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A Survey of the Antibacterial Activity of Some New Zealand Honeys

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Abstract—To assess the variation in antibacterial activity of honey a survey was carried out on 345 samples of unpasteurized honey obtained from commercial apiarists throughout New Zealand. Most of the honeys were considered to be monofloral, from 26 different floral sources. The honeys were tested against *Staphylococcus aureus* in an agar well diffusion assay, with reference to phenol as a standard. Antibacterial activity was found to range from the equivalent of < 2% (w/v) phenol to 58% (w/v) phenol, with a median of 13·6 and a standard deviation of 12·5. Neither the age of the honey samples nor whether they had been processed by the apiarist was associated with lower activity. However, the difference between floral sources in the antibacterial activity was very highly significant. Kanuka (*Kunzea ericoides* (A. Rich.) J. Thompson. Family: *Myrtaceae*), manuka (*Leptospermum scoparium* J. R. et G. Forst. Family: *Myrtaceae*), ling heather (*Calluna vulgaris* (L.) Hull. Family: *Ericaceae*) and kamahi (*Weinmannia racemosa* Linn. f. Family: *Cunoniaceae*) were shown to be sources likely to give honey with high antibacterial activity. When antibacterial activity was assayed with catalase added to remove hydrogen peroxide, most of the honeys showed no detectable antibacterial activity. Only manuka and vipers bugloss (*Echium vulgare* L. Family: *Boraginaceae*) honeys showed this type of activity in a significant proportion of the samples. The high antibacterial activity of manuka honey was in many cases due entirely to this non-peroxide component.

Honey has been used as a wound dressing since ancient times (see Ransome 1937; Majno 1975), but it was not until the twentieth century that it was found to have antibacterial properties (Sackett 1919). The antibacterial activity has been attributed to the high osmolarity, the acidity and particularly the hydrogen peroxide content of honey (White 1966).

The use of honey as a topical antibacterial agent is gaining acceptance for the treatment of surface infections such as ulcers and bed sores (Hutton 1966; Blomfield 1973; Bloomfield 1976; Keast-Butler 1980), and those resulting from burns, injuries and surgical wounds (Bulman 1955; Cavanagh et al 1970; Lawrence 1976; Armon 1980; Braniki 1981; Dumronglert 1983; Effem 1988; Green 1988; McInerney 1990). Its usage in medical practice has been reviewed recently by Zumla & Lulat (1989), who concluded that "the time has now come for conventional medicine to lift the blinds off this 'traditional remedy' and to give it its due recognition".

A common feature of all of the reports on the use of honey as an antibacterial agent is that little consideration is given to the selection of the type of honey that is used. Although two millenia ago it was recommended that honey collected in specific regions and seasons (and therefore presumably from different floral sources) be used for the treatment of different ailments (Aristotle ca. 350 B.C.; Dioscorides ca. 50 A.D., in Gunther 1934), in recent times these recommendations have gone unnoticed. Little notice has been taken either of research reports demonstrating differences between honeys in their antibacterial potency (Dold & Witzenhausen 1955; Duisberg & Warnecke 1959; Stomfay-Stitz & Kominos 1960; White & Subers 1963; White et al 1963; Smith et al 1969; Dustmann 1971, 1979; James et al 1972; Daghie et al 1983; Bogdanov 1984; Radwan et al 1984; Tomlinson & Williams 1985; Roth et al 1986; Bogdanov et al 1987; Dolezal et al 1988).

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A preliminary study of some New Zealand honeys (Molan et al 1988) revealed some very marked differences in the potency of their antibacterial activities. Although the small number of samples surveyed limited statistical analysis of the data, there was a significant association of potency with floral type, with manuka honey being noted for its high activity. Also, a correlation was found between the honeys with high activity and the content of antibacterial activity that was not due to hydrogen peroxide (Molan & Russell 1988).

The present report has subsequently been extended to a much larger number of samples of honey, using a standardized assay technique. The original samples (Molan et al 1988) were also included in this assay. Activity was assessed both with and without their hydrogen peroxide. The honeys surveyed were obtained from commercial apiarists so that the survey would reveal the variation likely to be found in the activity of honey that may be purchased for therapeutic usage.

Materials and Methods

Collection of honeys

Honey samples were obtained from commercial apiarists throughout New Zealand. These honey samples were collected during the 1982–1989 flowering seasons. The majority were considered by the apiarists to be monofloral specimens, with the floral source of each honey being identified by the apiarist supplying it. Identification was based on the flavour, colour and aroma of each honey, also the season and location of its production. The sources of the honeys were considered to be:

Barberry (*Berberis vulgaris* L. Family: *Berberidaceae*), buttercup (*Ranunculus repens* L. or *Ranunculus sardous* Crantz. Family: *Ranunculaceae*), catsear (*Hypochoeris radicata* L. Family: *Asteraceae*), clover (*Trifolium repens* L., or *Trifolium pratense* L. Family: *Fabaceae*), dandelion (*Taraxacum officinale* G. Weber. Family: *Asteraceae*), fennel (*Foeniculum vulgare* Miller. Family: *Apiaceae*), gooseberry (*Ribes uva-crispa* L. Family: *Grossulariaceae*), honeydew (from *Nothofagus solandri* (Hook. f.) Oerst. Family: *Fagaceae*), kamahi (*Weinmannia racemosa* Linn. f. Family: *Cunoniaceae*), kanuka (*Kunzea ericoides* (A. Rich.) J. Thompson. Family: *Myrtaceae*), ling heather (*Calluna vulgaris* (L.) Hull. Family: *Ericaceae*), manuka (*Leptospermum scoparium* J. R. et G. Forst. Family: *Myrtaceae*), nodding thistle (*Carduus nutans* L. Family: *Asteraceae*), oilseed rape (*Brassica napus* L. Family: *Brassicaceae*), penny royal (*Mentha pulegium* L. Family: *Lamiaceae*), pohutukawa (*Metrosideros excelsa* Sol. ex Gaertn. Family: *Myrtaceae*), ragwort (*Senecio jacobæa* L. Family: *Asteraceae*), rata (*Metrosideros robusta* A. Cunn. Family: *Myrtaceae*), rewarewa (*Knightia excelsa* R. Br. Family: *Proteaceae*), spanish heath (*Erica lusitanica* Rudolphi. Family: *Ericaceae*), tawari (*Ixerba brexioides* A. Cunn. Family: *Escalloniaceae*), thyme (*Thymus vulgaris* L. Family: *Lamiaceae*), towai (*Weinmannia silvicola* Sol. ex A. Cunn. Family: *Cunoniaceae*), vine rata (*Metrosideros fulgens* Sol. ex. Gaertn. Family: *Myrtaceae*), vipers bugloss (*Echium vulgare* L. Family: *Boraginaceae*), white rata (*Metrosideros perforata* (J. R. et G. Forst.) A. Rich, or *Metrosideros diffusa* Hook. f. Family: *Myrtaceae*), willow (*Salix fragilis* L. Family: *Salicaceae*).

The honeys were supplied and stored in airtight glass or plastic containers at 10°C in the dark. The honeys assayed were unpasteurized, and were from 1 month to 6 years old. Most were centrifugally extracted from the comb, but some were supplied scraped from the comb or in the form of broken comb.

Preparation of honey samples

All samples were prepared aseptically, and were handled away from direct sunlight. Samples of each honey (10 g, avoiding any visible pieces of wax), were added to sterile purified water and stirred at 37°C for 30 min to aid mixing. The 50% (w/v) solutions of each honey sample thus prepared were diluted to 25% (w/v) by taking 1 mL of each and adding it to either 1 mL of sterile purified water or 1 mL of catalase solution. The catalase solution was prepared by adding 40 mg catalase (Sigma, C-10: 4000 units mg^{-1}) to 20 mL of sterile purified water. The samples were assayed immediately after dilution.

Solutions of some honeys were also prepared for the production of standard curves of activity vs honey concentration. The 50% (w/v) solutions of each honey sample prepared as above were diluted with sterile purified water to give a series of ten concentrations in the range 5–50% (w/v).

Phenol standards

Solutions of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10% (w/v) phenol in purified water were prepared to use as standards.

Assay of antibacterial activity

The antibacterial activity of the honeys was assayed by the agar well diffusion method. The method was adapted from the punch plate assay for inhibitory substances described in the microbiology standard laboratory methods manual for the New Zealand dairy industry (Anon. 1982).

Large square plates (Nunc Bioassay Dishes—243 × 243 × 18 mm) seeded with *Staphylococcus aureus* (ATCC 25923) were prepared by adding 100 μL of an 18 h culture of the bacteria in Trypticase Soy Broth (BBL) to 150 mL of sterilized nutrient agar (made with 8 g L^{-1} BBL nutrient broth plus 15 g L^{-1} Difco agar) cooled to 45°C. The plates were poured on a level surface immediately after mixing, and stored for 24 h at 4°C before being used.

Sixty-four wells were cut in the agar using a cooled flamed 8 mm cork borer, and using a quasi-Latin square as a template. The template was prepared on a black card 243 × 243 mm. A 25 mm grid was drawn on the card, 34 mm away from the sides, and the wells were centred on the intersections of the grid. The wells were numbered just above the intersections using a quasi-Latin square which enabled the samples to be placed randomly on the plate.

The honey samples were tested in quadruplicate by adding 100 μL to each of four wells with the same number. For each assay one plate was used to test the honey samples for total activity (i.e. 25% w/v honey in water), and the other plate was used to test for non-peroxide activity (i.e. 25% w/v honey in catalase solution). A blank of water or catalase solution was used in two wells of each plate.

The plates were incubated for 18 h at 37°C and were then placed over the template. Using vernier callipers, the diameter of the clear zones was measured along the horizontal line on the template and recorded in mm. The diameter of each zone was then measured at 90° to the first measurement and entered on a new sheet to prevent any recording bias. All measurements were recorded without reference to the identity of the samples in the wells.

The mean diameter of the clear zone around each phenol standard was calculated and squared. A standard graph was then plotted of % phenol against the square of the mean diameter of the clear zone. A best-fit straight line was plotted and the equation of this line was used to calculate the activity of each honey sample from the square of each measurement of the diameter of the clear zone. Activity was then expressed as the equivalent phenol concentration (1% w/v).

Verification of removal of hydrogen peroxide

An experiment was conducted to confirm that when antibacterial activity was detected with catalase added it was not due to the catalase being inhibited by components of the honey. Solutions were prepared as above, using a manuka honey with high non-peroxide activity (M7). In addition other solutions were prepared in a similar way to contain 25% (w/v) honey in a solution of 45 mmol L^{-1} hydrogen peroxide, 25% (w/v) honey plus catalase in a solution of 45 mmol L^{-1} hydrogen peroxide, and 45 mmol L^{-1} hydrogen peroxide alone. These solutions were assayed in the wells of an assay plate in the same way as the honey samples were assayed.

Results

Total antibacterial activity of the honeys

The results of the survey of the honeys are shown in a

Table 1. Statistical analysis and summary of the data on total antibacterial activity (i.e. measured without catalase present) of the 345 honeys surveyed. The honeys have been grouped according to floral source, and the median, mean and s.d. of the antibacterial activity found in each group is shown (expressed as the concentration of phenol, % w/v, with equivalent activity in the agar well diffusion assay, calculated for the undiluted honey). For each floral source the proportion of samples which had activity greater than the overall median (13·6) is shown. The 90% confidence intervals for the medians and means are also shown.

Source	Activity (% phenol) Median	Mean	s.d.	No. of samples	% ≥ 13·6	90% CI for median	90% CI for mean
Barberry	41·4	42·1	1·8	3	100	(40·80, 44·20 at 75%)	39·07, 45·19
Pennyroyal	25·3	24·2	13·4	5	80	10·05, 35·15	11·46, 36·98
Oilseed rape	22·0	21·35	3·6	6	100	17·40, 24·48	18·37, 24·33
Kanuka	21·95	21·6	8·0	20	95	17·59, 24·53	18·45, 24·67
Rewarewa	20·9	20·9		1	100		
Willow	18·6	18·6		1	100		
Ling heather	16·6	13·4	8·3	21	62	11·68, 17·80	10·26, 16·53
Fennel	15·5	15·5		1	100		
Manuka	15·45	16·2	10·5	50	64	13·83, 19·07	13·68, 18·66
Kamahi	14·3	13·8	6·3	12	67	12·88, 18·84	10·55, 17·10
Ragwort	14·0	14·0		1	100		
Clover	12·0	13·9	12·7	39	44	5·87, 15·21	10·51, 17·37
Buttercup	10·7	13·2	12·7	3	33	(2·00, 27·00 at 75%)	−8·16, 34·63
Tawari	9·9	7·3	5·9	11	9	2·00, 10·22	4·11, 10·56
White rata	8·65	8·65	9·4	2	50	(2·00, 15·30 at 50%)	−33·34, 50·64
Towai	8·0	8·9	1·9	3	0	(7·70, 11·10 at 75%)	5·76, 12·11
Vipers bugloss	5·85	8·0	6·8	16	19	3·17, 9·14	5·01, 11·00
Catsear	2·7	2·7	1·0	2	0	(2·00, 3·40 at 50%)	−1·72, 7·12
Spanish heath	2	8·2	10·7	3	33	(2·00, 20·60 at 75%)	−9·90, 26·30
Thyme	2	6·3	6·3	25	28	2·00, 4·84	4·12, 8·44
Nodding thistle	2	4·9	5·3	24	12·5	2·00, 2·00	2·44, 6·14
Dandelion	2	2		1	0		
Gooseberry	2	2		1	0		
Honeydew	2	2		1	0		
Pohutukawa	2	2		1	0		
Rata	2	2	0·0	2	0	(2·00, 2·00 at 50%)	2·00, 2·00
Vine rata	2	2	0·0	2	0	(2·00, 2·00 at 50%)	2·00, 2·00
Pasture (mixed source)	21·4	19·5	5·1	3	100	(13·70, 23·30 at 75%)	10·90, 28·04
Native bush (mixed source)	12·1	12·1		1	0		
Clover and nodding thistle	8·35	8·35	9·0	2	50	(2·00, 14·70 at 50%)	−31·75, 48·45
Clover and kamahi	2	2		1	0		
Clover and vipers bugloss	2	2	0·0	2	0	(2·00, 2·00 at 50%)	2·00, 2·00
Unidentified source	17·4	19·1	16·3	79	58	11·31, 21·20	16·00, 22·12

summarized form in Table 1, with the honeys grouped according to their assumed floral source and ranked according to the median values. For the purpose of summarizing the data and for statistical analysis a value of 2% (w/v) phenol was arbitrarily assigned in all instances where the activity was found to be below the level of detection in the assay (lower limit of detection ~2% phenol).

Statistical analysis of the data was carried out to determine any correlation of total antibacterial activity (i.e. activity without catalase present) with floral source, age, and processing of the honeys. Honeys of dual floral source were omitted when samples were grouped according to floral source for statistical analysis of results.

The mean and median activities without catalase present of the 345 samples assayed were equivalent to 14·1 and 13·6% (w/v) phenol respectively. The standard deviation was 12·5. Analysis of variance between the groups of samples from each floral source showed that there were very significant differences between the groups in their mean activity ($F = 3·62$, $P < 0·007$).

A scatter graph of age of sample vs activity without catalase present plotted for all samples of known age showed no correlation of activity with age ($r^2 = 0·003$). Analysis of variance on the activity without catalase present showed no difference between the extracted and comb honeys (means 14·38 and 14·44, respectively; $F = 0·01$, $P = 0·968$).

The distribution of age of the samples, and of their form (extracted or comb honey), was not evenly associated with the various floral sources. Because of this there was the possibility that the preponderance of a particular age or form in samples from a floral source giving very high or low activity could have masked any correlation when the total data were used to look for correlation between activity and age or form. Therefore single floral sources were examined to look for such correlations.

Scatter graphs of age of sample vs activity were plotted for each of the floral sources from which there were 20 or more samples. These showed that there was little or no relationship between the age of a sample and its antibacterial activity. Examination of the proportion of samples from each floral source that had an activity greater than the overall median revealed that in most cases the proportions were very similar, and that there was no trend towards a higher proportion in either form.

The data from individual groups which had a reasonable number of both extracted and comb samples in them (kanuka, kamahi, ling heather, manuka, nodding thistle, thyme and vipers bugloss) were analysed using the Mann–

Table 2. Summary of the data on non-peroxide antibacterial activity (i.e. measured with catalase present) of the honeys in which it was detectable. The honeys have been grouped according to floral source, and the median, mean and s.d. of the non-peroxide antibacterial activity found in each group is shown (expressed as the concentration of phenol, % w/v, with equivalent activity in the agar well diffusion assay, calculated for the undiluted honey). The proportion of the total antibacterial activity in these honeys that was due to non-peroxide activity is also shown.

Source	No. of samples	Activity (%)			Proportion (%) of total antibacterial activity	
		Median	Mean	s.d.	Mean	s.d.
Manuka	19	15·5	18·6	8·0	89·5	15·6
Clover	1	15·1	15·1		100	
Unidentified source	2	13·75	13·75	13·1	97·5	3·5
Vipers bugloss	4	4·35	4·35	0·1	75	2·4
Kanuka	1	4·2	4·2		19	

Whitney U-test. This still failed to show any significant difference between extracted and comb honeys, except for manuka honey where the extracted samples were more active ($0{\cdot}01 > P > 0{\cdot}001$) and thyme honey where the comb samples were more active ($0{\cdot}05 > P > 0{\cdot}01$).

Non-peroxide antibacterial activity of the honeys

Non-peroxide activity was detectable in samples from very few floral sources, being found only in single samples from sources other than manuka and vipers bugloss (also in two samples of unidentified source). Non-peroxide activity was detected in 38% of the manuka samples and 25% of the vipers bugloss samples. The results for the samples with detectable non-peroxide activity are summarized in Table 2.

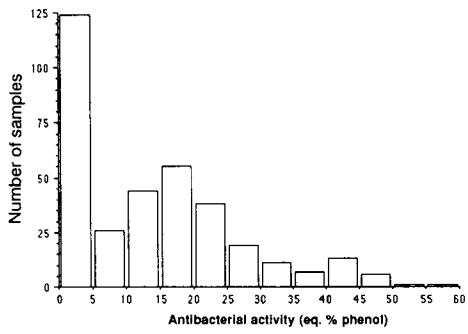

FIG. 2. Distribution of antibacterial activity amongst the honey samples surveyed.

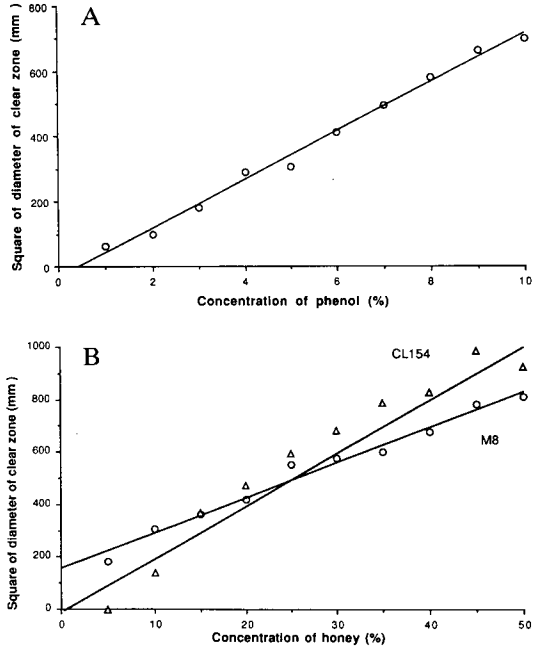

FIG. 1. A. Standard curve for the phenol standard used in the agar well diffusion assay of antibacterial activity. B. Standard curve for honey in the agar well diffusion assay of antibacterial activity: a honey with a high peroxide activity, CL154; a honey with a high non-peroxide activity, M8.

Table 3. The antibacterial activity of manuka honey (M7) and added hydrogen peroxide measured with and without catalase added to remove the hydrogen peroxide. The antibacterial activity is expressed as the area (mm^2) of the clear zones on the agar plate in a well diffusion assay.

Solution tested	Activity without catalase	Activity with catalase
(a) With 25% (w/v) honey and 45 mmol L^{-1} hydrogen peroxide		
H_2O_2	998 ± 147	not detectable
Honey	539 ± 97	555 ± 79
Honey + H_2O_2	817 ± 122	490 ± 113
(b) With 12·5% (w/v) honey and 22·5 mmol L^{-1} hydrogen peroxide		
H_2O_2	677 ± 56	not detectable
Honey	287 ± 29	304 ± 28
Honey + H_2O_2	597 ± 51	297 ± 21

Linearity of the assay of antibacterial activity

Standard curves obtained with phenol solutions and honey solutions are illustrated in Fig. 1. The relationship between concentration and area of the clear zone is linear over the full range tested. Taking into account that the honey samples in the survey were assayed at 25% (w/v) of their original activity, this means that all of the activities shown in Table 1 are within the linear part of the standard curve.

Verification of removal of hydrogen peroxide

The results of the experiment in which hydrogen peroxide

was added to manuka honey to confirm that catalase was not being inhibited are shown in Table 3. Because the antibacterial activities recorded were so high, the experiment was repeated with all solutions diluted to half of their original concentration to bring the activities into a more sensitive range of the assay. Since the high antibacterial activity due to the hydrogen peroxide added was removed in the presence of catalase, it can be concluded that the antibacterial activity found in the manuka honey with catalase present is due to a substance other than hydrogen peroxide. In addition, the manuka honey itself was active in removing some of the antibacterial activity due to the hydrogen peroxide added.

Discussion

Like the preliminary study of Molan et al (1988), this survey has revealed that there is a very large variation in antibacterial activity between various New Zealand honeys. Such large variations have not been shown before. In the present work the comparison with a concurrently determined linear dose-response for phenol (see Fig. 1A) allowed direct quantitative comparison of the honey samples. The validity of this comparison is confirmed by the linearity of the dose-response plot obtained for honeys with high levels of both types of activity (see Fig. 1B).

The agar well diffusion assay used is of relatively low sensitivity (James et al 1972) because the samples are further diluted as soon as they diffuse out into the agar (Cooper 1963). However, this method did allow a much larger number of samples to be studied than could have been done by more sensitive methods.

Also it is considered to be the more appropriate assay for topical antibacterial agents because it takes into account the diffusibility of the agent into infected areas with little or no blood supply (Heggers et al 1987). However, it may be because of the insensitivity of the method that the antibacterial activity of many honeys in this study were found to be below the level of detection (equivalent to $< 2\%$ w/v phenol, approx., in the original honey). Antibacterial activity may have been detectable in these if a less osmotolerant species than *S. aureus* had been used for the assay. However, the clinical interest still remains in the more potent honeys which range in activity up to a level equivalent to 58% (w/v) phenol in the original honey.

The low levels of antibacterial activity seen in many samples in the present study could be the result of loss of activity during processing and handling of the honey by the apiarists. The glucose oxidase that generates hydrogen peroxide when honey is diluted is denatured by heat (White & Subers 1964a; White 1966; Bogdanov et al 1987) and light (Duisberg & Warnecke 1959; White & Subers 1964b; Dustmann 1979). Revathy & Banerji (1980) concluded that processing of raw honey before marketing reduces its antibacterial activity. However, their study was carried out on only 13 samples. In the present study with 345 samples, the lack of correlation of activity with age and whether or not the honeys had been extracted from the comb suggests that storage and processing were not responsible for loss of activity.

As there is no obvious reduction in antibacterial activity resulting from extraction of honey from the comb, the full antiseptic potential may be obtained in a form that is much more convenient to use. Honey that is commercially extracted from the comb is likely to be much more homogeneous and therefore the bulk will have the same antibacterial activity as a sample taken for assay. As there is seen to be no marked deterioration of antibacterial activity with age, a bulk supply of honey may be purchased when a suitable honey is available, and stored for future use, as long as it is kept cool and protected from light.

In view of the finding that most of the antibacterial activity of honey is due to hydrogen peroxide, a correlation of activity with some floral sources was expected because it is believed that the variation in the levels of hydrogen peroxide results from different amounts of catalase activity being present from different types of plants (Schepartz 1966; Schepartz & Subers 1966; Dustmann 1971). Another explanation for this may lie in the finding that in honeys from some floral sources there are unidentified substances present which make glucose oxidase more sensitive to denaturation by light (Dustmann 1979).

The present survey was in general agreement with the survey of Molan & Russell (1988) in that manuka honey has a marked non-peroxide antibacterial activity. In many samples of manuka honey in the present survey this type of activity was typically equivalent to 15–30% (w/v) phenol, whereas it was very low or non-detectable in other honey types. The demonstration that catalase was active in removing hydrogen peroxide in the presence of a manuka honey with a high activity of this type shows that this antibacterial activity is a real non-peroxide activity and not an artefact.

This non-peroxide antibacterial activity in manuka honey is of particular significance for the therapeutic use of honey in light of the findings of Hodgson (1989). He compared the effectiveness of manuka honey with a ling heather honey that had activity due primarily to hydrogen peroxide, and found that whereas *S. aureus* and *Pseudomonas aeruginosa* were inhibited by both honeys, inhibition of *Citrobacter freundii*, *Escherichia coli*, *Proteus mirabilis* and *Streptococcus faecalis* was not seen with the ling heather honey yet the manuka honey inhibited all of these species. Consequently, it may be of advantage to select manuka honey for therapy of infections.

However, the present survey has shown not all samples said to be manuka honey can be relied upon to provide this antibacterial activity. Likewise there was a very marked variation in the level of hydrogen peroxide activity within a single floral source. This could be the result of differences in handling and storage of the honeys by the apiarists, or could more likely be the result of misidentification of some of the honeys.

More definite identification of honeys by mellisopalynology is expensive and is rarely done. In the present study it could have led to a clearer correlation being seen of high or low activity with particular floral types of honey. Even so, this knowledge would only be useful for the pre-selection of honeys with potential for therapeutic use. Because of possible seasonal variations and differences in handling and storage of honeys it is important that even authentic monofloral honey be assayed for its antibacterial activity if it is to be used as an antibacterial agent in medicine.

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