

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

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

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11 ABSTRACT:

12 The flavonoid components of New Zealand mānuka (*Leptospermum scoparium*) honey have been
13 quantified in a series of 31 honeys of varying non-peroxide antibacterial activity to clarify
14 discrepancies between previous studies reported in the literature. Total flavonoid content was
15 1.16 mg/100 g honey. The principal flavonoids present were pinobanksin, pinocembrin, luteolin
16 and chrysin and together these represented 61% of the total flavonoid content. **1**, 2-formyl-5-(2-
17 methoxyphenyl)-pyrrole, which was weakly correlated with the non-peroxide antibacterial activity,
18 was isolated from the flavonoid fraction and separately synthesised. **1** did not display inhibitory
19 activity against *S. aureus* in vitro and thus the origin of the correlation, which is still unknown, is not
20 a direct contribution.

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22 INTRODUCTION:

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

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

52 Pyrroles in honey can arise from Maillard reactions or from the pyrolysis of amino acids when
53 honey is heated (Jerković, Mastelić, Marijanović, Klein & Jelić, 2007). 1*H*-Pyrrole has been found in
54 *Robinia pseudoacacia* L., *Castanea sativa* L. and *Salvia officinalis* L (Jerković *et al.*, 2007, Jerković,
55 Mastelić & Marijanović, 2006). 1*H*-Pyrrole-2-carboxylic acid has been found in *Paliurus spina-*
56 *christi* (Jerković, Tuberoso, Marijanović, Jelić & Kasum, 2009). 2-Acetylpyrrole has been found in
57 *abbamele*, a honey-based Sardinian product (Jerković, Kasum, Marijanović & Tuberoso, 2011) and
58 1*H*-pyrrole-3,4-diacetic acid has been found in pine honey (*Pinus brutia* Ten) (Eraslan, Kanbur, Silici
59 & Karabacak, 2010). Phenyl substituted pyrroles are also found in natural products, pyrrolnitrin is a
60 tryptophan-derived, antifungal antibiotic isolated from *Pseudomonas pyrocinia* (van Pée & Ligon,
61 2000). Both pentachloropseudilin and pentabromopseudilin, which are produced by an
62 *Actinoplanes* sp. strain, are strongly active against Gram-positive bacteria. The latter is also known
63 to inhibit a number of different enzyme systems and has high *in vitro* activity against leukemia and
64 melanoma cell lines (van Pée & Ligon, 2000).

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

72

73 MATERIALS AND METHODS

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

79 **3** was used as a model compound and **4** as an analogue for the characterisation of **1**. **3**, 5-(2-
80 nitrophenyl)-2-furaldehyde, was obtained from Acros Organics. **4**, 2-Formyl-5-phenyl-pyrrole, was
81 generously donated by Dr J. T. Reeves of the Department of Chemical Development, Boehringer
82 Ingelheim Pharmaceuticals Inc., Ridgefield, CT 06877-0368, USA.

83 Methanol was redistilled from drum grade or HPLC grade (Scharlau), Milli-Q water was obtained
84 from a Barnstead E-pure system (18.2 MΩcm). Dried and purified diethyl ether, hexanes,
85 tetrahydrofuran and dichloromethane were obtained from a Pure Solvent Purification System
86 (Model: PS-SD-5) as needed and used promptly. Dimethyl sulfoxide-*d*₆ (99.9 atom% D containing
87 0.03% v/v TMS) and chloroform-*d*₁ (99.8 atom% D) were obtained from Sigma-Aldrich Inc. Lithium
88 diisopropylamide (LDA), *o*-methoxyacetophenone, oxazole-4-carboxaldehyde, diisobutylaluminium
89 hydride (DIBALH) and triethylamine (Et₃N) were obtained from Sigma-Aldrich Inc., methane
90 sulphonyl chloride (MsCl) was obtained from Riedel-de Haën. Caffeic acid (≥98%), (+)-catechin
91 hydrate (98%), chlorogenic acid (predominantly *trans*) (≥ 95%), chrysin, *trans*-cinnamic acid (99+%),
92 *p*-coumaric acid (≥ 98%), (-)-epicatechin (97%), galangin (no stated purity), gallic acid (97%), luteolin
93 (≥ 98%), myricetin (approx 85%), pinocembrin (95%), quercetin dihydrate (98%), (+)-rutin hydrate
94 (95%) and syringic acid (98%) were obtained from Sigma-Aldrich Inc., isorhamnetin (pure),
95 kaempferol (pure) and naringin (pure) were obtained from Indofine Chemical Company Inc.

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

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

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

110 *Semi-preparative High Performance Liquid Chromatography (HPLC):* Semi-preparative HPLC of bulk
111 fractions of honey for isolation of flavonoids was carried out using two Waters 515 pumps, an
112 Alltech Elite Degassing System, a Rheodyne 7725i injector fitted with a 500 µL sample loop, and a
113 Waters 996 photodiode array detector. A reversed phase octadecylsilane Waters Xterra Prep
114 column (PR₁₈, 10 µm, 7.8 mm x 300 mm) was used in this system. A binary mobile phase was used;
115 this consisted of solvent A which was Milli-Q water acidified with acetic acid (0.075% v/v) and
116 methanol (5% v/v) and solvent B which was methanol. The overall flow rate was 4 mL/min. The
117 gradient used was held at 60% A for 5 min, decreasing to 50% A at 10 min and 20% A at 60 min and
118 finally 0% A at 60.2 min.

119 *Analysis of flavonoids and isolation of 1 by High Performance Liquid Chromatography (HPLC):*
120 Analysis of flavonoid extracts and isolation of **1** was carried out using two Waters 515 HPLC pumps
121 with their flows combined through a static mixer (Grace Binary Large Volume Mixer SS Housing
122 with 350 μ L Mixer Cartridge). Both solvents were degassed prior to entering the pumps by passing
123 through a Waters In-Line Degasser AF. Samples and standards were warmed (40 $^{\circ}$ C) and injected
124 manually using a Rheodyne 7725i injector system fitted with a Rheodyne loop (5 μ L for analyses, 50
125 μ L for isolations). Separation was achieved using a Waters Symmetry Shield™ octadecylsilane HPLC
126 column (RP18, 5 μ m, 3.0 x 250 mm) with a Waters Universal Sentry™ Guard SymmetryShield™
127 Column. A binary gradient was used, solvent A was Milli-Q water and methanol (5% v/v), acidified
128 with acetic acid (0.075% v/v), solvent B was methanol. The gradient was run at a constant
129 combined flow rate of 0.3 mL/min. Detection was achieved using a Waters 996 Photodiode Array
130 Detector (240-400 nm). Gradient method 1 (for analyses and initial isolation of **1**) consisted of 70%
131 A for 15 min, decreasing to 40% A at 20 min and 0% A at 60 min and held for 10 min. Gradient
132 method 2 (for final purification of **1**) consisted of 70% A for 5 min, decreasing to 55% A at 10 min,
133 20% A at 50 min and 0% A at 52 min and held for 8 min.

134 *Gas Chromatography-Mass-Spectrometry (GC-MS):* GC-MS was carried out on a HP 6890 series GC
135 fitted with a Phenomenex ZB-5 5% phenyl-methylsiloxane) column (30 m x 0.25 mm x 0.025 μ m)
136 interfaced to a HP 5973 mass selective detector. Conditions used were 120 $^{\circ}$ C (0.75 min), 50
137 $^{\circ}$ C/min up to 200 $^{\circ}$ C, and 10 $^{\circ}$ C/min up to 295 $^{\circ}$ C (held 15 min). The mass spectrometer was
138 operated in either total ion chromatogram (TIC) or a selected ion monitoring (SIM) mode using m/z
139 201 (M^{+}), 158 and 130 ions in the case of **1**.

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

144 *Nuclear Magnetic Resonance Spectroscopy (NMR)*: 1D- and 2D- ^1H and ^{13}C NMR spectra of samples
145 were obtained using a Bruker Avance DRX-400 spectrometer. ^1H and ^{13}C experiments were carried
146 out at 400.13 and 100.62 MHz respectively using a 5 mm inverse $^{13}\text{C}/^1\text{H}$ probe head except for the
147 ^{13}C spectrum of **1**, which was acquired using a 5 mm dual $^{13}\text{C}/^1\text{H}$ probe head. Samples were
148 dissolved in dimethylsulfoxide- d_6 or chloroform- d_1 for NMR analysis. Operation of the NMR
149 spectrometer and processing of spectra were performed using Bruker Topspin 1.3 software.

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

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

158 For a growth study, **1** was diluted in dimethyl sulfoxide (DMSO) and then further serial dilutions
159 were made in DMSO. Each dilution was added in duplicate to a 96-well plate to a final DMSO
160 concentration of 2%. An overnight culture was diluted to 1×10^8 cfu/mL (determined by back-
161 titration on tryptic soy agar plates) and 150 μL was added to each well of the 96-well plates.
162 Controls included a serial dilution of Lincomycin (to assess plate-to-plate variation), a positive
163 control with bacteria alone in CaMHB with 2% DMSO and a negative (no bacteria) with 150 μL
164 CaMHB containing 2% DMSO. Plates were incubated in a shaking incubator at 37 $^\circ\text{C}$ for 22 h and
165 absorbance was measured at a wavelength of 595 nm using a plate reader (Biotek Synergy HT).

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

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

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

177 *3-hydroxy-1-(2-methoxyphenyl)-3-(oxazol-4-yl) propan-1-one (5)*: To THF (20 mL) at -84 °C was
178 added LDA (2.5 mL, 5.5 mmol). The resultant solution of LDA was added dropwise to a solution of
179 *o*-methoxyacetophenone (0.6 mL, 4.5 mmol). The reaction mixture was stirred at -84 °C for 30
180 minutes before a solution of oxazole-4-carboxaldehyde (0.5 g, 5.5 mmol) in a minimal amount of
181 THF was added dropwise at -84 °C. The reaction mixture was stirred for 30 minutes at -84 °C,
182 quenched with saturated aqueous NH₄Cl (20 mL) and allowed to warm to room temperature. The
183 layers were allowed to separate, and the aqueous layer was extracted with EtOAc (2 x 20 mL). The
184 combined organic layers were dried (MgSO₄), filtered and concentrated. The product was
185 recrystallized from CH₂Cl₂/hexanes yielding **5** as pale yellow crystals (0.751 g, 68%), Mpt: 104 – 107
186 °C, HRMS: found: 270.0735, calculated for C₁₂H₁₂NO₄Na [M+Na]⁺: 270.0737, ¹H NMR (400.13 MHz,
187 DMSO-*d*₆) δ ppm 3.87 (s, 2-OCH₃), 5.36 (d, *J* = 5.4 Hz, OH), 3.26 (dd *J* = 8.2, 16.2 Hz, H_a-2'), 3.37 (dd
188 *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*
189 = 7.5 Hz, H-4), 7.03 (t, *J* = 7.5 Hz, H-5), 7.52 (d, *J* = 8.6 Hz, H- 6), ¹³C NMR (100.62 MHz, DMSO-*d*₆) δ
190 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
191 (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 Et₃N (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 NaHCO₃ solution, extracted with EtOAc (2 x 20 mL) and the organic layer dried
197 (MgSO₄), 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 C₁₂H₁₁NO₂Na [M+Na]⁺: 224.0682, ¹H NMR (400.13
203 MHz) and ¹³C 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 C₁₃H₁₁NO₃Na [M+Na]⁺: 252.0631, ¹H
208 NMR (400.13 MHz, DMSO-*d*₆) δ ppm 3.86 (s, 2-OCH₃), 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), ¹³C 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-OCH₃). 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.

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216

217 RESULTS AND DISCUSSION

218 *Flavonoid profiles:* The flavonoid profiles of 31 different mānuka honey samples were analysed.
219 XAD-2 extraction was used according to the method of Ferreres *et al.* (1991) but modified to use 5
220 g rather than 50 g samples, which modification was made to allow flavonoid profiling of a much
221 larger set of honey samples. To validate this modification multiple extractions of 50 g (3
222 replicates), 20 g (3 replicates) and 5 g (5 replicates) samples were performed and the total area of
223 the chromatogram (340 nm) in the phenolic region was measured. The results, $(1.7 \pm 0.8) \times 10^8$,
224 $(1.9 \pm 1.0) \times 10^8$ and $(2.1 \pm 1.0) \times 10^8$ for 50, 20 and 5 g respectively, indicate that any variation due
225 to sample size is insignificant compared with the intra-sample size variation. Phenolic acids and
226 flavonoids were separated using Sephadex-LH20 (Bohm, 1998). The HPLC chromatogram of the
227 flavonoid fraction of a typical mānuka honey is shown in Fig.1. Flavonoids and phenolic acids were
228 identified by HPLC co-elution with an authentic standard and for some confirmed by isolation and
229 characterisation by NMR and UV spectroscopy (caffeic acid, *p*-coumaric acid, pinobanksin and 8-
230 methoxykaempferol). Several compounds were identified as flavonoids by their characteristic UV
231 spectra, Table 1, but isolation and identification has so far proven elusive. Unknown flavonoids 1,
232 2 and 5 showed only a Band II absorption for λ_{\max} indicating that they are flavanones and/or
233 dihydroflavonols while unknown flavonoids 3, 4 and 6 showed both Band I and Band II absorptions
234 indicating that they are flavones and/or flavonols. Quantification of the flavonoids was achieved by
235 comparing their absorbance in the HPLC chromatograms to four external standards. Pinocembrin
236 at 290 nm was used to quantify all flavanones and dihydroflavonols, chrysin at 340 nm was used to
237 quantify flavones and flavonols with unsubstituted B rings and kaempferol at 340 nm was used for
238 those flavones and flavonols with a singly oxygenated B ring, while quercetin at 340 nm was used
239 for all other flavones and flavonols, Table 2. Total flavonoid content was 1.16 mg/100 g honey with
240 a 95% confidence interval of 0.16 mg/100 g honey and a range of 0.594-2.235 mg/100 g honey.
241 This value is considerably higher than the 0.0147 mg/100 g total flavonoid content reported by
242 Weston *et al.* (2000) and closer to that reported by Yao *et al.* (2003), that is 3.06 mg/100 g of honey

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

245 The principal flavonoids present in the HPLC chromatograms were pinobanksin (PB, 0.27 ± 0.04
246 mg/100 g honey), pinocembrin (PC, 0.17 ± 0.02 mg/100 g honey), luteolin (L, 0.14 ± 0.02 mg/100 g
247 honey) and chrysin (C, 0.13 ± 0.02 mg/100 g honey) and together these represented 61% of the
248 total flavonoid content. Hitherto Oelschlagel, Gruner, Wang, Boettcher, Koelling-Speer and Speer
249 (2012) have qualitatively reported the presence of luteolin in mānuka honey extracts analysed by
250 UPLC while Daher and Gülaçar (2010) have observed the presence of pinocembrin in two mānuka
251 honey samples examined by SPME followed by GC-MS.

252 In this study in addition to the four principal flavonoids five other known flavonoids quercetin (Q) ,
253 8-methoxykaempferol (8MK), isorhamnetin (IR), kaempferol (K) and galangin (G) and six unknown
254 flavonoids 1-6 (F1-6) were detected at lower levels. In addition to the well known propolis-derived
255 flavonoids pinocembrin, pinobanksin, chrysin and galangin, as found for example in the studies of
256 19 mānuka honeys by Weston, Mitchell and Allen(1999) and Weston *et al.* (2000), we detected the
257 presence of a further five known flavonoids by comparison with standards. The identification of
258 chrysin and luteolin as major components of the flavonoid fraction of mānuka honey is consistent
259 with the results of Yao *et al.* (2003) which showed quercetin (13.8%), isorhamnetin (12.9%), an
260 unknown flavanone (12.7%), chrysin (12.6%) and luteolin (12.6%) as the main components of two
261 mānuka honey samples; isorhamnetin and quercetin were however only found at low levels in the
262 present study. The quercetin content of the 31 mānuka honeys in the present study was found to
263 be highly variable with levels ranging from 0.000 to 0.115 mg/100 g honey. This high variability
264 suggests that any quercetin found in mānuka honey probably originates from floral sources
265 (introduced by nectar or pollen) other than mānuka trees. Pinobanksin, which was the main
266 component of the mānuka honey flavonoids in the present study, was not observed in the two
267 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 ^1H NMR spectrum of **1**
296 in $\text{DMSO-}d_6$ gave a 3 proton singlet at 3.96 ppm typical of a $-\text{OCH}_3$ or $-\text{COOCH}_3$ 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 $-\text{CHO}$ 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 ^1H NMR spectrum of 5-(2-

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

321 GCMS of **1** showed a base peak at m/z 201 and a much smaller peak at m/z 202. Although aldehydes
322 typically show a large $[M-H]^+$ ion in this case it seemed likely that m/z 201 was the molecular ion and
323 202 was the corresponding isotope peak. The molecular mass of **2** is 202 and the substitution of N for O
324 in the five-membered ring would yield a molecular mass of 201 and be consistent with the presence of
325 an exchangeable proton in **1**.

326 **3** was utilised as a model compound to assist in identification of **1**. The HMBC spectrum of **1**
327 determined with a correlation (mixing) time of 65 msec did not include correlations which defined the
328 point at which the proposed pyrrole ring was linked to the methoxyl substituted ring. A series of HMBC
329 experiments were run using a specimen of **3** in which the mixing time was varied from 35 to 200 msec
330 and the intensity of the correlation between H-6' and C-5 was determined, this showed a maximum at
331 50 msec. Using this optimised mixing time the HMBC spectrum of **1** revealed the presence of a 3J
332 correlation between the aromatic proton at 7.77 ppm (H-6') and a quaternary carbon (C-5) at 136.5
333 ppm in the five-membered ring moiety. Other structurally significant HMBC correlations observed for **1**
334 are depicted in Fig. 4.

335 GC-MS analysis of **3**, under identical conditions used for **1**, afforded a peak which had a retention time
336 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
337 attributable to a $(M - CHO)^+$ ion. The observation that the retention time of **3** was greater than that
338 determined for **1** is consistent with the conclusion that **1** was a less polar, lower molecular weight
339 variant of **3**. It was concluded that **1** was 2-formyl-5-(2-methoxyphenyl)-pyrrole. Reeves, Song, Tan,
340 Lee, Yee & Senanayake (2007) have reported the synthesis of another analogue of **1**, 2-formyl-5-phenyl-
341 pyrrole, **4**. The structure of **4** only differs from that proposed for **1** in that it lacks the aryl ring methoxy
342 substituent. A sample of **4** was generously donated by Dr J.T. Reeves.

343 The ^1H and ^{13}C NMR assignments for **1** are given in Table 4 and compared with those which were
344 determined for the specimen of **4** supplied by Dr Reeves. Notable points of similarity in the ^1H
345 spectrum include the broad signal for the exchangeable proton attached to N, the chemical shift of the
346 aldehyde proton and the coupling constant (3.9 Hz) of the *cis*-protons in the pyrrole ring. When **1** was
347 subsequently synthesised (see below) the ^1H NMR spectrum was also recorded in chloroform- d_1 and
348 the two pyrrole protons (H-3' and H-4') which were doublets in DMSO- d_6 were found to be a doublet of
349 doublets in chloroform- d_1 . Homonuclear decoupling irradiating the NH proton signal reduced H-3' and
350 H-4' to doublets with $J = 3.9$ Hz. GC-MS analysis of **4**, under identical conditions to those used for **1** and
351 **3** afforded a peak which had a retention time of 8.23 min and showed a M^+ ion at m/z 171, together
352 with strong m/z 170 $[\text{M}-\text{H}]^+$, 142 $[\text{M} - \text{CHO}]^+$ and 115 $[\text{M} - \text{C}_2\text{H}_2\text{ON}]^+$ fragment ions.

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

362 *Bioactivity of luteolin and 1*: Notwithstanding the low yield (0.2%) of **1**, the quantity of this compound
363 that was isolated from the crude mixture by chromatography was sufficient for its bioactivity against
364 *S. aureus* to be determined. **1** was found to be inactive at the concentrations screened (20 $\mu\text{g}/\text{mL}$ -
365 0.1325 $\mu\text{g}/\text{mL}$). On the other hand luteolin and its 4'-*O*-glucoside has been shown to have
366 bactericidal activity against *Staphylococcus aureus* and *Escherichia coli* with MIC = 5.0×10^{-2} - 1.0×10^{-1}
367 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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397 REFERENCES

- 398 Adams, C. J., Boulton, C. H., Deadman, B. J., Farr, J. M., Grainger, M. N. C., Manley-Harris, M., Snow,
399 M. J. (2008) Isolation by HPLC and characterization of the bioactive fraction of New Zealand
400 manuka (*Leptospermum scoparium*) honey. *Carbohydrate Research*, 343 (4), 651-659.
- 401 Adams, C.J., Manley-Harris, M., Molan, P.C. (2009) The origin of methylglyoxal in New Zealand
402 manuka (*Leptospermum scoparium*) honey. *Carbohydrate Research*, 344 (8), 1050-3.
- 403 Allen, K. L., Molan, P. C., Reid, G. M., (1991) A survey of the antibacterial activity of some New
404 Zealand honeys. *Journal of Pharmacy and Pharmacology*, 43 (12), 817-22.
- 405 Anklam, E. (1998) A review of the analytical methods to determine the geographical and botanical
406 origin of honey. *Food Chemistry*, 63 (4), 549-562.
- 407 Benoit, G.-E., Carey, J. S., Chapman, A. M., Chima, R., Hussain, N., Popkin, M. E., Roux, G., Tavassoli,
408 B., Vaxelaire, C., Webb, M. R., Whatrup, D. (2007) Large-scale preparation of 2-methyloxazole-4-
409 carboxaldehyde. *Organic Process Research & Development*, 12 (1), 88-95.
- 410 Bohm, B. A., *Introduction to flavonoids*, Harwood Academic Publishers: Amsterdam, Holland 1998,
411 pp. 193-194.
- 412 Cushnie, T. P. T., Lamb, A. J. (2005) Antimicrobial activity of flavonoids. *International Journal of*
413 *Antimicrobial Agents*, 26 (5), 343-356.

414 D'Arcy, B. R. *Antioxidants in Australian Floral Honeys - Identification of health-enhancing nutrient*
415 *components*, Australian Government Rural Industries Research and Development Corporation:
416 Barton, May 2005.

417 Daher, S., Gülaçar (2010) Identification of new aromatic compounds in the New Zealand manuka
418 honey by gas chromatography-mass spectrometry. *Journal of Chemistry*, 7(S1) S7-S14.

419 Eraslan, G., Kanbur, M., Silici, S., Karabacak, M. (2010) Beneficial effect of pine honey on trichlorfon
420 induced some biochemical alterations in mice. *Ecotoxicology & Environmental Safety*, 73 (5), 1084-
421 1091.

422 Ferreres, F., Tomas-Barberan, F. A., Gil, M. I., Tomas-Lorente, F. (1991) An HPLC technique for
423 flavonoid analysis in honey. *Journal of the Science of Food and Agriculture*, 56 (1), 49-56.

424 Havsteen, B. H. (2002) The biochemistry and medical significance of the flavonoids. *Pharmacology*
425 *& Therapeutics*, 96 (2-3), 67-202.

426 Hosoya, T., Aoyama, H., Ikemoto, T., Kihara, Y., Hiramatsu, T., Endo, M., Suzuk, M., (2003)
427 Dantrolene analogues revisited: General synthesis and specific functions capable of discriminating
428 two kinds of Ca²⁺ release from sarcoplasmic reticulum of mouse skeletal muscle. *Bioorganic &*
429 *Medicinal Chemistry*, 11 (5), 663-673.

430 Jerković, I., Kasum, A., Marijanović, Z., Tuberoso, C. I. G. (2011) Contribution to the characterisation
431 of honey-based Sardinian product abbamele: Volatile aroma composition, honey marker
432 compounds and antioxidant activity. *Food Chemistry*, 124 (1), 401 - 410.

433 Jerković, I., Mastelić, J., Marijanović, Z. (2006) A Variety of Volatile Compounds as Markers in
434 Unifloral Honey from Dalmatian Sage (*Salvia officinalis* L.). *Chemistry & Biodiversity*, 3 (12), 1307-
435 1316.

436 Jerković, I., Mastelić, J., Marijanović, Z., Klein, Z., Jelić, M. (2007) Comparison of hydrodistillation
437 and ultrasonic solvent extraction for the isolation of volatile compounds from two unifloral honeys
438 of *Robinia pseudoacacia* L. and *Castanea sativa* L. *Ultrasonic Sonochemistry*, 14 (6), 750 - 756.

439 Jerković, I., Tuberoso, C. I. G., Marijanović, Z., Jelić, M., Kasum, A. (2009) Headspace, volatile and
440 semi-volatile patterns of *Paliurus spina-christi* unifloral honey as markers of botanical origin. *Food*
441 *Chemistry*, 112 (1), 239 - 245.

442 Jurd, L., Spectral properties of flavonoid compounds. In *The chemistry of flavonoid compounds*,
443 Geissman, T. A., Ed. The Macmillan Company: New York, 1962, pp. 107-155.

444 Kumarasamy, Y., Nahar, L., Byres, M., Delazar, A., Sarker, S. D. (2005) The assessment of biological
445 activities associated with the major constituents of the methanol extract of 'wild carrot' (*Daucus*
446 *carota* L.) seeds. *Journal of Herbal Pharmacotherapy* 5(1), 61-72.

447 Mabry, T. J., Markham, K. R., Thomas, M. B., *The systematic identification of flavonoids*. Springer-
448 Verlag: New York, 1970, pp.41-56, 165-171, 227-229.

449 Markham, K. R., Mabry, T. J., Ultraviolet-visible and proton magnetic resonance spectroscopy of
450 flavonoids. In *The flavonoids*, Harborne, J. B., Mabry, H., Mabry, T. J., Eds. Chapman and Hall:
451 London, England 1975, pp. 46-56.

452 Mavric, E., Wittmann, S., Barth, G., Henle, T. (2008) Identification and quantification of
453 methylglyoxal as the dominant antibacterial constituent of manuka (*Leptospermum scoparium*)
454 honeys from New Zealand. *Molecular Nutrition & Food Research*, 52 (4), 483-489.

455 Oelschlaegel, S., Gruner, M., Wang, P.N., Boettcher, A., Koelling-Speer, I., Speer, K. (2012)
456 Classification and characterization of manuka honeys based on phenolic compounds and
457 methylglyoxal. *Journal of Agricultural & Food Chemistry*, 60(29):7229-37.

458 Reeves, J. T., Song, J. J., Tan, Z., Lee, H., Yee, N. K., Senanayake, C. H. (2007) A general synthesis of
459 substituted formylpyrroles from ketones and 4-formyloxazole. *Organic Letters*, 9 (10), 1875-1878.

460 Tomas-Barberan, F. A., Ferreres, F., Garcia-Viguera, C., Tomas-Lorente, F. (1992) Flavonoid analysis
461 in the determination of the geographical and botanical origin of honey. *Bulletin de Liaison - Groupe*
462 *Polyphenols*, 16 (Pt. 2), 233-6.

463 Tomas-Barberan, F. A., Ferreres, F., Garcia-Viguera, C., Tomas-Lorente, F. (1993) Flavonoids in
464 honey of different geographical origin. *Zeitschrift für Lebensmittel-Untersuchung und -Forschung A*
465 *(Berlin)*, 196 (1), 38-44.

466 van Pée, K.-H., Ligon, J. M. (2000) Biosynthesis of pyrrolnitrin and other phenylpyrrole derivatives
467 by bacteria. *Natural Product Reports*, 17 (2), 157-164.

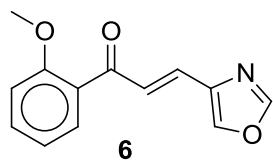
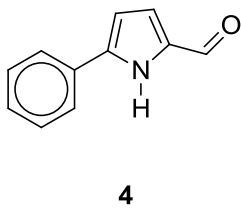
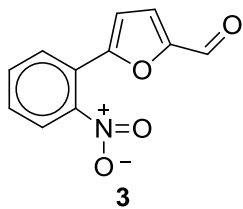
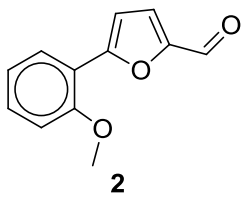
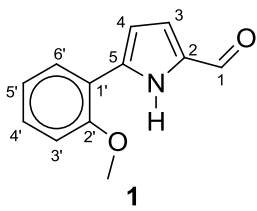
468 Venugopal, S., Devarajan, S. (2011) Estimation of total flavonoids, phenols and antioxidant activity
469 of local and New Zealand manuka honey. *Journal of Pharmacy Research* 4(2) 464-466.

470 Weston, R. J., Brocklebank, L. K., Lu, Y. (2000) Identification and quantitative levels of antibacterial
471 components of some New Zealand honeys. *Food Chemistry*, 70 (4), 427-435.

472 Weston, R. J., Mitchell, K. R., Allen, K. L. (1999) Antibacterial phenolic components of New Zealand
473 manuka honey. *Food Chemistry*, 64 (3), 295-301.

474 Yao, L., Datta, N., Tomas-Barberan, F. A., Ferreres, F., Martos, I., Singanusong, R. (2003) Flavonoids,
475 phenolic acids and abscisic acid in Australian and New Zealand *Leptospermum* honeys. *Food*
476 *Chemistry*, 81 (2), 159-168.

477



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)

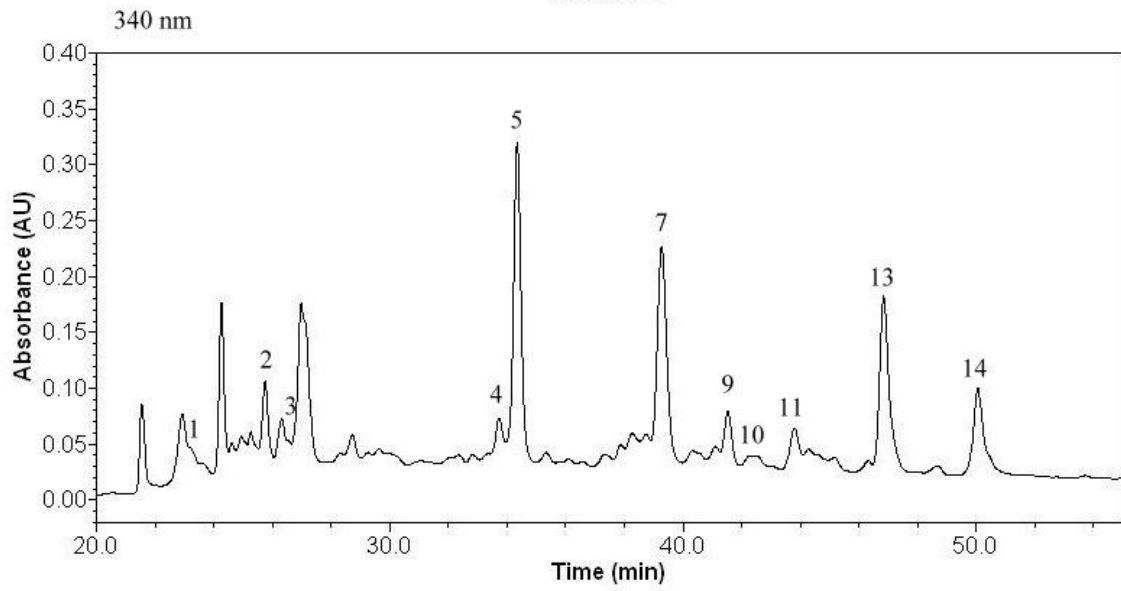
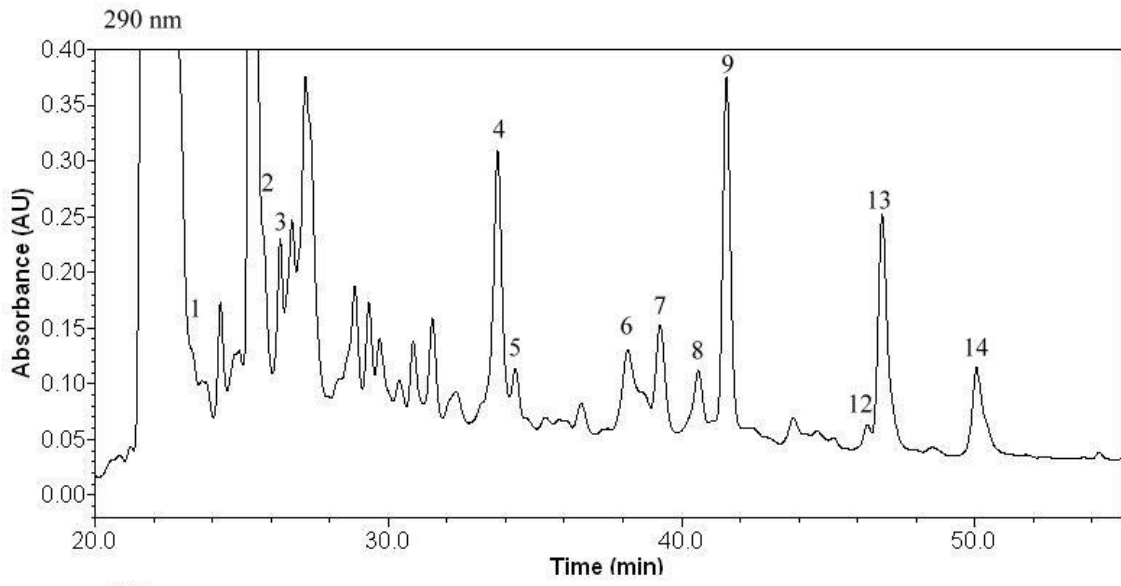
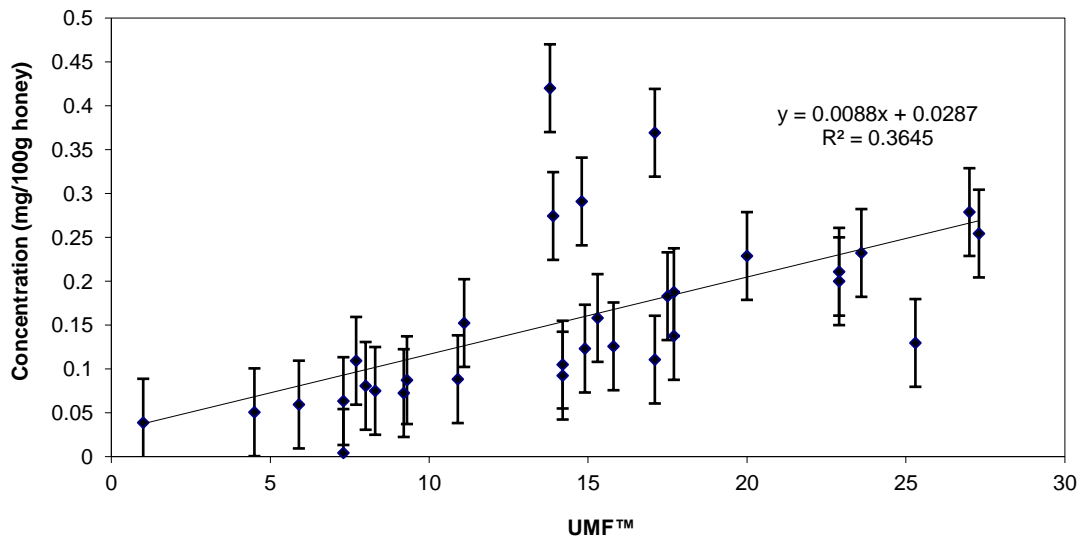
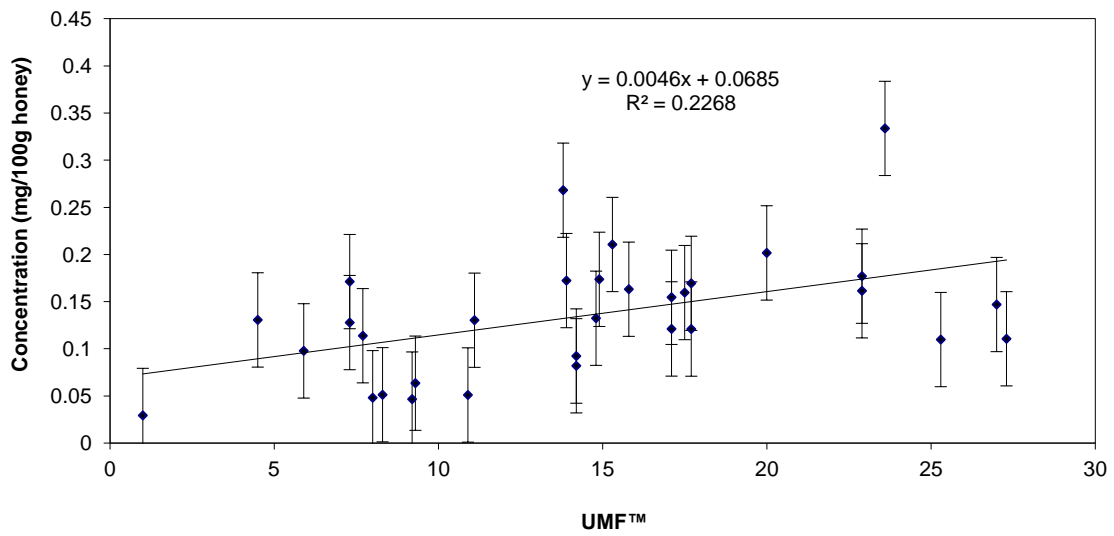


Figure 2

(a)



(b)



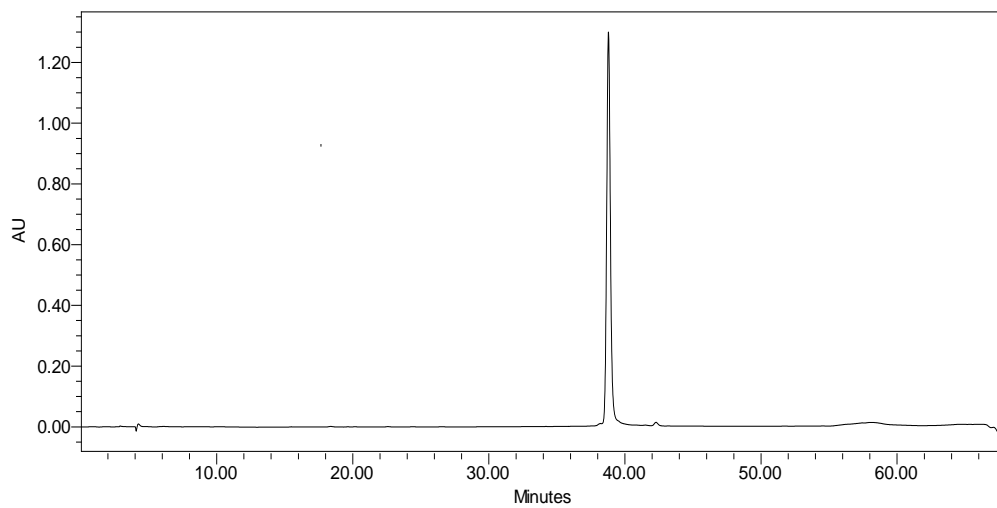


Figure 3

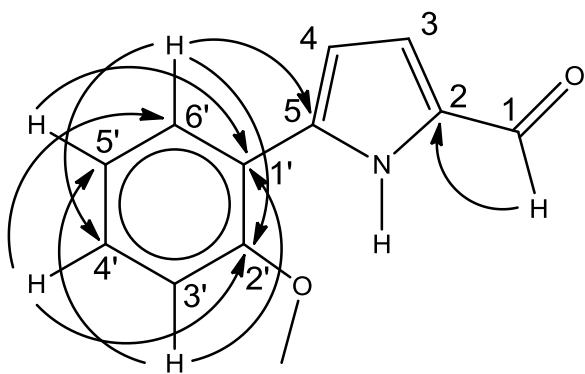
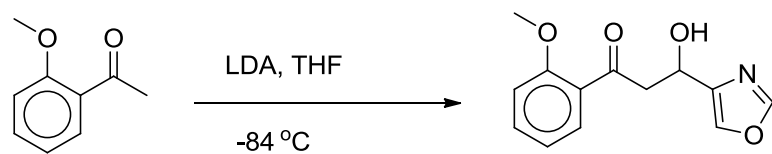
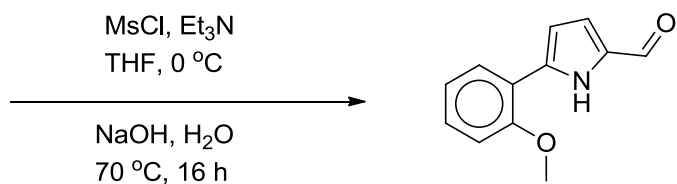


Figure 4



5



1

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 |
| <i>p</i> -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 |

^aMean and 95% confidence interval from 48 determinations.

^bMean 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, <i>J</i> = 3.9 Hz) | 121.9 | 7.08 (dd, <i>J</i> = 3.9, 2.3 Hz) |
| 4 | 111.4 | 6.77 (d, <i>J</i> = 3.9 Hz) | 108.9 | 6.79 (dd, <i>J</i> = 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, <i>J</i> = 8.0 Hz) |
| 3' | 112.1 | 7.14 (dd, <i>J</i> = 8.5, 1.2 Hz) | 128.7 | 7.42 (t, <i>J</i> = 8.0 Hz) |
| 4' | 129.4 | 7.33 (ddd, <i>J</i> = 8.5, 7.3, 1.7 Hz) | 127.9 | 7.32 (t, <i>J</i> = 8.0 Hz) |
| 5' | 120.7 | 7.02 ^b (~td, <i>J</i> = 8.3, 1.2 Hz) | 128.7 | 7.42 (t, <i>J</i> = 8.0 Hz) |
| 6' | 128.4 | 7.77 (dd, <i>J</i> = 7.8, 1.7 Hz) | 125.5 | 7.89 (d, <i>J</i> = 8.0 Hz) |
| 2'-OCH ₃ | 55.6 | 3.96 s | | |

^asample kindly supplied by Dr Reeves; ^bsignals partly overlapped