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ORIGINAL RESEARCH ARTICLE

The antimicrobial efficacy of Fijian honeys against clinical isolates from diabetic foot ulcers

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Summary

A diverse range of illnesses has been treated with honey since ancient civilizations. There has been growing interest by health care professionals in wound care products based on New Zealand manuka honey and Australian honey of similar *Leptospermum* spp. In Fiji, local honeys have been used in homes to treat diabetic foot ulcers which have failed to heal by conventional therapeutic methods. This suggests that Fiji honeys may confer antimicrobial activity against the isolates from diabetic foot ulcers and this inference was tested in this study. The antimicrobial activity of 30 natural and two processed honeys was determined using some clinical isolates from diabetic foot ulcers, namely: *Staphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Candida albicans*. The antimicrobial activity of the natural honeys, determined by an agar well diffusion assay and expressed as the concentration of phenol with equivalent activity, was found to be between 4.1 and 14.5% w/v phenol. The mean inhibitory concentrations (MIC) of the honeys determined by an agar incorporation technique, was found to range from 4.8% to more than 9.1% (v/v) honey (9.1% being the highest concentration tested). In comparison, the activities of two processed honeys were between 4.5 - 8.9% phenol equivalence and did not inhibit the clinical isolates from diabetic foot ulcers at the highest concentration of honey tested (9.1%). The results demonstrate that Fijian honeys could be utilized as a herbal remedy for the treatment of diabetic foot ulcers. However, to assess the potential of Fijian honeys on diabetic foot ulcers, there is a need for clinical trials on these wounds.

Keywords: Diabetic foot ulcers, antibacterial activity, mean inhibitory concentration, honey, *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis, Klebsiella pneumonia, Candida albicans.*

Introduction

Although honey is known as a food, there is growing interest in the medicinal properties of honey and its role in the treatment of many different health problems. Honey has many therapeutic properties; however the antibacterial property of honey is one which has evoked great interest among researchers. The antimicrobial property of honey is dependent on several contributing factors. Low water content, high osmolarity (high sugar content) and low pH prevent the growth of many bacteria but do not fully account for the activity of the honeys. The hydrogen peroxide and non-peroxide phytochemical) components of honey contribute to its additional activity (Molan, 1992). About 70% of honey's natural sugars are made up of glucose and fructose. The enzyme glucose oxidase which is introduced to the honey during nectar collection acts on glucose and produces gluconic acid and hydrogen peroxide upon dilution (White *et al.*, 1963). In many honeys, heating at elevated temperatures destroys this hydrogen peroxide activity and it is also lost in the presence of catalase (an enzyme that degrades hydrogen peroxide and is present in wound fluid). Honeys that retain activity in the presence of catalase are said to have non-peroxide antibacterial activity, manuka honey from New Zealand being an example of a

honey with a high level of such activity (Allen, *et al.*, 1991).

There is increased development of resistance to every antibiotic introduced in clinical practice (Payne *et al.,* 2007). Wound infections caused by drug-resistant organisms are common and lead to increased costs, morbidity and mortality. There is an urgent need for the discovery of new antibiotics with novel modes of action. Honey has been utilized as a wound care product and its usage as a wound healing agent is reported in the treatment of venous leg ulcers (Gethin & Cowman, 2008; Jull *et al.*, 2008), burns (Subramanian, 1993), chronic leg ulcers (Oluwatosin *et al.,* 2000), pressure ulcers (Weheida *et al.,* 1991) and exit sites of central venous catheters (Johnson *et al.,* 2005). There has been growing interest by health care professionals in wound-care products based on New Zealand Manuka honey and Australian honey of similar *Leptospermum* spp. (Molan and Betts, 2004). In Fiji, local honeys have been used in homes to treat diabetic foot ulcers (DFU) which have failed to heal by conventional therapeutic methods. This suggests that Fiji honeys may confer antimicrobial activity against the species infecting DFU and this inference was tested in this study.

Infected foot ulcers are a common cause of morbidity in diabetic patients leading to complications like gangrene and amputations. In Fiji, one in every five people has diabetes and the incidence of foot problems and amputations remains high. Full thickness penetration of the dermis of the foot of diabetic people allows colonization of microbial species and initiates a complex series of reactions which leads to transient wound contamination or clinical infection. The initial microbial burden is low in DFU; however lack of proper care promotes microbial density and diversity. Most of the DFU are polymicrobial in nature (Brodsky *et al.*, 1991; Ramani *et al.*, 1991; Criado *et al.*, 1992; Pathare *et al.,* 1998; Chincholikar & Pal, 2002; Viswanathan *et al.*, 2002). It has been reported that the prevalence of *Staphylococcus aureus* amongst Gram-positive bacteria and *Pseudomonas aeruginosa* amongst Gram-negative bacteria were the most dominant of the flora of DFU, followed by *Klebsiella pnuemoniae*, *Escherichia coli*, *Proteus mirabilis* and *Candida albicans*, (Bansal *et al.*, 2008).

There are many published reports on the antimicrobial potency of honey against different microbes and some of these studies have included *S. aureus* (Cooper *et al.*, 1999; Cooper *et al.*, 2002; Miorin *et al.*, 2003; Al Waili, 2004; French *et al.*, 2005; Basualdo *et al.*, 2007; Mercan *et al.*, 2007), *P. aeruginosa* (Cooper *et al.*, 2002; Estrada *et al.,* 2005; Boukraa *et al.*, 2007; Mercan *et al.*, 2007; Mullai & Menon., 2007), *E. coli* (Estrada *et al.*, 2005; Fangio *et al.*, 2007;), *K. pnuemoniae* (Al Waili, 2004; Estrada *et al.*, 2005; Mercan *et al.*, 2007), *P. mirabilis* (Al Waili, 2004; Estrada *et al.*, 2005) and *Candida* species (Estrada *et al.,* 2005; Mercan *et al.*, 2007).

The aim of the present study was to compare the antimicrobial capacity of 30 unprocessed Fijian honeys produced by

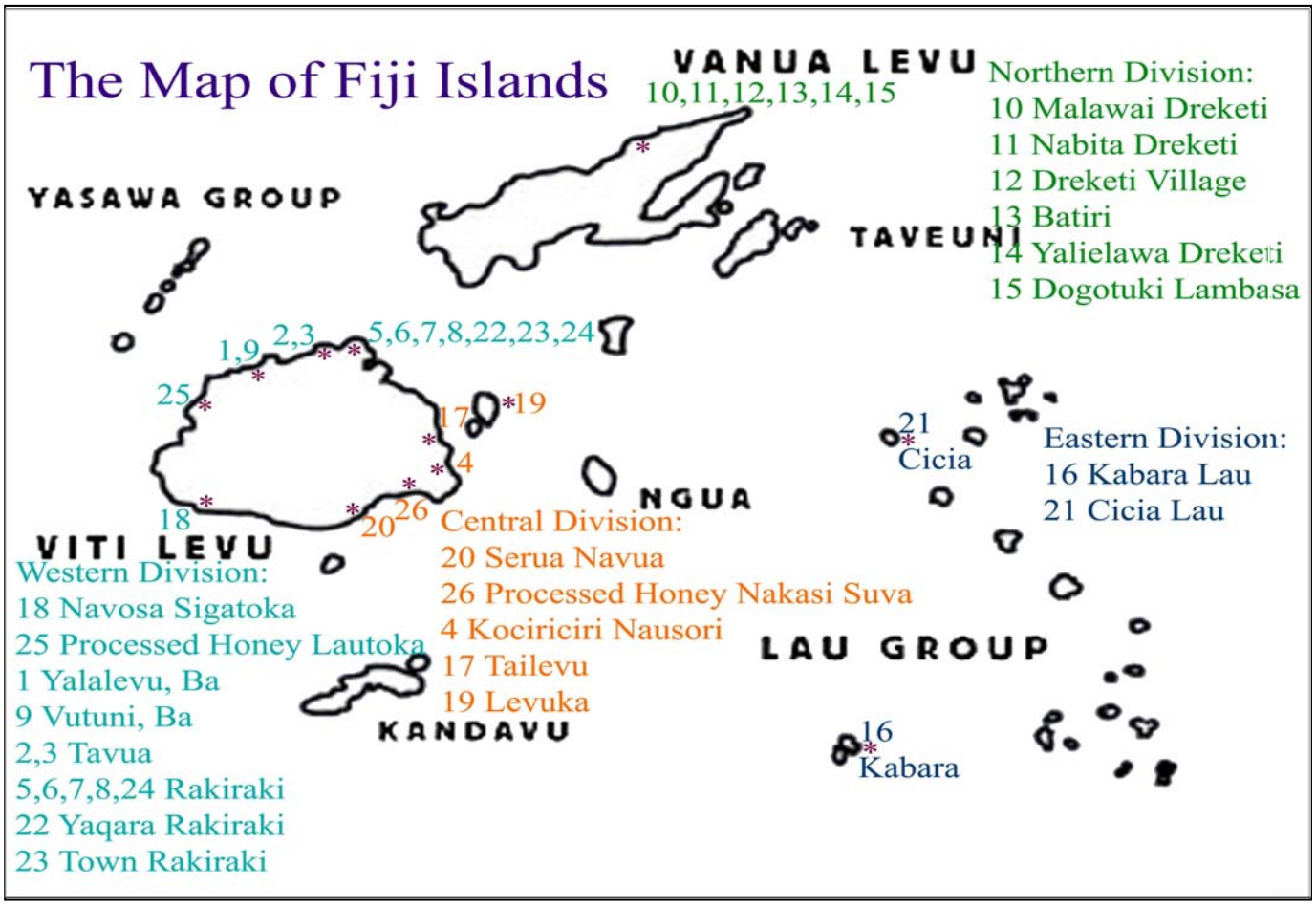

Fig. 1. The map of Fiji Islands with marked locations showing the origin of the honey samples.

honey bees (*Apis mellifera*) and two processed Fijian honeys through measurement of the zones of inhibition in an agar well diffusion assay, with reference to phenol as a standard antiseptic, testing them against the clinical isolates from DFU. The minimum inhibitory concentrations (MIC) of the 32 honeys were also determined against the same isolates.

Materials and methods

Honey Samples

Thirty samples of raw honey were collected by the beekeepers and the National Apicultural Coordinator from different geographical regions in Fiji as shown on the map in Fig. 1. Two samples of processed honey were obtained from the supermarket. The samples represented prominent bee farming industries in Fiji. The floral sources of the honeys were identified by the beekeepers (Table.1). Two samples from different suppliers of the same floral source were analyzed for comparison purposes. All of the samples were stored at room temperature and in the dark for 2 months until they were tested.

Antimicrobial Activity

The procedure used for evaluating antimicrobial activity was described by Allen and colleagues but without the inclusion of catalase (Allen *et al.,* 1991). Whereas that method used only *S. aureus,* in this study six clinical isolates were used. The test organisms: *S. aureus*, *E. coli*, *P. aeruginosa*, *P. mirabilis*, *K. pneumoniae* and *C. albicans* were isolated from DFU of patients from CWM Hospital. The information on their identities and antibiotic sensitivities was provided by the Head Microbiologist. The organisms were stored on plates at 4^0C. Cultures were also stored in glycerol at -80^0C and sub cultured when required.

Inoculum preparation: Each of the clinical isolates were inoculated into 10 ml of Trypticase Soy broth and grown at 37^0C for 18 hours. A further working culture was prepared by inoculating 200 µL from the overnight culture into 10 mL of Trypticase Soy broth and adjusted to 0.5 McFarland Standard (equivalent to 10^6 Colony Forming Unit (CFU)/mL), and diluted further to set an inoculum density of $1x10^4$ CFU/mL which was used for the test.

Plate preparation: To prepare the assay plates 20 mL of nutrient agar (Merck) for each plate was sterilized and seeded with 100 µL inoculum adjusted to $1x10^4$CFU/mL.

Preparation of honey samples: A 40% (v/v) stock was prepared which was further diluted to obtain other concentrations of (20%, 10%, 5%, and 2.5%) v/v respectively.

Preparation of phenol standards: Standards 1% - 19% were prepared from a 20% w/v solution of phenol (BDH analar grade reagent) in water.

Sample and standard application: Wells with similar spacing were cut into the agar. Each sample and standard was tested in triplicate by adding to each well.

Plate incubation: After application of samples and standards, the plates were incubated at 37^0C for 24 hrs compared to a control plate that had no honey. The zones of inhibition were observed for various concentrations of honey and phenol standards after the incubation period.

Calculation of the antibacterial activity of honey

The mean diameter of the clear zone around each phenol standard was measured and squared. From the graph of % phenol against the square of the mean diameter of the clear zone, the activity of each diluted honey sample was calculated. The activity was expressed as the equivalent phenol concentration (% w/v).

Minimum Inhibitory Concentration (MIC)

The MIC was determined as described by Mulu, Belay & Fetene (2004). This paper tested MIC of honey against all the clinical isolates except for *C. albicans*.

Mueller Hinton Agar (Merck) was sterilized and held in a water bath ($45\text{-}50^40^0$C). Honeys were briefly heated to 40^0C to reduce viscosity and known volumes of honey were measured into 20 mL of molten media to give final concentrations of 2.4%, 3.6%, 4.8%, 5.9%, 7.0%, and 9.1% (v/v). The plate was poured, allowed to set and seeded with bacteria adjusted at $1x10^4$ CFU/ mL before incubation at 37^0C for 24h. The plates were observed for growth and the results of the MIC were reported as the lowest concentration of honey that completely inhibited visible growth. For the plates where growth was visible, the results were recorded as >9.1% v/v (highest concentration of honey tested). Plans were to select honeys with lower MIC values and include those in clinical trials, therefore the maximum concentration of honey used was 9.1%.

Table 1. Antimicrobial activities of Fijian Honeys against clinical isolates from DFU, measured by an agar well diffusion assay. The results are shown the concentration of a solution of phenol with the equivalent microbial activity. Values shown are the mean (± SD) of three determinations. ND = Not Detectable, (less than 4.1% (w/v) phenol equivalent).

Total Antimicrobial activity as % (w/v) phenol equivalent							
Sample	**Floral Source**	***S.aureus***	***E.coli***	***P.aeruginosa***	***K.pneumoniae***	***P.mirabilis***	***C.albicans***
1	Mango	4.9	6.4	ND	5.7	ND	ND
2	Coconut	6.7	5.2	ND	5.7	5.2	ND
3	Lantana	12.7	6.8	11.9	13.8	ND	5.8
4	Henna Plant	5.1	ND	ND	7.2	5.8	ND
5	Mile a minute	ND	ND	ND	6.1	7.6	ND
6	Mile a minute	ND	ND	ND	7.3	ND	ND
7	Pawpaw	9.1	ND	ND	11.3	ND	ND
8	Balsam	ND	7.2	ND	5.7	4.8	ND
9	Bush Thumbergia	12.4	1.3	6.9	5.7	5.6	4.9
10	Dilo	8.0	ND	ND	4.8	ND	ND
11	Mango	4.9	5.2	11.3	ND	ND	ND
12	Vaivai	5.2	10.3	5.7	5.7	ND	ND
13	Dilo	7.8	ND	7.6	5.2	ND	ND
14	Balsam	5.6	4.7	ND	5.2	ND	4.2
15	Mint Weed	13.5	ND	4.5	13.4	ND	4.3
16	Mint Weed	14.5	11.0	4.8	5.7	6.8	6.8
17	Bush Thumbergia	10.3	ND	ND	11.4	8.9	8.5
18	Lantana	12.1	7.8	10.2	10.1	10.3	6.4
19	Pawpaw	9.4	5.1	4.9	9.3	9.5	7.8
20	Passion Fruit	4.9	13.6	5.4	13.4	8.2	8.7
21	Vaivai	5.4	10.1	4.1	12.6	14.1	7.2
22	Marigold	7.3	11.8	4.9	6.1	7.6	9.7
23	Orange	9.9	ND	4.5	10.0	9.9	9.2
24	Coconut	6.5	ND	8.1	11.1	5.9	ND
25	Hibiscus	8.2	12.9	ND	7.8	6.1	5.5
26	Orange	9.4	11.5	7.4	7.2	ND	4.9
27	Henna Plant	5.0	ND	5.3	5.7	5.6	6.1
28	Hibiscus	8.6	ND	ND	ND	ND	4.4
29	Marigold	9.5	ND	4.9	5.4	4.7	ND
30	Passion Fruit	4.8	ND	6.2	4.8	ND	4.7
31	Processed A	5.3	6.5	4.7	4.9	6.1	ND
32	Processed B	4.5	5.1	ND	5.3	8.9	5.9
Mean	-	7.6	5.2	4.8	7.3	4.6	4.4
SD	-	0.3	0.1	0.5	0.4	0.8	0.6

Table 2. The Mean Inhibitory Concentrations (MIC) of Fijian Honeys as determined by the agar incorporation technique. The results are shown as the minimum concentration of each honey that gave complete inhibition of each isolate from DFU. The values > 9.1% indicate that there was no inhibition at the highest concentration tested.

Mean Inhibitory Concentration (% v/v)							
Sample	**Floral Source**	***S.aureus***	***E.coli***	***P.aeruginosa***	***K.pneumoniae***	***P.mirabilis***	***C.albicans***
1	Mango	>9.1	>9.1	>9.1	>9.1	>9.1	>9.1
2	Coconut	>9.1	>9.1	>9.1	>9.1	>9.1	>9.1
3	Lantana	9.1	>9.1	4.8	7.0	>9.1	>9.1
4	Henna Plant	>9.1	>9.1	>9.1	>9.1	>9.1	>9.1
5	Mile a minute	>9.1	>9.1	>9.1	>9.1	>9.1	>9.1
6	Mile a minute	>9.1	>9.1	>9.1	>9.1	>9.1	>9.1
7	Pawpaw	9.1	>9.1	>9.1	9.1	>9.1	>9.1
8	Balsam	>9.1	>9.1	>9.1	>9.1	>9.1	>9.1
9	Bush Thumbergia	9.1	>9.1	>9.1	>9.1	>9.1	>9.1
10	Dilo	>9.1	>9.1	>9.1	>9.1	>9.1	>9.1
11	Mango	>9.1	>9.1	5.9	>9.1	>9.1	>9.1
12	Vaivai	>9.1	>9.1	>9.1	>9.1	>9.1	>9.1
13	Dilo	>9.1	>9.1	>9.1	>9.1	>9.1	>9.1
14	Balsam	>9.1	>9.1	>9.1	>9.1	>9.1	>9.1
15	Mint Weed	9.1	>9.1	>9.1	7.0	>9.1	>9.1
16	Mint Weed	7.0	9.1	>9.1	>9.1	>9.1	>9.1
17	Bush Thumbergia	9.1	>9.1	>9.1	9.1	>9.1	>9.1
18	Lantana	9.1	>9.1	7.0	9.1	9.1	>9.1
19	Pawpaw	9.1	>9.1	>9.1	9.1	9.1	>9.1
20	Passion Fruit	>9.1	9.1	>9.1	7.0	>9.1	>9.1
21	Vaivai	>9.1	9.1	>9.1	9.1	7.0	>9.1
22	Marigold	>9.1	9.1	>9.1	>9.1	>9.1	>9.1
23	Orange	9.1	>9.1	>9.1	9.1	9.1	>9.1
24	Coconut	>9.1	>9.1	>9.1	9.1	>9.1	>9.1
25	Hibiscus	>9.1	9.1	>9.1	>9.1	>9.1	>9.1
26	Orange	9.1	9.1	>9.1	>9.1	>9.1	>9.1
27	Henna Plant	>9.1	>9.1	>9.1	>9.1	>9.1	>9.1
28	Hibiscus	>9.1	>9.1	>9.1	>9.1	>9.1	>9.1
29	Marigold	9.1	>9.1	>9.1	>9.1	>9.1	>9.1
30	Passion Fruit	>9.1	>9.1	>9.1	>9.1	>9.1	>9.1
31	Processed A	>9.1	>9.1	>9.1	>9.1	>9.1	>9.1
32	Processed B	>9.1	>9.1	>9.1	>9.1	>9.1	>9.1
SD	-	0.5	0.3	0.4	0.6	0.3	0.6

Results

Antimicrobial Activity

The antimicrobial activity of the 32 Fijian honeys (natural and processed) against the clinical isolates of DFU is shown in Table 1, expressed as the concentration of phenol with activity equivalent to that of the undiluted honey (Allen *et al.*, (1991). The lowest concentration of phenol standard which was able to produce detectable antimicrobial activity in this assay was 4.1% (w/v). The absence of zones of inhibition indicated that the activity could not be detected (ND) for these honeys as it was lower than 4.1% (w/v) phenol equivalent.

Minimum Inhibitory Concentration

The MIC's of the 32 Fijian honeys tested against the clinical isolates from DFU showed marked variation between microorganisms (Table 2). The 2 processed honeys did not inhibit any of the microbes at 9.1% v/v, which is the highest concentration used for this assay. None of the honeys were able to inhibit *C.albicans* at 9.1% (v/v). The MIC values of the 30 natural honeys against the six microbes were between 4.8 and higher than 9.1% (v/v).

Discussion

In this study we have demonstrated that all the 32 Fijian honeys display antimicrobial activity against clinical isolates of DFU. More than half of the honeys demonstrated activity equivalent to that of phenol between 4% and 12% (w/v) (Table 1). The MIC values indicate that most of the 30 natural honeys (Table 2) failed to inhibit the DFU clinical isolates tested here, (19 honeys against *S. aureus,* 24 honeys against *E. coli,* 27 honeys against *P. aeruginosa,* 20 honeys against *K. pneumoniae,* 26 honeys against *P. mirabilis,* and all the 30 honeys against *C. albicans)*. The two processed honeys did not inhibit any of the microbes from DFU at the maximum concentration tested (9.1% v/v). These values for the antibacterial activity of Fijian honeys tested against *S. aureus* can be compared with those obtained by Allen *et al.* (1991) from testing 345 samples of New Zealand honeys by the same method. They found the median MIC value to be equivalent to 13.6% (w/v) phenol. The mean value from the testing against *S. aureus* in the present study, equivalent to 7.6% phenol, indicates that Fijian honeys are not as potently antibacterial as New Zealand honeys. This is also indicated by the finding that the most active of the 30 samples of unprocessed Fijian honey had an activity equivalent to only 14.5% (w/v) phenol, little more than the median activity for the New Zealand honeys. However, a larger number of samples would have to be tested before this could be concluded with certainty.

The MIC values found in the present study for Fijian honeys can be compared with those found by other authors for other honeys tested against *S. aureus* and *P. aeruginosa*, the dominant microbes in DFU (Bansal *et al.*, 2008). Cooper *et al.* (1999) used a manuka honey (non-peroxide activity) and a pasture honey (hydrogen peroxide activity), each with a near median level of activity for that type of activity, testing it against 58 strains of coagulase-positive *S. aureus*, and found the MIC to range from 2% to 3% (v/v) for the manuka honey and 3% to 4% (v/v) for the pasture honey. Similar honeys were also tested against 20 clinical isolates of *Pseudomonas* spp from infected wounds (Cooper, Molan and Harding, 2002) and against 17 strains of *P. aeruginosa* isolated from infected burns. (Cooper, Halas and Molan, 2002). Mean values for the MIC for both types of honey were 7% (v/v) for both studies. In comparison, the MIC values found in the present study in testing against *S. aureus* ranged from 7.0- >9.1% (v/v) and against *P. aeruginosa* were in the range 4.8- >9.1% v/v. The comparison again indicates that the Fijian honeys do not have a high level of antibacterial activity. However, the MIC values found indicate that it would be possible to select Fijian honeys that would completely inhibit the growth of bacteria even if the honey became diluted ten-fold when in use on infected ulcers.

A clinical trial would be needed using Fijian honey to discover whether the locally produced honey is suitable for resolving the problem of infected DFU in Fiji. There is no evidence from randomized controlled trials to evaluate the effectiveness of honey in the eradication of infection or healing of DFU. However, it was reported by Sahel (2004) that honey treatment of DFU gave better control of infection and reduced mean healing time and amputation rates, much better than that achieved with Povidone iodine / hydrogen peroxide. Gethin and Cowman (2008) recruited 180 patients with venous leg ulcers. The patients represented some with diabetic ulcers. It was observed that manuka honey increased incidence of healing, gave effective desloughing and a lower incidence of infection than the control. In a non-comparative study of the use of honey as a wound dressing, Efem (1988) treated wounds of various etiology, including diabetic ulcers, with honey therapy. The results of this study indicated that honey promoted granulation and epithelization of the wounds, reduced odour and had a dehydrating effect on the wounds. Case studies have also shown that honey has rapid healing effects on DFU (Eddy *et al.,* 2008).

The faster healing of DFU dressed with honey also may result from the anti-inflammatory and de-sloughing effects of honey. There are numerous reports of these effects being observed clinically in a variety of types of wound as a reduction in odema and pain (Molan, 1999) and improvement in healing outcomes (Gethin & Cowman, 2008). These beneficial effects of honey, and its lack of adverse effects on wounds (Molan, 1999), considered along with the findings from the present study, indicate that Fijian honeys can be regarded as a possible treatment option for DFU**.** The authors are currently recruiting participants for a randomized controlled trial to assess the efficacy of honey against DFU.

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