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Antibacterial phenolic components of New Zealand manuka honey

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Abstract

This paper describes several methods for isolation of the antibacterially active phenolic fraction of honey derived from the native New Zealand manuka tree, *Leptospermum scoparium* (Myrtaceae). This fraction consists of phenolic derivatives of benzoic acids, cinnamic acids and flavonoids, all of which have been identified previously in honeys which do not exhibit non-peroxide residual antibacterial activity. The flavonoids had not previously been identified in manuka honey. Furthermore, the flavonoids were different from those found in the leaves of manuka trees but were the same as those found in European honeys and propolis. While most of these phenolic products possess antibiotic activity, they do not individually or collectively account for the antibacterial activity of 'active' manuka honey. Essentially all of this activity is associated with the carbohydrate fraction of the honey. © 1998 Elsevier Science Ltd. All rights reserved.

1. Introduction

The antibacterial activity of honey was reviewed extensively by Molan (1992). The principal factor in honey, responsible for this activity is hydrogen peroxide that is formed from the oxidation of glucose by the enzyme glucose oxidase, during the period when honey is ripened. While the monosaccharides in honey are acquired from flower nectar, the enzyme originates from the glands of bees (White, 1978).

If the hydrogen peroxide in an aqueous solution of honey is destroyed by addition of the enzyme catalase, residual non-peroxide antibacterial activity is observed in several honeys (Molan and Russell, 1988). A survey of such activity in a number of unifloral New Zealand honeys was published by the Waikato group (Allen et al., 1991). The greatest activity was observed in manuka honey, a very popular and economically important product derived from the native manuka tree, *Leptospermum scoparium* (Myrtaceae). The non-peroxide antibacterial activity of honeys is believed to be due to honey components derived from the floral source (Molan and Russell, 1988). This deduction is supported by the observation that not all manuka honey possessed non-peroxide antibacterial activity. Instead, the bioactivity was recorded in manuka honey, only from specific localities (Molan, 1995).

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Studies of the use of honey as a topical antibiotic have been published (Effem, 1988), as have *in vitro* studies of the use of manuka honey as an antibiotic against *Helicobacter pylori,* the bacterium which causes stomach ulcers (Somal et al., 1994).

Molan's group have attempted to isolate and characterise the chemical components which are responsible for the residual non-peroxide antibacterial activity of manuka honey (Russell et al., 1990). They isolated, by thin-layer chromatography, a number of aromatic acid derivatives, of which syringic acid and phenyllactic acid were the most abundant. These acids were later shown by Wilkins et al. (1993) to be so abundant amongst the extractable organic components that they proposed these two acids as phytochemical fingerprints of manuka honey. However, methyl syringate is abundant in rape and clover honey (Joerg and Sonntag, 1993). Furthermore, syringic acid and especially phenyllactic acid are the major acids in several European honeys (Steeg and Montag, 1987). The Waikato group also showed that the extractable components of manuka honey were not present in manuka flowers but instead were probably derived from the honeydew which characteristically coats the trunks of manuka trees (Tan et al., 1988).

The aromatic acids isolated from manuka honey exhibit antibiotic activity towards a range of bacteria, but since these same acids occur in other honeys, then the acids clearly are not the only substances responsible

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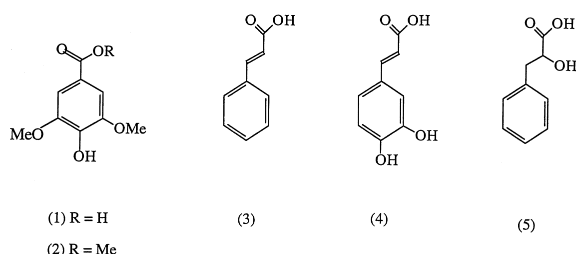
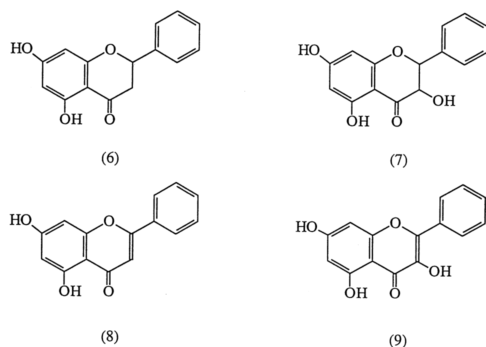
for the high residual non-peroxide antibacterial activity characteristic of manuka honey. This paper describes the isolation of additional substances which might be responsible for this antibacterial activity of manuka honey.

The major task in this work was to separate the monosaccharides from the antibacterial material and to establish an appropriate separation medium. Whereas Russell et al. (1990) used preparative thin-layer chromatography for this purpose, the present work employs a number of alternative chromatographic media.





(1) Syringic acid
(2) Methyl syringate
(3) Cinnamic acid
(4) Caffeic acid
(5) Phenyllactic acid
(6) Pinocembrin
(7) Pinobanksin
(8) Chrysin
(9) Galangin

2. Materials and methods

2.1. Materials

'Active' manuka honey (code M61) was obtained from the Honey Research Unit, Department of Biological Sciences, Waikato University, Hamilton.

2.2. Methods

2.2.1. Antibiotic activity

All antibiotic activity discussed in this paper, refers to *in vitro* non-peroxide antibacterial activity, determined by well-diffusion bioassays on agar plates inoculated with *Staphylococcus aureus*. The procedures for these bioassays are described by Allen et al. (1991). Results of the bioassays are reported as the diameter of the area of inhibition of growth of the bacteria. The concentration is that used for the bioassay. For the active honey, used as a standard, the minimum inhibitory concentration (MIC) was 125 g litre^{-1}.

2.2.2. High performance liquid chromatography (hplc)

Hplc was performed on a LiChrospher 100 RP-18 (5 μm) column (12×0.4 cm) with UV detection at 270 nm. Solvents consisted of A: 5% formic acid in water and B: methanol. The solvent programme began with a linear gradient from 65%A, decreasing to 55%A at 10 min, held for 10 min and then decreased to 20%A at 55 min and again to 5%A at 60 min. The flow-rate was 1 ml min^{-1} at 30°C.

2.2.3. Chromatography of honey on poly(capryl) amide

Polyamide (360 g) was slurried with water overnight. The mixture was then packed into a column (5×81 cm) affording a bed volume of 1.6 litre. Honey (4.5 g; which consisted of 3.6 g solids) in water (10 ml) was filtered through glass wool and applied to the column which was developed with 4 column volumes of water (6.0 l). Fractions of 500 ml were collected.

Fraction	Mass (mg)	MIC[a] (g litre^{-1})	Activity[b]
4	1526	250	b
5	2097	250	a
6	6	4.5	a
8	4	5.3	b
Honey	—	125	a

All other fractions contained less than 4 mg and were not bioassayed.
[a] MIC = minimum inhibitory concentration.
[b] a = bacteriocidal and b = bacteriostatic.

2.2.4. Chromatography of honey on sephadex G-10

Honey (8.5 g; 7.0 g solids) was dissolved in water (6 ml) and the solution was applied to a column (3.3×51 cm; bed volume 440 ml) of Sephadex G-10 (200 g) which was developed with water (2 column volumes; 900 ml). 18 fractions (50 ml) were collected and combined according to their UV spectra.

Sample	Fraction	Mass (mg)	MIC (g litre^{-1})	
			Bacteriostatic	Bacteriocidal
A	1–3	3	—	—
B	4	29	38	> 77
C	5	183	> 267	> 267
D	6–8	6823	267	> 267
E	9–12	152	269	269
F	13–18	12	> 32	> 32
G(MeOH)	—	13	35	35
Honey	—	—	133	266

The most active sample G, which was eluted with methanol, was analysed by hplc and found to contain methyl syringate (75%), phenyllactic acid (12%) cinnamic acid (2%) and the flavonoids, pinobanksin and pinocembrin. The chromatography was not continued long enough to detect chrysin or galangin.

2.2.5. Separation of honey on XAD-2 resin

XAD-2 resin(54 g; approx 100 ml) was soaked in a mixture of water (200 ml) and methanol (200 ml) overnight. The liquid was decanted from the resin which was then washed with water and packed into a column to give a bed volume of 2×25 cm. The column was rinsed with water (1 litre).

Active honey (200 g) was dissolved in a mixture of water (1 litre) and concentrated hydrochloric acid (10 M, 1 ml). The solution was filtered through glass wool and then added slowly to the column, followed by acidified water (pH, 2; 100 ml), distilled water (300 ml) and methanol (300 ml). The methanol extract was concentrated under vacuum at 40°C and freeze-dried, to afford a fraction that was analysed by hplc.

This procedure was repeated for inactive manuka honey (183 g). The methanol extracts were suspended in water (20 ml) and the solution was extracted twice with diethyl ether (20 ml). The aqueous and ether extracts were concentrated and the residual material was analysed by hplc and subjected to a bioassay.

Sample	Mass (mg)	Percentage of honey (%)	MIC (g litre^{-1})
A: Active honey MeOH extract	538	0.27	N/D[a]
B: Inactive honey MeOH extract	469	0.26	N/D
C: Active honey ether extract	148	0.07	9.3
D: Inactive honey ether extract	242	0.13	27.8
E: Active honey aqueous extract	341	0.17	68
F: Inactive honey aqueous extract	177	0.10	31.3
Honey	—	—	125

[a] Not determined.

Hplc data for samples C and D are listed in Table 1 and the chromatogram. of sample C is shown in Fig. 1.

2.2.6. Chromatography of honey on XAD-2 resin

Active manuka honey (150 g) was dissolved in acidified water (1 litre) and applied to a column of XAD-2 resin as above. The column was developed with acidified water (pH = 2; 110 ml) distilled water (300 ml), methanol/water (30:70; 250 ml) and methanol (250 ml).

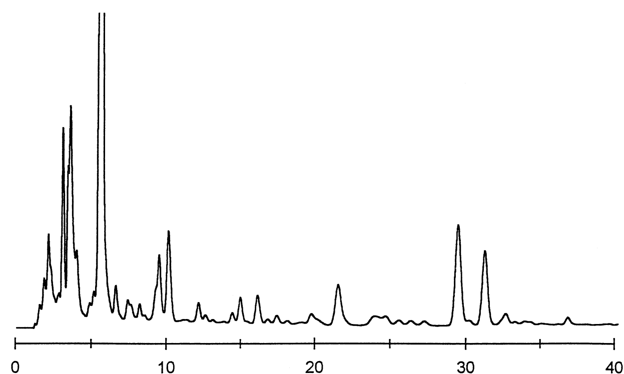


Fig. 1. High performance liquid chromatogram of sample 1 (Table 1).

Fraction	Mass (mg)	MIC (g litre^{-1})
Polar (MeOH/water)	162	28
Non-polar (MeOH)	294	7
Honey	—	125

2.2.7. Identification of methyl syringate

Thin-layer chromatography of the active ether extract (C, from section separation of honey on XAD-2 resin, above), on silica gel in a mixture of benzene/ethyl acetate/formic acid (7:2:1) and visualisation with UV light at 254 nm or by a ferric chloride/ferricyanide spray indicated that it contained a number of minor products together with one major product.

The active ether extract (C, above) was therefore fractionated by preparative thin-layer chromatography on plates (20×20 cm, coated with silica gel 60 F_{254} 0.25 mm). Development in a mixture of benzene/ethyl acetate/formic acid (7:2:1) yielded three bands. The least polar band was methyl syringate which was identified by GC-MS, proton and carbon NMR spectra and glc of an authentic sample. The middle band contained phenyl lactic acid which was identified by the same means and the most polar band contained insufficient material for identification.

2.2.8. Separation of honey on XAD-2 under neutral conditions

Honey (145 g) was dissolved in distilled water (1 litre) and filtered through XAD-2 as above. Polar material was eluted with water (400 ml) and the active material was eluted with ethanol (300 ml). The non-polar extract (376 mg) was dissolved in water and extracted three times with diethyl ether. The ethereal solution yielded 168 mg of crystalline material which was analysed by hplc (see Table 1).

Table 1
HPLC data for the composition (%) of selected fractions of manuka honeys

R_t (min)[a]	Component	Structure	Sample[b]			
			1	2	3	4
2.2	Caffeic acid	4	2.6	4.8	2.0	1.8
3.1	Unknown		3.6	—	3.4	3.1
3.6	Phenyllactic acid	5	8.9	26.2	6.8	5.9
4.1	Unknown		0.7	—	1.5	1.4
5.1	Unknown		0.5	—	0.5	—
5.6	Methyl syringate	2	46.3	21.1	48.6	47.1
7.4	Unknown		0.8	—	0.8	1.7
9.5	Cinnamic acid	3	2.7	—	2.9	2.6
10.2	Pinobanksin	7	3.1	3.2	2.9	2.8
21.4	Pinocembrin	6	2.3	—	2.4	2.3
29.2	Chrysin	8	5.1	—	4.5	4.5
31.1	Galangin	9	3.7	—	2.9	2.9

[a] Under these conditions, the following retention times were also recorded: Gallic acid: 1.4 min; Methyl gallate: 1.9 min; Syringic acid: 2.4 min; Leptospermone: 47.0 min.

[b] Samples 1: Ether extract (C) of active honey; 2: Ether extract (D) of inactive honey; 3: Ether extract of active honey; 4: 2-Butanol extract of active honey.

2.2.9. *Isolation of antibacterial material with 2-butanol*

Active honey (150 g) was dissolved in water (1litre). This solution was saturated with sodium chloride and then filtered through glass wool. This solution was extracted with 2-butanol (2×100 ml and 1×50 ml) and the alcoholic extract was concentrated to dryness, slurried with water and then freeze-dried. The dried material was shaken with methanol. The alcoholic extract was filtered, concentrated and the syrup was dissolved in water and then extracted three times with diethyl ether. The ethereal solution was dried and concentrated to yield (160 mg, 0.11%) material which was analysed by hplc (see Table 1).

2.2.10. *Comparative bioassays of honey extracts*

The antibacterial activity of the solvent extract obtained from separation of active honey on XAD resin under neutral conditions and also the butanol/ether extract were compared with that of honey and phenyllactic acid by well-diffusion assays of these materials adsorbed on paper discs. Solutions were prepared by dissolving the extracts (120 mg) in a mixture of methanol 120 µl and diethyl ether (120 µl) to give a concentration of 0.33 mg μl^{-1}. Honey was prepared as a 25% solution in water and phenyllactic acid (20 mg) was dissolved in methanol (100 µl) as was methyl syringate. Whatman filter paper (No. 52) with a circular diameter of 7 mm was used for bioassays.

Sample	Volume (µl)	Mass (mg)	Inhibition (mm)
Honey	130	32	18
MeOH/ether blank	10/10	—	0
Phenyllactic acid (0.16 mg μl^{-1})	0.25	0.04	0
	0.50	0.08	0
	2.5	0.4	12
	5.0	0.8	17
	10.0	1.6	20
XAD extract from neutral chromatography (0.33 mg/µl)	1.0	0.33	11
	2.0	0.66	15
	4.0	1.33	18
	10.0	3.33	21
Butanol/ether extract (0.33 mg μl^{-1})	1.0	0.33	9
	2.0	0.66	11
	4.0	1.33	14
	10.0	3.33	18

Methyl syringate failed to inhibit the growth of the bacterium at levels of 0.04–1.6 mg $disc^{-1}$.

2.2.11. *Comparison of the bioactivity of the noncarbohydrate extracts of active and inactive honeys*

The non-carbohydrate material from three samples of manuka honey (A,B,C) known to have strong non-peroxide antibacterial activity and three samples of manuka honey (E,F,G) without activity, together with a honeydew honey (D; of unknown antibacterial activity) was extracted on XAD-2 resin and into diethyl ether as described above. The hplc composition of these extracts is provided in Table 2.

The extracts were dissolved in a mixture of methanol and diethyl ether (2:1) to provide solutions with a concentration of 20 mg ml^{-1}. Twenty five µl (0.5 mg) of these solutions were then applied to paper discs for bioassay. The solutions were then evaporated to dryness and redissolved at a concentration of 500 mg ml^{-1}. Ten µl (5 mg) of these solutions were then applied to discs for bioassay.

Sample	Honey (g)	Extract (mg)	Yield (%)	Dose (0.5 mg) Inhibition	Dose (5 mg) (mm)
A	100	77	0.08	9.0	20
B	105	119	0.11	8.5	19
C	104	68	0.07	8.5	18
D	229	76	0.03	9.0	20
E	101	99	0.10	8.0	18
F	102	79	0.08	9.0	20
G	104	159	0.15	8.5	21

2.2.12. *Chromatography of honey on XAD-2*

An aqueous solution of honey (25 g in a total of 100 ml) was filtered through a column of XAD-2 resin (100 ml) which was then developed with mixtures (250 ml) of increasing proportions of methanol in water.

Table 2
HPLC data for the composition (%) of ether extracts of selected manuka honeys

R_t (min)	Component	Structure	Sample						
			1	2	3	4	5	6	7
2.2	Caffeic acid	4	2.5	0.8	2.3	11.9	0.6	6.5	0.9
3.0	Unknown		3.5	3.2	3.3	1.4	2.5	5.1	5.9
3.2	Unknown		7.7	17.9	12.3	2.4	8.3	15.5	24.2
3.6	Phenyllactic acid	5	4.5	—	3.7	—	1.7	—	—
4.1	Unknown		—	—	—	6.4	—	—	—
5.1	Unknown		25.0	23.7	29.0	28.7	22.6	16.8	26.3
5.6	Methyl syringate	2	29.1	29.0	30.8	13.4	24.2	20.0	31.3
7.4	Unknown		0.7	0.4	0.5	11.4	0.5	2.3	—
9.5	Cinnamic acid	3	2.5	0.4	1.6	0.8	1.8	1.7	0.5
10.2	Pinobanksin	7	1.8	1.3	1.2	0.5	1.0	1.8	0.7
21.4	Pinocembrin	6	0.9	—	—	0.3	—	0.7	—
29.2	Chrysin	8	2.4	1.7	2.1	2.7	1.6	3.4	0.7
31.1	Galangin	9	1.7	0.9	1.2	1.1	0.8	2.0	0.5

Sample: 1,2,3: Active manuka honeys from selected locations; 4: A honeydew honey (beech); 5,6,7: Inactive manuka honeys from selected locations.

The fractions were concentrated under vacuum at 40°C until the methanol had been removed and then made up to 100 ml with water and bioassayed. The data for the honey in the following table, is shown graphically in Fig. 2.

Fraction	Solvent	Inhibition (mm)
1	Water	17
2	Water/methanol (95:5)	0
3	Water/methanol (90:10)	0
4	Water/methanol (80:20)	0
5	Water methanol (50:50)	0
6	Methanol	0
Honey	Concentration (%)	
	25	17
	20	16
	15	13
	10	11
	5	0

2.2.13. Chromatography of honey on Biogel P-2

A solution of honey (10 g) in water (10 ml) was filtered through a column of Biogel-P-2 (Biorad) (100 g) which had a bed volume (2×87 cm) of 270 ml. The column was developed with water at a flow-rate of 40 ml h^{-1} and seven fractions of 40 ml were collected. Glucose was expected to be eluted last and it was monitored by tlc on cellulose plates in n-BuOH/pyridine/water (6:4:3) and visualised with a spray of phenol (3 g) and concentrated sulphuric acid (5 ml) in ethanol (95 ml). The fractions were concentrated to 10 ml for bioassay.

Standard Honey Concentration (C) (g/dl)	log C	D (mm)	D^2
25	1.398	17	289
20	1.301	16	256
15	1.176	13	169
10	1.000	11	121
5	0.699	0	0

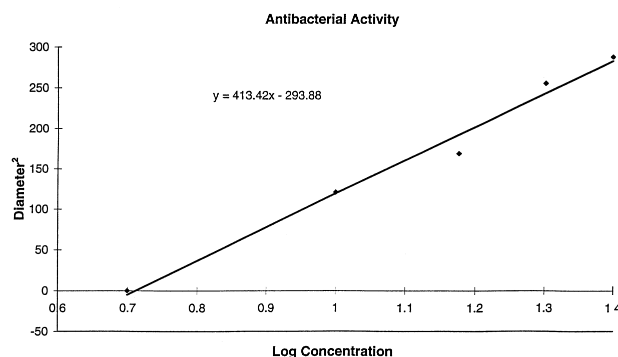


Fig. 2. Correlation of honey concentration and inhibition of bacterial growth (D).

Fraction	Mass (g)	Inhibition (mm)
A	0	0
B	0.007	0
C	0.003	0
D	0.214	0
E	4.161	13
F	3.427	14
G	0.283	0
Total	8.095	

TLC indicated that monosaccharides were in fractions E and F.

3. Results and discussion

Russell et al. (1990) qualitatively identified several antibacterial phenolic compounds in manuka honey after fractionation of the honey by thin-layer chromatography. The present work has identified and quantified these and further antibacterial components of manuka honey, after isolation on alternative media.

Determination of the minimum inhibitory concentration (MIC) of fractions from chromatography of antibacterially active manuka honey on polyamide and Sephadex G-10 indicated that active material was located in late fractions.

Analysis of this material by HPLC under conditions used earlier in a study of manuka propolis, (Markham et al., 1996) suggested that flavonoids were present in this honey. Because the antibacterial activity of propolis

has been attributed to the flavonoids in that material (Metzner et al., 1979; Grange and Davey, 1990; Houghton et al., 1995; Marcucci, 1995) it was possible that these flavonoids might also be responsible for the non-peroxide antibacterial activity of manuka honey. Analysis of their distribution and abundance in manuka honey was therefore undertaken.

The phenolic fraction of manuka honey was isolated by the method developed by Ferreres et al. (1994). The ether extract of active manuka honey, obtained by this method, had a MIC of 9 g $litre^{-1}$. Literature values for the MIC of the ethanolic extract of propolis, against *Staphylococcus aureus* range from 0.1 g $litre^{-1}$ (Bonvehi et al., 1994) and 1.5 g $litre^{-1}$ (Metzner et al., 1979) to 10 g $litre^{-1}$ (Meresta and Meresta, 1980). Values in the range of 1–10 g $litre^{-1}$ appear most frequently and the variation will reflect the differences in chemical composition of the propolis samples. Values of the MIC of extracts or fractions from 'active' manuka honey, recorded in the present work all fall in the range of 1–10 g $litre^{-1}$ and these extracts therefore clearly correspond to a propolis extract.

The major product in the phenolic extract of 'active' manuka honey was isolated by preparative thin-layer chromatography and was shown to be methyl syringate. A minor product was identified as phenyllactic acid. Because the isolation procedure utilises acidified methanol, the methyl syringate was initially thought to be an artefact, but this was disproved by repetition of the procedure under neutral conditions and use of ethanol as a solvent.

HPLC analysis of the 'active' phenolic extracts of manuka honey indicated that methyl syringate constituted more than 45% of the total extract. The ester was identified in earlier work (Russell et al., 1990) but only qualitatively. The present work established the ester as a major component of the phenolic extract of manuka honey.

Wilkins et al. (1993) found that phenyllactic acid was a major component of manuka honey, along with syringic acid and proposed these two acids as phytochemical markers, which could distinguish manuka honey. However, methyl syringate is abundant in rape and clover honeys (Joerg and Sonntag, 1993) and phenyllactic acid is very common in many European honeys (Steeg and Montag, 1987).

Methylation of the carboxyl group of syringic acid confers a degree of lipophilicity on this ester compared to the parent acid and such a property is known to be an important factor in the antibiotic activity of phenolic products (Schmalreck et al., 1975; Boyd and Beveridge, 1979).

To determine whether methyl syringate and phenyllactic acid were significant contributors to the non-peroxide antibacterial activity of manuka honey, a range of samples of these compounds, together with two extracts of honey, were adsorbed onto paper discs for bioassay by diffusion on agar plates. Methyl syringate failed to inhibit the growth of the bacteria at levels of 0.04–1.6 mg $disc^{-1}$, due probably to its insolubility in the aqueous diffusion medium. The same degree of inhibition of bacterial growth was recorded for 0.8 mg of phenyllactic acid, 1.33 mg of a chromatographically isolated extract and 3.33 mg of a (2-butanol) solvent extract. These data suggested that neither methyl syringate nor phenyllactic acid were significant contributors to the antibacterial activity of the honey.

Furthermore, HPLC data (Table 2) indicated that methyl syringate was equally abundant in both active and inactive honey and therefore it cannot be responsible for the observed non-peroxide antibacterial properties of active manuka honey.

This conclusion is also supported by the work of Joerg and Sonntag (1993) who found abundant quantities of methyl syringate in honeys which do not exhibit non-peroxide antibacterial activity.

In view of this result, it was essential to determine whether the phenolic fraction of the honey as a whole, was responsible for the non-peroxide antibacterial activity of the honey. The phenolic extracts from three 'active' and three 'inactive' manuka honeys together with that from a honeydew honey were adsorbed onto paper discs at two levels (0.5 and 5.0 mg). The degree of inhibition of bacterial growth was the same for all honeys (at each dose level). This experiment demonstrated that while the phenolic components of manuka honey individually and collectively were antibacterially active, they were not responsible for the *observed* antibacterial activity of manuka honey. This conclusion was also demonstrated by chromatography of 'active' manuka honey on XAD-2 resin. The fractions were dissolved in a volume of water equivalent to a 25% solution of honey, for the bioassay. In this way, all the antibacterial activity of the honey was shown to be eluted with the carbohydrates and no significant activity was recorded for the fractions (5 and 6) which contained the phenolic material. A similar result was observed when the honey was fractionated on polyacrylamide (Biogel P-2). In this case, only those fractions (E and F) which contained the carbohydrates, were antibacterially active.

Since the monosaccharides (glucose and fructose) have no antibacterial properties, this work suggests that an antibacterial substance is being 'carried' by the monosaccharides which form the bulk of the mass of honey.

The flavonoid components of the phenolic extract were those which had been identified earlier in New Zealand propolis (Markham et al., 1996) and while these flavonoids have antibiotic properties, their abundance is too low to contribute significantly to the antibacterial activity of manuka honey. Most European non-active honeys also contain these same flavonoids at

similar levels (Ferreres et al., 1991; Tomas-Barberan et al., 1993a,b). The Spanish group (Gil et al., 1995) have recently demonstrated that flavonoids originate in honey by way of contact with propolis in the hive. Since propolis is derived from the resin of poplar trees (Greenaway et al., 1991), it is not surprising that the flavonoids in manuka honey are those which also occur in most other honeys, but are different from those found in parts of the manuka tree (Mayer, 1990; Haberlein and Tschiersch, 1994).

Leptospermone, an antibacterial substance which occurs characteristically in *Leptospermum* (manuka) trees (Perry et al., 1997), has a retention time of 47.0 min in the hplc system used here and was not detected as a component of the phenolic extracts of manuka honey.

In conclusion, phenolic products in manuka honey, from nectar, pollen or propolis, are only partly responsible for the observed non-peroxide antibacterial properties of the 'active' New Zealand manuka honey.

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