 3.9 Hz. GC-MS analysis of **4**, under identical conditions to those used for **1** and **3** afforded a peak which had a retention time of 8.23 min and showed a M^+ ion at *m/z* 171, together with strong *m/z* 170 $[M-H]^+$, 142 $[M - CHO]^+$ and 115 $[M - C_2H_2ON]^+$ fragment ions.

*Synthesis of **1**:* The synthesis of **1** was undertaken following the method of Reeves *et al.* (2007), Scheme 1. The synthesis of **5** proceeded readily with 67.5% yield but the subsequent conversion to **1** gave predominantly the by-product (*E*)-1-(2-methoxyphenyl)-3-(oxazol-4-yl)-prop-2-en-1-one, **6**, and a lesser amount of its (*Z*)-isomer, which are the outcome of the dehydration of **5**, together with only a small amount of **1** . A significant quantity of *o*-methoxyacetophenone from the degradation of **5** was also present in the product mixture. Based on the mechanism proposed by Reeves *et al.* (2007) **6** is an intermediate in the formation of **1** from **5**. It is probable that the subsequent nucleophilic attack by the N lone pair upon the carbonyl carbon is sterically hindered by the presence of the *ortho* methoxyl group and this prevents **1** from forming.

*Bioactivity of luteolin and **1**:* Notwithstanding the low yield (0.2%) of **1**, the quantity of this compound that was isolated from the crude mixture by chromatography was sufficient for its bioactivity against *S.aureus* to be determined. **1** was found to be inactive at the concentrations screened (20 µg/mL-0.1325 µg/mL). On the other hand luteolin and its 4'-*O*-glucoside has been shown to have bactericidal activity against *Staphylococcus aureus* and *Escherichia coli* with MIC = 5.0×10^{-2}-1.0×10^{-1} mg/mL), luteolin was also active against *Bacillus cereus* and *Citrobacter freundii* (MIC = 5.0×10^{-2}

368 mg/mL) and luteolin 3'-*O*-glucoside was active against *Bacillus cereus* and *Lactobacillus plantarum* with
369 MIC = 2.5 × 10-1 mg/mL and 5 × 10-1 mg/mL respectively (Kumarasamy, Nahar, Byres, Delazar & Sarker,
370 2005). However the mean concentration at which luteolin is present in mānuka honey (0.14 ± 0.02
371 mg/100 g honey) shows that it would be unlikely to attain any of these minimum inhibitory
372 concentrations in a well-diffusion assay as used in this study.

373 CONCLUSIONS

374 Analysis of the flavonoid profiles of a large sample set of mānuka honeys gave a mean total flavonoid
375 content of 1.16 mg/100g of honey with pinobanksin, pinocembrin, luteolin and chrysin as the principal
376 flavonoids present. Although the phenolic acids and flavonoids did not contribute directly to the non-
377 peroxide antibacterial activity of the honey, luteolin and non-flavonoid compound **1** were weakly
378 correlated with measured activity. **1** and the principal flavonoid which does not derive from propolis,
379 luteolin, are potential marker compounds for mānuka monofloral honey and their correlation with non-
380 peroxide antibacterial activity could be explained if one assumes that the activity of the honey is
381 directly related to the proportion of mānuka in the blend.

382 **1** was isolated and characterised by NMR and MS methods and its structure was confirmed by
383 synthesis. **1** showed no bioactivity against *S. aureus*. The lack of bioactivity indicates that the moderate
384 correlation (R^2 = 0.36) which **1** exhibited with non-peroxide antibacterial activity does not arise by a
385 direct contribution from **1**. At the concentration in which it is present in mānuka honey, luteolin is also
386 unlikely to make a direct contribution to non-peroxide antibacterial activity. Given that the total
387 phenolic fraction was inactive at the concentrations normally present in mānuka honey, there is no
388 indication of synergy between **1** and other phenolics including luteolin.

389

390

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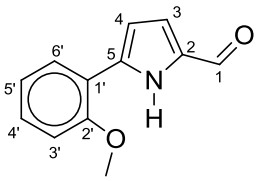

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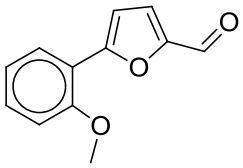

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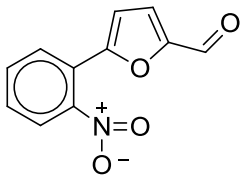

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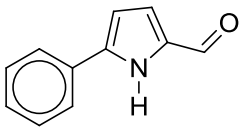

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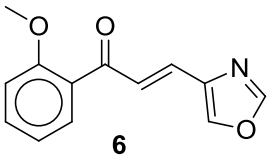

6

Captions for Tables and Figures

Fig. 1: HPLC chromatogram (gradient method 1) of flavonoid fraction of mānuka honey at 290 and 340 nm: (1) caffeic acid, (2) isoferulic acid, (3) *p*-coumaric acid, (4) pinobanksin, (5) unknown compound **1**, (6) unknown flavonoid 01, (7) luteolin, (8) unknown flavonoid 02, (9) pinocembrin, (10) unknown flavonoid 03, (11) unknown flavonoid 04, (12) unknown flavonoid 05, (13) chrysin, and (14) galangin.

Fig. 2: Scatterplot of (a) unknown compound **1** and (b) luteolin concentration *versus* UMF™ non-peroxide antibacterial activity.

Fig. 3: HPLC chromatogram (gradient method 2, 340 nm) of purified **1**.

Fig. 4: HMBC correlations observed in **1**.

Scheme 1: Synthesis of **1**

Table 1: Retention times of phenolic acids and flavonoids found in the flavonoid fraction of mānuka honeys : pinobanksin (3,5,7-trihydroxyflavanone), unknown compound **1**, unknown flavonoid 1, quercetin (3,5,7,3′,4′-pentahydroxyflavone), luteolin (5,7,3′,4′-tetrahydroxyflavone), unknown flavonoid 2, 8-methoxykaempferol (3,5,7,4′-tetrahydroxy-8-methoxyflavone), pinocembrin (5,7-dihydroxyflavone), unknown flavonoid 3, isorhamnetin (3,5,7,4′-tetrahydroxy-3′-methoxyflavone), unknown flavonoid 4, kaempferol (3,5,7,4′-tetrahydroxyflavone), unknown flavonoid 5, chrysin (5,7-dihydroxyflavone), galangin (3,5,7-trihydroxyflavone), unknown flavonoid 6.

Table 2: Quantitation of flavonoids in mānuka honeys (UMF = non-peroxide antibacterial activity, mean of 12 determinations, other abbreviations as for Table 1)

Table 3: Non-peroxide antibacterial activity of fractions from XAD-2 separation of mānuka honey

Table 4: NMR signals assignments for **1** and **4** (δ ppm in DMSO-d_6)

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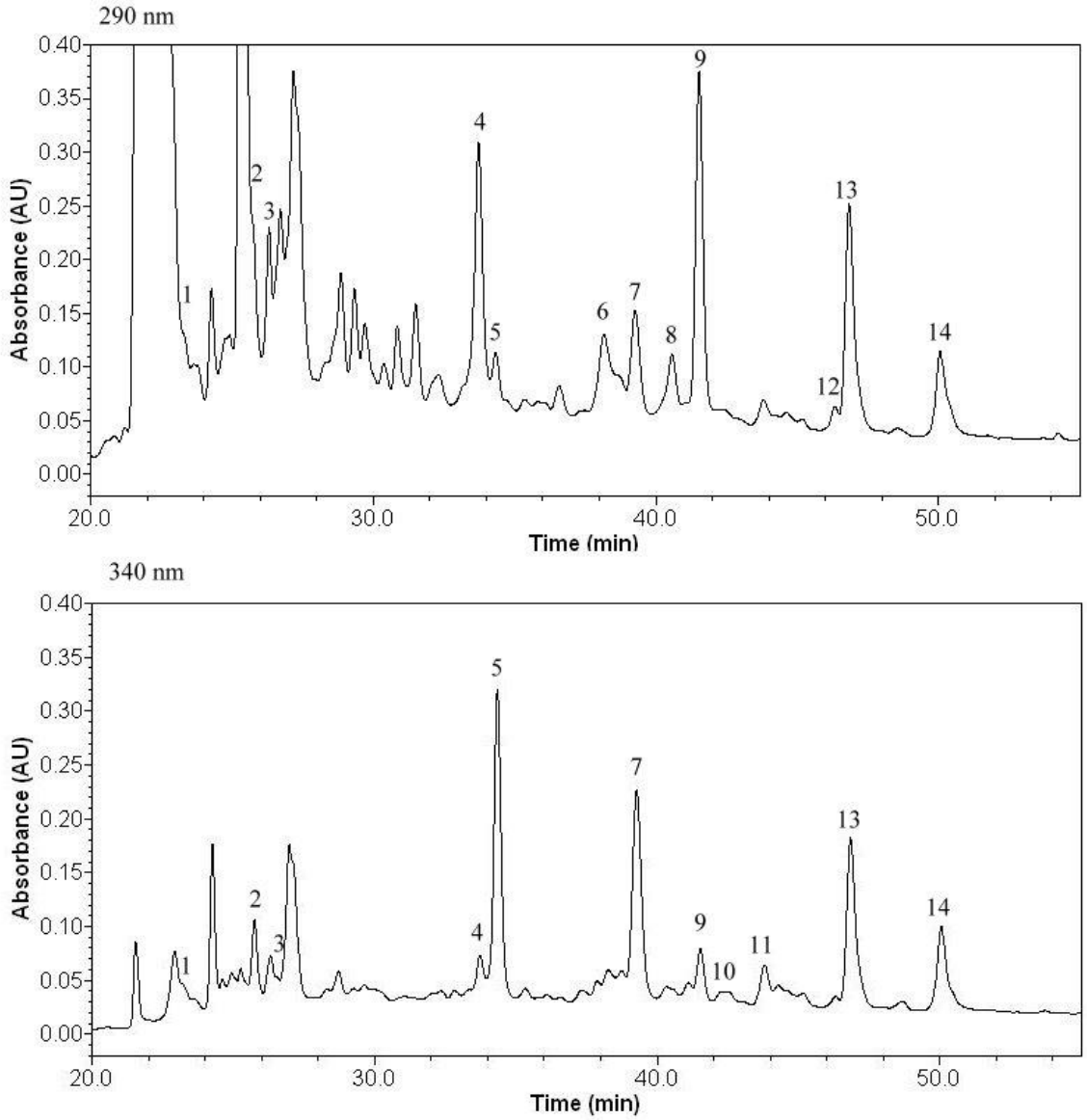
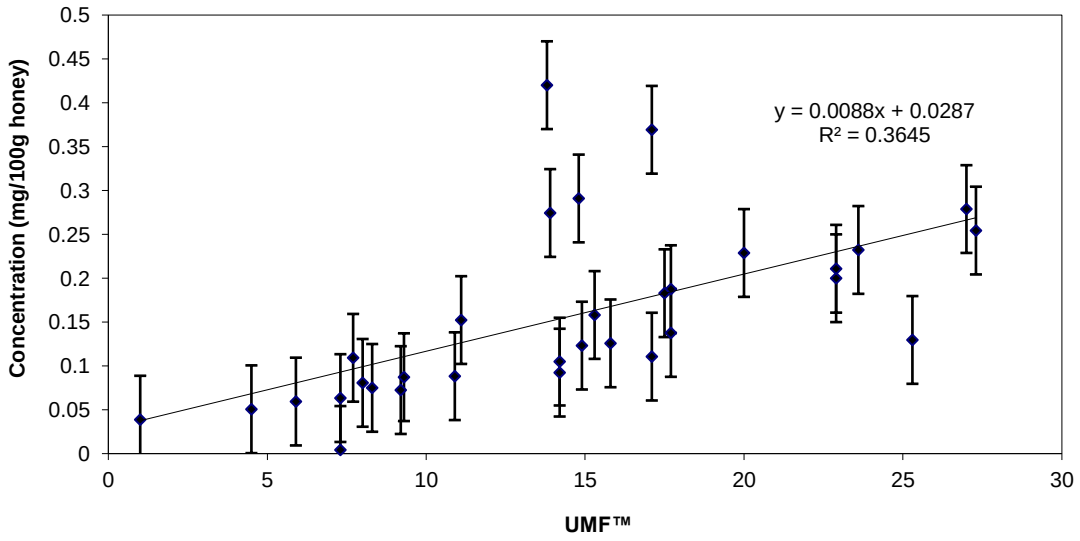
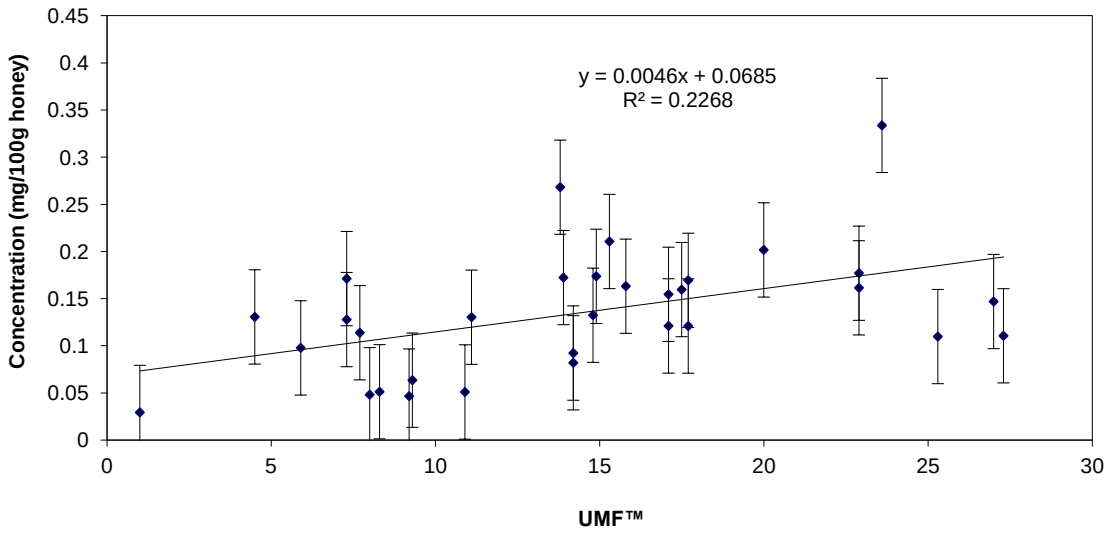

290 nm
Absorbance (AU)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
20.0
30.0
40.0
50.0
Time (min)
1
2
3
4
5
6
7
8
9
12
13
14
340 nm
Absorbance (AU)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
20.0
30.0
40.0
50.0
Time (min)
1
2
3
4
5
7
9
10
11
13
14

Figure 2

(a)


y = 0.0088x + 0.0287
R² = 0.3645
Concentration (mg/100g honey)
UMF™
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
0.5
0
5
10
15
20
25
30

(b)


y = 0.0046x + 0.0685
R² = 0.2268
Concentration (mg/100g honey)
UMF™
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
0
5
10
15
20
25
30

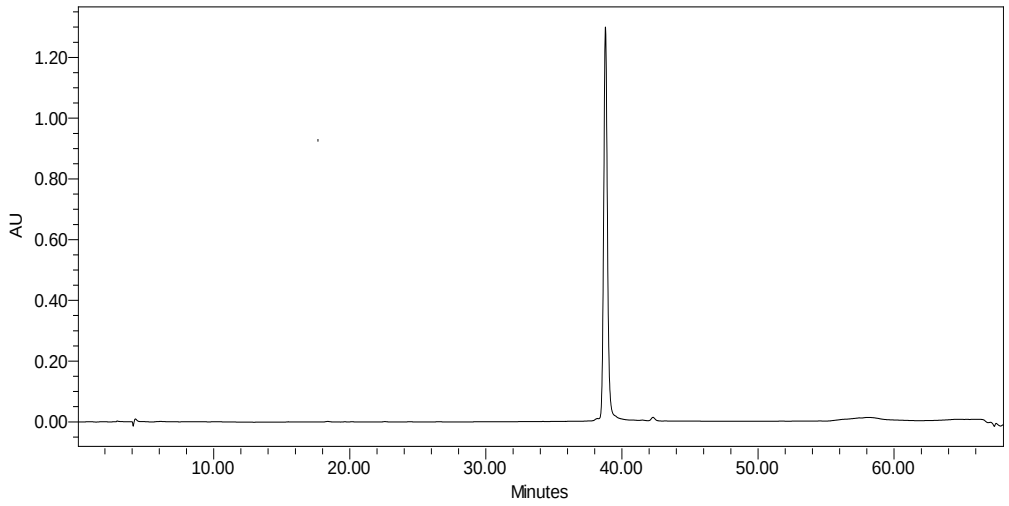

AU
1.20
1.00
0.80
0.60
0.40
0.20
0.00
10.00
20.00
30.00
40.00
50.00
60.00
Minutes

Figure 3

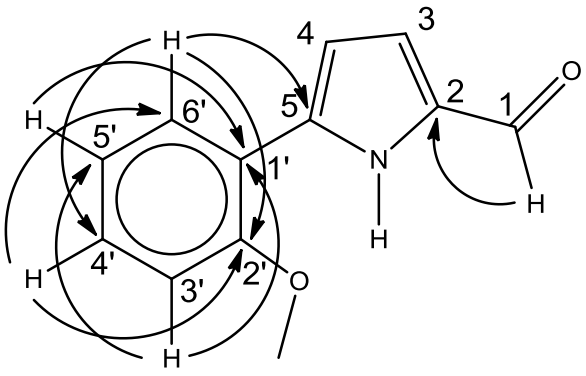
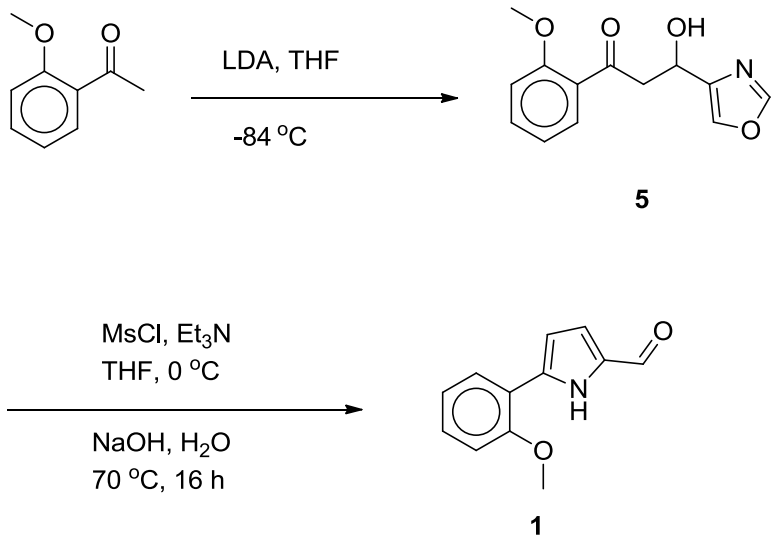


Figure 4



Scheme 1

Table 1

Name	Identification[a]	Quantification[b]	R_t (min)[c]	λ_{max} (nm)[d]
Caffeic acid	I	[e]	23.3	325
Isoferulic acid	I	[e]	35.7	324
p-Coumaric acid	I	[e]	26.5	310
Pinobanksin (PB)	I	P	33.9	292
1	I	Q	34.4	342
Unknown flavonoid 1(F1)	NA	P	38.3	286
Quercetin (Q)	S	Q	38.7	367, 256
Luteolin (L)	S	Q	39.3	350, 255
Unknown flavonoid 2 (F2)	NA	P	40.4	292
8-Methoxykaempferol (8MK)	I	K	41.2	375, 272
Pinocembrin (PC)	S	P	41.5	290
Unknown flavonoid 3 (F3)	NA	Q	42.4	360, 258
Unknown flavonoid 4 (F4)	NA	K	43.7	361, 253
Isorhamnetin (IR)	S	K	44.3	373, 255
Kaempferol (K)	S	K	45.5	364, 255
Unknown flavonoid 5 (F5)	NA	P	46.4	291
Chrysin (C)	S	C	46.7	314, 269
Galangin (G)	S	C	50.0	360, 266
Unknown flavonoid 6 (F6)	NA	C	50.6	311, 269

[a] Method used to identify the compound: S = authentic standard, I = isolation and characterisation and NA = not identified.

[b] Quantification standard used: Q = quercetin, K = kaempferol, P = pinocembrin and C = chrysin.

[c] HPLC retention time.

[d] Peak maxima in the UV absorbance spectrum (240 – 400 nm).

[e] Phenolic Acids were not quantified as their presence in this fraction represents only a portion of their total presence in the honey.

		Concentration of flavonoids (mg/100g of honey)																
Sample	**UMF**	**PB**	**1**	**F1**	**Q**	**L**	**F2**	**8MK**	**PC**	**F3**	**F4**	**IRM**	**K**	**F5**	**C**	**G**	**F6**	**Total**
1	7.7	0.343	0.109	0.058	0.000	0.114	0.037	0.013	0.200	0.013	0.016	0.020	0.017	0.006	0.102	0.027	0.009	1.083
2	11.1	0.313	0.152	0.065	0.020	0.130	0.036	0.017	0.213	0.013	0.029	0.026	0.019	0.009	0.118	0.052	0.016	1.229
3	15.8	0.365	0.126	0.071	0.031	0.163	0.040	0.025	0.206	0.023	0.046	0.030	0.038	0.014	0.207	0.083	0.014	1.482
4	17.1	0.392	0.240	0.062	0.115	0.138	0.042	0.004	0.231	0.018	0.092	0.034	0.146	0.012	0.442	0.051	0.016	2.036
5	22.9	0.224	0.205	0.043	0.040	0.169	0.028	0.008	0.128	0.023	0.036	0.030	0.072	0.005	0.246	0.020	0.007	1.285
6	27	0.200	0.279	0.039	0.016	0.147	0.015	0.010	0.112	0.013	0.016	0.016	0.012	0.006	0.081	0.021	0.008	0.990
7	13.8	0.585	0.420	0.094	0.037	0.268	0.040	0.036	0.337	0.030	0.044	0.042	0.027	0.016	0.168	0.072	0.020	2.235
8	17.7	0.297	0.188	0.039	0.049	0.121	0.023	0.020	0.157	0.018	0.024	0.024	0.072	0.004	0.151	0.033	0.011	1.231
9	20	0.166	0.229	0.047	0.006	0.202	0.027	0.010	0.129	0.015	0.020	0.017	0.013	0.008	0.081	0.038	0.013	1.021
10	27.3	0.144	0.254	0.058	0.002	0.111	0.022	0.014	0.137	0.010	0.016	0.016	0.006	0.012	0.086	0.023	0.011	0.923
11	25.3	0.062	0.130	0.027	0.013	0.110	0.015	0.005	0.070	0.007	0.018	0.009	0.007	0.004	0.075	0.034	0.008	0.594
12	23.6	0.262	0.232	0.075	0.014	0.334	0.062	0.019	0.227	0.020	0.040	0.025	0.022	0.020	0.168	0.078	0.024	1.622
13	17.5	0.279	0.183	0.068	0.020	0.160	0.033	0.009	0.146	0.023	0.032	0.028	0.021	0.006	0.103	0.043	0.008	1.162
14	17.7	0.240	0.138	0.082	0.043	0.170	0.043	0.016	0.127	0.020	0.042	0.017	0.016	0.003	0.070	0.030	0.005	1.062
15	13.9	0.310	0.274	0.066	0.051	0.172	0.045	0.016	0.197	0.018	0.037	0.031	0.019	0.010	0.104	0.053	0.013	1.417
16	14.8	0.254	0.291	0.064	0.000	0.133	0.047	0.011	0.191	0.015	0.029	0.023	0.016	0.009	0.090	0.042	0.010	1.225
17	14.9	0.197	0.123	0.048	0.010	0.174	0.058	0.000	0.142	0.007	0.025	0.015	0.012	0.009	0.082	0.047	0.011	0.960
18	10.9	0.141	0.088	0.060	0.012	0.051	0.026	0.005	0.110	0.006	0.017	0.009	0.006	0.003	0.043	0.016	0.006	0.599
19	15.3	0.125	0.158	0.067	0.035	0.211	0.035	0.015	0.102	0.012	0.022	0.013	0.016	0.003	0.052	0.021	0.005	0.894
20	14.2	0.273	0.105	0.112	0.000	0.082	0.063	0.000	0.176	0.000	0.012	0.013	0.000	0.003	0.038	0.017	0.005	0.899
21	14.2	0.336	0.092	0.129	0.000	0.092	0.098	0.009	0.312	0.017	0.017	0.025	0.000	0.010	0.099	0.054	0.011	1.302
22	1	0.340	0.039	0.034	0.017	0.029	0.018	0.083	0.239	0.010	0.017	0.017	0.011	0.003	0.051	0.016	0.004	0.927
23	9.2	0.291	0.073	0.081	0.000	0.047	0.029	0.004	0.186	0.000	0.010	0.014	0.000	0.006	0.047	0.017	0.008	0.813
24	8.3	0.235	0.075	0.075	0.000	0.051	0.017	0.000	0.164	0.000	0.008	0.014	0.000	0.007	0.046	0.015	0.005	0.711
25	9.3	0.211	0.087	0.052	0.000	0.064	0.010	0.002	0.129	0.006	0.008	0.011	0.002	0.003	0.032	0.011	0.004	0.631
26	7.3	0.325	0.004	0.056	0.017	0.128	0.023	0.007	0.185	0.014	0.018	0.017	0.016	0.004	0.064	0.022	0.004	0.904
27	7.3	0.276	0.063	0.054	0.000	0.171	0.050	0.011	0.248	0.011	0.026	0.024	0.021	0.016	0.133	0.054	0.018	1.178
28	8	0.284	0.081	0.059	0.000	0.048	0.026	0.007	0.177	0.005	0.012	0.015	0.031	0.005	0.060	0.022	0.005	0.837
29	5.9	0.269	0.059	0.057	0.000	0.098	0.057	0.008	0.181	0.007	0.009	0.015	0.004	0.007	0.048	0.018	0.003	0.841
30	4.5	0.309	0.051	0.044	0.000	0.131	0.061	0.003	0.018	0.004	0.012	0.021	0.008	0.005	0.063	0.020	0.004	0.754
31	0	0.264	0.064	0.049	0.000	0.144	0.045	0.005	0.198	0.007	0.016	0.019	0.021	0.004	0.061	0.024	0.005	0.926
Mean		0.273	0.157	0.061	0.024	0.136	0.038	0.012	0.174	0.013	0.029	0.022	0.032	0.008	0.131	0.035	0.010	1.155
S.D.		0.095	0.096	0.021	0.043	0.062	0.018	0.014	0.064	0.008	0.029	0.009	0.051	0.005	0.132	0.020	0.005	0.461
95% C.I.		0.034	0.034	0.008	0.015	0.022	0.006	0.005	0.023	0.003	0.010	0.003	0.018	0.002	0.047	0.007	0.002	0.164
CV (%)		34.9	61.3	34.7	175.8	45.7	47.4	120.7	36.6	60.8	99.5	43.3	161.5	60.3	100.4	56.4	56.8	39.9
% of total		23.6	13.6	5.3	2.1	11.8	3.3	1.0	15.1	1.2	2.5	1.9	2.7	0.7	11.4	3.0	0.8	

Table 2

Table 3

Fraction from XAD-2	Non-Peroxide Antibacterial Activity (% Phenol Equivalents)
Aqueous sugar fraction	30.5 ± 0.7[a]
MeOH phenolic fraction 1:1	No detectable activity [a]
MeOH phenolic fraction 25:1	37 ± 1 [b]
Manuka honey	30.6 ± 0.8 [a]
Clover honey	No detectable activity [b]

[a]Mean and 95% confidence interval from 48 determinations.

[b]Mean and 95% confidence interval from 32 determinations.

Table 4

	1		**4**[a]	
	^{13}C	^{1}H	^{13}C	^{1}H
1-NH		11.79 (br s)	12.39 (br s)	
2	132.5		133.8	
3	120.5	7.04[b] (d, *J* = 3.9 Hz)	121.9	7.08 (dd, *J* = 3.9, 2.3 Hz)
4	111.4	6.77 (d, *J* = 3.9 Hz)	108.9	6.79 (dd, *J* = 3.9, 2.3 Hz)
5	136.5		139.5	
2-CHO	178.8	9.52 s	178.8	9.50 s
1'	119.2		130.9	
2'	156.2		125.5	7.89 (d, *J* = 8.0 Hz)
3'	112.1	7.14 (dd, *J* = 8.5, 1.2 Hz)	128.7	7.42 (t, *J* = 8.0 Hz)
4'	129.4	7.33 (ddd, *J* = 8.5, 7.3, 1.7 Hz)	127.9	7.32 (t, *J* = 8.0 Hz)
5'	120.7	7.02[b] (~td, *J* = 8.3, 1.2 Hz)	128.7	7.42 (t, *J* = 8.0 Hz)
6'	128.4	7.77 (dd, *J* = 7.8, 1.7 Hz)	125.5	7.89 (d, *J* = 8.0 Hz)
2'-OCH_3	55.6	3.96 s		

[a]sample kindly supplied by Dr Reeves; [b]signals partly overlapped