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# Absence of bacterial resistance to medical-grade manuka honey

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Abstract: Clinical use of honey in the topical treatment of wounds has increased in Europe and North America since licensed wound care products became available in 2004 and 2007 respectively. Honey-resistant bacteria have not been isolated from wounds, but there is a need to investigate whether honey has the potential to select for honey resistance. Two cultures of bacteria from reference collections (Staphylococcus aureus NCTC 10017 and Pseudomonas aeruginosa ATCC 27853) and four cultures isolated from wounds (Escherichia coli, MRSA, Pseudomonas aeruginosa and Staphylococcus epidermidis) were exposed to sub-lethal concentrations of manuka honey in continuous and stepwise training experiments to determine whether susceptibility to honey diminished. Reduced susceptibilities to manuka honey in the test organisms during long-term stepwise resistance training were found, but these changes were not permanent and honey-resistant mutants were not detected. The risk of bacteria acquiring resistance to honey will be low if high concentrations are maintained clinically.

Response to Reviewers: Dear Editor,
Thank you for the reviewers' comments on our paper entitled "Absence of bacterial resistance to medical-grade manuka honey". We were pleased that they liked the design and execution of our study and we understood the reservations that reviewer had about our conclusions. We have made the following changes in response to each specific observation, which we feel strengthen our paper:
Reviewer 1.
We have inserted line numbers into the paper.
In order to rebut the reservations about our conclusions we have recently performed further tests on the four clinical isolates that were collected at the end of the recovery period and stored at -80$^0$C. For each of the thawed cultures, MICs and MBCs were determined in duplicate on three separate occasions. We found that three cultures (P.aeruginosa, S. epidermidis and MRSA) had returned to pre-training levels of susceptibility and that the MIC of E. coli was only 1.4 times higher than at time 0. That information is included as Table 2 (line 370) and described in lines 201 to 209. We respectfully maintain that honey-resistant mutants were not recovered. To support this conclusion as advised we noted (lines 241-243) that antibiotic-resistance training led to MICs that increased by factors of either 32 or 64 (citation 17-Blair et al, 2009), while honey resistance training led to an increase of 1.4. We

also used the EUCAST definition of clinical resistance to show that honey susceptibility which had increased by a factor of 1.4 is unlikely to lead to therapeutic failure when wound care products normally contain at least 80% manuka honey and normally 95%(w/v) (lines 258 to 260).
We have removed the statement "gradually increased towards pre-treatment levels".
We have made clear the proportional changes in MICs (lines 195 to 197 and 208 and 251). We have commented on the need to maintain high concentrations of manuka honey in wounds to avoid the selection of resistant strains (lines 278 to 284).
We have modified the Abstract (lines 42 to 45) to explain that reduced susceptibility was found during long-term training and we have commented on the need to maintain high concentrations of manuka honey during clinical use (lines 45 to 46). In the concluding paragraph we also note that prolonged exposure to antimicrobial agents should be avoided.
We have deleted statements about the development of antibiotic resistance from the Introduction and the Discussion.
We have inserted "preceding days' culture" into line 169, and "day" into line 194.
We have removed the reference to high sugar content, low water content and acidity.
We have changed the captions to Figure 1 and 2.
Reviewer 2.
We were encouraged that our conclusions were considered to be robust.
We have changed MIC against manuka honey to "MICs to manuka honey" throughout the paper.
We have clarified which honey was used in which experiments by changing "preliminary" to "short-term"(line 91) and explained the activity associated with each honey (lines 100 to 103).
We have inserted "preceding days' culture" in line 133.
In order to clarify the number of MIC tests performed and the experimental design we have inserted a brief explanation in lines 210 to 217.
We hope that you will now be able to accept our manuscript for publication,

Yours sincerely,
Rose Cooper

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# Absence of bacterial resistance to medical-grade manuka honey

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**Abstract**

Clinical use of honey in the topical treatment of wounds has increased in Europe and North America since licensed wound care products became available in 2004 and 2007 respectively. Honey-resistant bacteria have not been isolated from wounds, but there is a need to investigate whether honey has the potential to select for honey resistance. Two cultures of bacteria from reference collections (*Staphylococcus aureus* NCTC 10017 and *Pseudomonas aeruginosa* ATCC 27853) and four cultures isolated from wounds (*Escherichia coli*, MRSA, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*) were exposed to sub-lethal concentrations of manuka honey in continuous and stepwise training experiments to determine whether susceptibility to honey diminished. Reduced susceptibilities to manuka honey in the test organisms during long-term stepwise resistance training were found, but these changes were not permanent and honey-resistant mutants were not detected. The risk of bacteria acquiring resistance to honey will be low if high concentrations are maintained clinically.

**Introduction**

For thousands of years wounds were treated topically with relatively poorly characterised preparations of antimicrobial agents derived from animal, vegetable and mineral sources [1]. Apart from honey, many of those substances were too toxic for internal use [2]. During the nineteenth century, the chemical industry yielded a variety of antiseptic compounds that were utilised for preventing and treating wound infections [3], but in recent times cytotoxicity has limited their acceptance [4]. A systematic strategy of

discovering and evaluating promising chemotherapeutic antimicrobial agents was initiated by Paul Ehrlich at the beginning of the twentieth century; it paved the way for the advent of antibiotics, which revolutionised the treatment of infection. These antimicrobial agents with their specific mechanisms of inhibition exhibited selective toxicity against microbial species, rather than patients and were initially effective in controlling infections. Although not universally susceptible to all antibiotics, most microbial species were susceptible to at least one type of antibiotic. To date seventeen classes of antibiotic have been introduced, but the rate of discovery of new antibiotics has slowed within the past twenty years. The therapeutic value of many antibiotics has been curtailed by the acquisition of resistance in species that were once susceptible, as well as the emergence of species with resistance to multiple antibiotics. This is a real concern because species resistant to every known antibiotic have emerged [5], antiseptic resistant strains have been isolated and organisms with both antibiotic and antiseptic resistance determinants have been discovered [6]. The speed of emergence of antimicrobial resistance is unpredictable [7], but whenever a new inhibitory agent is introduced, resistant organisms emerge in due course. With fewer effective antimicrobial agents to manage wound infections, impacts such as increased morbidity, treatment costs and mortalities have ensued [8]. Novel antimicrobial remedies are needed, but there is always the possibility that organisms with resistance to these agents will arise.

Since medical-grade honey was first licensed for treating wounds in Australia in 1999, the clinical use of wound care products containing honey has

extended to Canada, Europe, Hong Kong, New Zealand and United States of America. As with antibiotics, extensive use of honey may well provide a selective pressure for the emergence of honey resistant strains of wound pathogens. This can be tested *in vitro* by either repeated cultivation of bacteria in a sub-lethal concentration of the agent, or by incubation in stepwise increasing concentrations. The aim of this study, therefore, was to determine whether bacteria continuously exposed to low concentrations of manuka honey changed their susceptibility to manuka honey and whether resistant strains were selected.

## Methods

### Organisms tested

In short-term resistance training experiments *Staphylococcus aureus* NCTC 10017 and *Pseudomonas aeruginosa* ATCC 27853 were utilised with a sample of sterile manuka honey provided by the New Zealand Natural Food Company. For the rest of the study clinical isolates of MRSA*, Staphylococcus epidermidis, Escherichia coli* and *Pseudomonas aeruginosa* were tested with a sample of manuka honey that was provided by Comvita UK (Manukacare 18+). This product is available as Medihoney in Canada and the United States. The clinical isolates were cultures of bacteria that had been isolated from patients with chronic wounds attending a local out-patient clinic at University Hospital of Wales, Cardiff. Using a bioassay [9], the antibacterial activity of New Zealand Natural Food company honey sample was found to be equivalent to 19%(w/v) phenol and Manukacare 18+ was equivalent to 18%(w/v) phenol.

### Determination of Minimum Inhibitory Concentrations (MICs)

MICs were determined in 96 well, flat bottomed microtitre plates (Nunc, Roskilde, Denmark) using manuka honey dissolved in either nutrient broth (NB; Oxoid, Basingstoke, UK) or isosensitest broth (ISB; Oxoid, Basingstoke, UK). Honey concentrations varying by 1%(w/v) were used in successive wells and all wells contained 200 µl. Wells were inoculated with 1µl undiluted overnight broth cultures of test organisms (approximately 1 x $10^6$ cfu/ml) and incubated at 37ºC for 24 h. Positive (broth and inoculum) and negative controls (broth and honey) were included. MICs were determined visually and confirmed by measuring absorbance at 400 nm in a microtitre plate reader (Anthos Labtec Instruments).

Short-term resistance training by exposure to a single sub-lethal concentration of manuka honey.

To one 100 ml conical flask with 20 ml NB containing 2.5%(w/v) manuka honey, 40 µl overnight broth culture of *S. aureus* was inoculated and to another 100 ml conical flask with 20 ml NB containing 8%(w/v) manuka honey, 40 µl *P. aeruginosa* was inoculated. Both flasks were incubated at 37ºC in a shaking water bath (100 cycles per min) for 24 h. On 10 successive days similar, freshly prepared flasks were inoculated with 40 µl from each preceding days' culture, respectively. MICs for each test organism were determined on days 0 and 10 and compared by the Mann-Whitney test using Minitab version 14.

Short-term resistance training by exposure to stepwise increasing concentrations of manuka honey.

Cultures were set up as above using an initial concentration of 1%(w/v) manuka honey in NB for *S. aureus* and 5%(w/v) manuka honey in NB for *P.*

*aeruginosa* and incubated at 37ºC with shaking for 24 h. At 24 h intervals on 10 consecutive days, freshly prepared broths with honey concentrations increased by 0.5%(w/v) for *S. aureus* and 1%(w/v) for *P. aeruginosa* were inoculated with 40 µl from the preceding days' culture. Throughout the training period the identity of each culture was confirmed daily by BBL Crystal kits (Becton Dickinson, Basingstoke, UK). For both test organisms MICs of manuka honey were determined before and after the training period.

Stepwise resistance training of clinical isolates by long-term exposure to sub-inhibitory concentrations of manuka honey.

MICs of manuka honey for each of the clinical isolates were determined before continuous exposure to honey. In a 96 well, flat bottomed microtitre plate for each of the 4 test organisms a range of 15 concentrations of manuka honey (Comvita manukacare 18+) in ISB that varied by 1%(w/v) intervals and included concentrations above and below the initial MIC of each test organism was prepared. Each well contained 200 µL ISB and honey and was inoculated on day 0 with 5 µl overnight broth culture, then incubated at 37ºC for 24 h. MIC was determined visually as the lowest concentration of honey to prevent growth. A fresh microtitre plate was prepared every 24 h with a new range of 15 honey concentrations contained in ISB that increased by 1%(w/v) increments and included the last MIC in the middle of the range. With time, increasingly higher concentrations of honey in ISB were required, but the range always included honey concentrations above and below the expected MIC. On each successive day of the experiment, wells in the new microtitre plate were inoculated with 5 µl of each respective test organism taken from the well of the previous microtitre plate containing the highest concentration of

honey with viable cells (i.e. one well below the actual MIC). Also, the purity of each inoculum was checked by dropping 20 µl onto nutrient agar plates and streaking out to achieve isolated colonies after incubation at $37^0$C for 24 hours. The experiment was continued for 28 successive days with each test organism using an appropriate range of honey concentrations above and below the last recorded MIC value. Cultures obtained after 28 days continuous exposure to a sub-lethal concentration of honey were frozen and stored at -$80^0$C for future reference.

Recovery of cultures of clinical isolates following long-term exposure to honey.

Each 'honey-trained' culture obtained after 28 days continuous exposure to manuka honey was inoculated into 20 ml isosensitest broth (ISB) contained within a 100 ml conical flask and incubated in a shaking water bath at $37^o$C for 24 hours. On each successive day, one of four fresh flasks of ISB were inoculated with 100 µl of each culture transferred from the preceding days' culture, respectively until 28 days had elapsed. At each daily transfer MIC against manuka honey was determined. Cultures obtained after the 28 day recovery period were frozen and stored at -80ºC.

Cultural characteristics.

Identity of the test organisms before the start of the long-term training regime, collected after the 28 day training period and after the 28 day recovery period was determined by Crystal kits (Becton Dickinson, Basingstoke, UK) and by RAPD typing.

**Results**

Short-term resistance training with reference cultures.

When *S. aureus* NCTC 10017 was cultured in a sub-lethal concentration of manuka honey for 10 consecutive days, susceptibility to manuka honey did not decrease significantly (Table 1). Conversely the MIC for *P. aeruginosa* ATCC 27853 increased significantly from 15.7 to 19.7%(w/v), indicating that the culture had become less susceptible to manuka honey. However, after 6 subcultures in honey free NB, there was no significant difference in MIC compared to the starting value.

When these two test organisms were successively cultured in stepwise increasing concentrations of honey, viable bacteria were not recovered above their starting MIC values. Hence honey resistance was not observed in either *S. aureus* or *P.aeruginosa*.

Resistance training of clinical isolates by long-term exposure to honey.

The susceptibility of the 4 clinical isolates tested in this study for manuka honey decreased during the 28 day resistance training period (Fig. 1), with the greatest proportional change observed for *E. coli* (2.1) and the least in *P. aeruginosa* (1.4). MICs of MRSA and *S. epidermidis* increased by factors of 1.6 and 1.7, respectively . However during the recovery period, MIC values returned to pre-training values within 9 and 14 days for *S. epidermidis* and *P. aeruginosa* respectively, demonstrating that honey-resistant strains of these bacteria had not been selected (Fig. 2). Neither MRSA nor *E. coli* returned to the pre-training MIC values within the 28 day recovery period. Several months later the cultures frozen at the end of the recovery period were thawed and MICs were determined in duplicate on three separate occasions (Table 2). MBCs were also determined by streaking out samples from microtitre plate

wells showing no growth onto nutrient agar. It was seen that the susceptibility of three of the clinical isolates was similar to those values obtained before resistance training (Fig. 1), but the MIC of manuka honey in *E.coli* remained 1.4 times higher. MBC values were close to MICs indicating that manuka honey was bactericidal for the recovered cultures.

It is perhaps worth noting that by exposing each test organism to a range of honey concentrations simultaneously during the long-term training experiment, MIC values were obtained daily at each transfer step. This meant that only one determination was obtained at each time point. MICs obtained later on stored cultures were done in replicate (Table 2). Unlike antibiotic testing where serial doubling dilutions series are normally utilised, here honey concentrations differing by 1%(w/v) intervals were used to get a more precise estimate of MIC.

Purity plates and biochemical characteristics confirmed that culture identities were maintained throughout the training and recovery periods; RAPD profiles remained constant.

## Discussion

The excessive use and misuse of antibiotics is known to favour the selection of resistant strains and to inhibit sensitive strains. In Norway, for example, where mupirocin was not available, mupirocin-resistant strains of *S. aureus* were not detected [10]. Restricting mupirocin use in an American medical centre caused an immediate reduction in the occurrence of mupirocin-resistant MRSA [11] and an association between exposure to fusidic acid and fusidic acid-resistance in *S.aureus* has been established [12]. Resistance to biocides used for skin and wound care has also been demonstrated [6].In the

first ‘acclimatization’ experiments in 1887, reduced sensitivity to mercuric chloride and boric acid was noted [13]; increased tolerance towards many other antibiotics and antiseptics has since been investigated. Inability to select for resistance to povidone iodine has been reported [13], yet *Pseudomonas stutzeri* was shown to develop stable resistance to each of chlorhexidine diacetate and cetylpyridinium chlorine when exposed to stepwise increases in concentration [14]. Bacterial resistance to silver, which is often used in wound care, is well defined and debated [15 and16]. Furthermore, short-term stepwise resistance training of *P. aeruginosa* ATCC 27853 to each of tetracycline, ciprofloxacin and honey, and of *S.aureus* ATCC 9144 to each of tetracycline, oxacillin and honey demonstrated acquisition of antibiotic resistance but not honey resistance [17]. In that study antibiotic-resistant strains recovered after stepwise resistance training to antibiotics exhibited antibiotic susceptibilities significantly reduced by factors of 32 and 64 [17].

Our study showed that continuous exposure to sub-lethal concentrations of manuka honey for up to 28 days failed to select for honey resistant mutants in any of two reference cultures or four clinical isolates. Reduced susceptibility to manuka honey was observed throughout the long-term training period in each of the clinical isolates. Unlike the large changes in susceptibilities seen in antibiotic resistance training [17], honey MICs increased in *P. aeruginosa*, MRSA, *S. epidermidis* and *E. coli* by factors of 1.4, 1.6, 1.7 and 2.1, respectively (Fig. 1). These changes, however, were not permanent because susceptibility to honey was found to increase during cultivation in honey-free media and during storage at -80ºC. MICs of final cultures (Fig. 2 and Table 2)

were close to pre-training MIC values (Fig. 1), except for *E. coli* which remained 1.4 times higher than at the start of the honey resistance training period. It is not known why susceptibility to honey increased in *E.coli* and MRSA during storage at -80ºC. The European consensus of a clinically resistant organism is one in which the level of antimicrobial susceptibility has a high likelihood of clinical failure [18]. Since the concentrations of medical-grade manuka honey that are contained in contemporary licensed wound care products usually exceed 80% (w/v) and many are 95% (w/v), the honey susceptibilities found in our experiments are not likely to give rise to clinical failure.

Topical application of honey to wounds will invariably result in dilution, depending on the extent of exudation, and concentrations *in vivo* must always exceed those shown to be inhibitory *in vitro* by a significant margin. Providing that the concentration of manuka honey in practice exceeds the highest MBC recorded here by a factor of three, the risk of selecting honey-resistant organisms should be low. Regular dressing changes will help to keep levels of manuka honey high, particularly in highly exuding wounds. Maintaining high levels of antimicrobial agents in the wound environment is always necessary to effectively inhibit viable bacteria before resistant strains emerge. For some biocides cytotoxicity may then become a limitation, but this should not be an issue with manuka honey.

Our findings confirm and extend those of Blair and colleagues [17]. With increasing topical use of honey in wounds, the possibility of selecting for honey-resistant wound pathogens is raised. This study suggests that this

outcome will be remote if high concentrations of manuka honey are maintained in clinical practice. Prolonged exposure to antimicrobial agents, including honey, should always be avoided. Hopefully manuka honey will continue to be used as a whole product and specific active components [19-21] will not be purified and used alone, because this is more likely to promote the development of honey-resistant bacteria.

**Acknowledgements**

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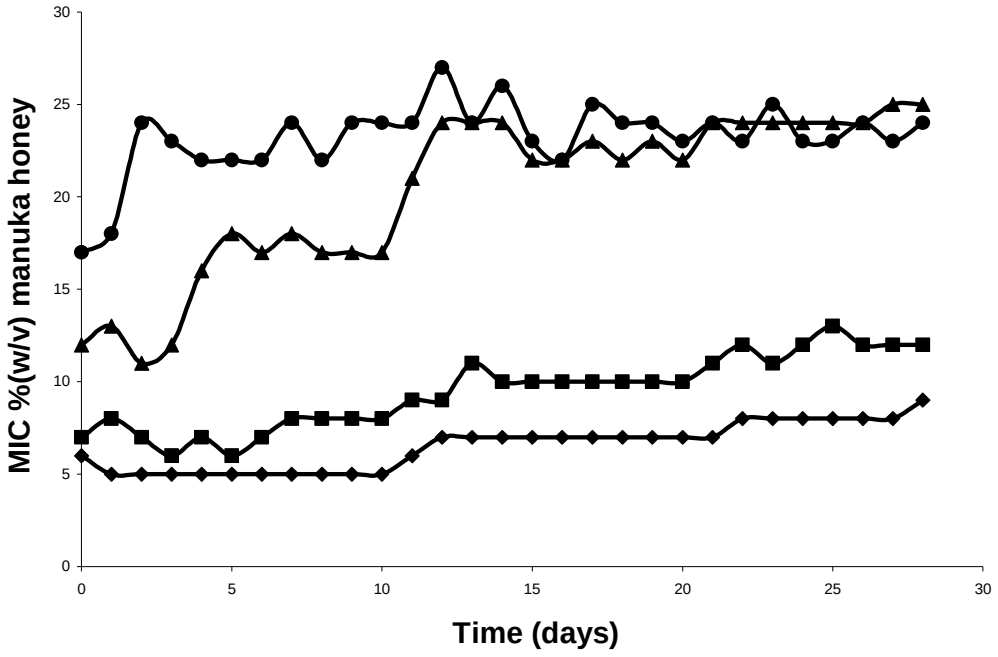
Table 1: Sensitivity of two reference cultures to manuka honey before and after culture in sub-lethal concentrations of honey for 10 successive days.

| | Mean MIC %(w/v) ± sd (n) | |
|---|---|---|
| | *P.aeruginosa* ATCC 27853 | *S.aureus* NCTC 10017 |
| Before training | 15.7 ± 2.0 (8) | 2.8 ± 0.4 (5) |
| After training | 19.7 ± 1.4 (7) | 3.8 ± 0.08 (5) |
| *p* | 0.03 | >0.05 |

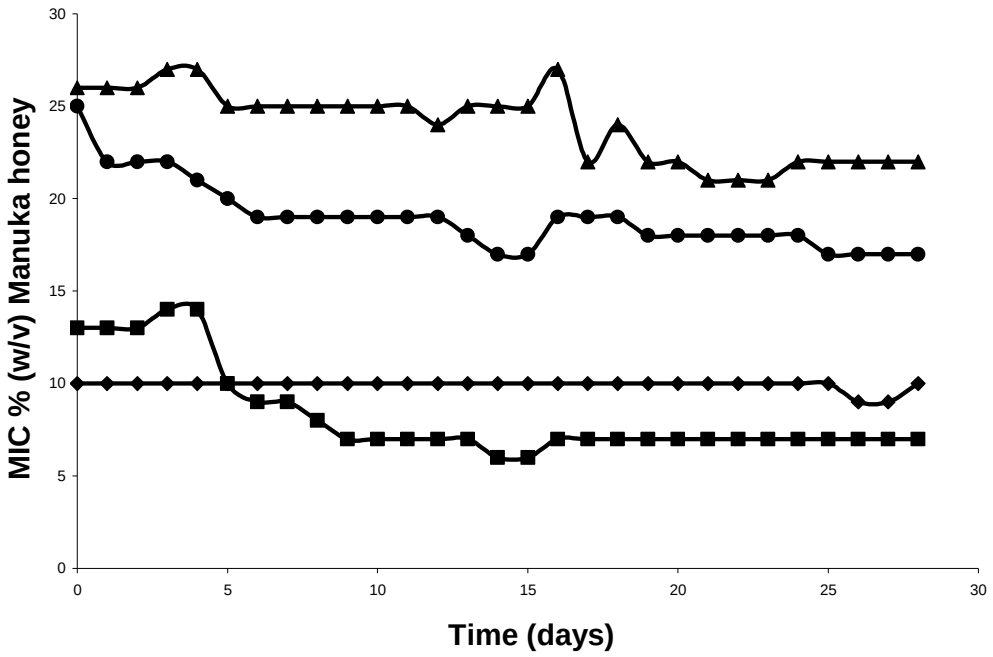
sd = standard deviation; n = number of assays

Fig.1: Sensitivity of four clinical isolates to manuka honey during exposure to sub-lethal concentrations of manuka honey for 28 days




MICs of manuka honey in subcultures of four clinical isolates during a long-term honey resistance training experiment. Cultures were isolated from patients with chronic wounds: *E. coli* (triangles), MRSA (diamonds), *P. aeruginosa* (circles) and *S. epidermidis* (squares). .

Fig. 2: Sensitivity of four clinical isolates to manuka honey during recovery by cultivation in nutrient broth following long-term honey-training




MICs of manuka honey in subcultures of four 'honey-trained' clinical isolates during a 28 day recovery period in honey-free media: *E. coli* (triangles), MRSA (diamonds), *P. aeruginosa* (circles) and *S. epidermidis* (squares)

Table 2: Sensitivity of clinical isolates to manuka honey following long-term exposure, recovery in honey free media and storage at -80ºC.

| Test organism | MIC* | MBC* |
|---|---|---|
| *E. coli* | 16.17 ± 1.47 | 18.0 ± 1.67 |
| MRSA | 5.83 ± 1.6 | 8.5 ± 2.5 |
| *P. aeruginosa* | 15.33 ± 2.25 | 15.67 ± 3.2 |
| *S. epidermidis* | 5.67 ± 1.63 | 8.33 ± 2.56 |

*Mean manuka honey concentration as %(w/v) ± standard deviation (tests were done in duplicate on three separate occasions)