Potential pathway of anti-inflammatory effect by New Zealand honeys

Abstract: The role of honey in wound healing continues to attract worldwide attention. This study examines the anti-inflammatory effect of four honeys on wound healing, to gauge its efficacy as a treatment option. Isolated phenolics and crude extracts from manuka (Leptospermum scoparium), kanuka (Kunzea ericoides), clover (Trifolium spp.), and a manuka/kanuka blend of honeys were examined. Anti-inflammatory assays were conducted in HEK-Blue™-2, HEK-Blue™-4, and nucleotide oligomerization domain (NOD)2-Wild Type (NOD2-WT) cell lines, to assess the extent to which honey treatment impacts on the inflammatory response and whether the effect was pathway-specific. Kanuka honey, and to a lesser extent manuka honey, produced a powerful anti-inflammatory effect related to their phenolic content. The effect was observed in HEK-Blue™-2 cells using the synthetic tripalmitoylated lipopeptide Pam3CSK4 ligand, suggesting that honey acts specifically through the toll-like receptor (TLR)1/TLR2 signaling pathway. The manuka/kanuka blend and clover honeys had no significant anti-inflammatory effect in any cell line. The research found that kanuka and manuka honeys have an important role in modulating the inflammatory response associated with wound healing, through a pathway-specific effect. The phenolic content of honey correlates with its effectiveness, although the specific compounds involved remain to be determined.

Keywords: Leptospermum scoparium, manuka, Kunzea ericoides, kanuka, Trifolium, clover, inflammatory response, phenolics, wound healing

Introduction

Honey has long been used as a natural treatment in wound repair and has increased in popularity with antibiotic resistance increase.\(^1^-^4\) Honey is effectual and cost-effective, and its healing properties are well documented.\(^5^-^6\) Honey can decrease healing time via a dual effect on the inflammatory response. It suppresses the production and proliferation of inflammatory cells at the wound site, to prevent a prolonged inflammatory response, and it stimulates proinflammatory cytokine production, enabling normal healing to occur.\(^7^-^11\) Wound healing is a tissue remodeling process, comprising a systematic progression of events involving multiple interactions that are regulated by biologically active cytokines, growth factors, and proteases.\(^12^-15\) The transcription factor nuclear factor-kappa beta (NF-KB) is an important marker of inflammation.\(^16\) It enhances proinflammatory activity, thereby contributing to an amplified inflammatory response, and activates genes encoding for proinflammatory cytokines – interleukin (IL)-6, IL-8, and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)).\(^8,17\) These proinflammatory cytokines stimulate nitric oxide production, an important mediator of inflammation. Nitric oxide production
and NF-KB activation are inhibited by the flavonoids present in honey.\textsuperscript{18} When healing is impaired, chronic wounds develop, characterized by proinflammatory cytokines and reactive oxygen species.\textsuperscript{8,19–22}

Honey’s effectiveness in wound care has been hypothesized to be largely due to its anti-inflammatory action.\textsuperscript{8} The specific compounds and the mechanisms involved are largely undetermined.\textsuperscript{23} However, it has been suggested that specific polyphenols, the flavonoids, and caffeic acid phenethyl ester, are important factors.\textsuperscript{24–26} The antioxidants found in honey are considered to be important determinants of its anti-inflammatory activity.\textsuperscript{2} An elevated inflammatory response results from hydrogen peroxide oxygen radicals present at the wound site, triggering NF-KB to enhance the inflammatory response.\textsuperscript{2,8} New Zealand honeys have been suggested to display significant anti-inflammatory activity, particularly kanuka and manuka honeys, by reducing neutrophil superoxide production.\textsuperscript{23} Manuka honey has been shown to specifically decrease the inflammatory response associated with ulcerative colitis, an inflammatory bowel disease characterized by an overexpression of inflammatory cells, possibly by increasing antioxidant activity.\textsuperscript{27–29}

Several studies have investigated the anti-inflammatory activity of New Zealand honeys in treating topical wounds. This study further investigated the anti-inflammatory properties of manuka and kanuka honey, and demonstrates the effectiveness of these New Zealand honeys in reducing the inflammatory response associated with healing, independent of its known topical effect. Furthermore, specific signaling pathways, through which these honeys are effective, were observed.

**Materials and methods**

**Preparation of honey extracts**

Four New Zealand honeys were used – manuka, kanuka, a manuka/kanuka blend (all supplied by Conviva New Zealand Ltd, Te Puke, NZ), and clover honey (supplied by Airborne Honey Ltd, Leeston, NZ). Honey extracts were fractionated by Dr Peter Brooks (University of the Sunshine Coast, Sippy Downs, QLD, Australia) to isolate their phenolic compounds. The percentages of phenolics were determined (manuka 59\%, kanuka 39\%, the manuka/kanuka blend 59\%, and clover 40\%). They were stored at 4°C. Both phenolic and crude extracts were tested. Figure 1 depicts the process flow for the preparation of the following materials and the accompanying observation steps.

**Reagents**

HEK-Blue\textsuperscript{TM}-2, HEK-Blue\textsuperscript{TM}-4, and nucleotide oligomerization domain (NOD)2-Wild Type (NOD2-WT) embryonic kidney cell lines were selected due to their accessibility and relatively high expression of matrix metalloproteinase (MMP)-1, -2, and -9. These were obtained from the Auckland Cancer Society Research Centre (Auckland, NZ). Dulbecco’s Modified Eagle’s Medium (DMEM), an antibiotic mixture (penicillin, streptomycin, L-glutamine), and fetal calf serum (FCS) were obtained from Life Technologies Corp (Carlsbad, CA, USA). Phorbol 12-myristate 13-acetate (PMA) and ibuprofen were purchased from Sigma-Aldrich Corp (St Louis, MO, USA). Lipopolysaccharide (LPS), Pam3CysSerLys4 (Pam3CSK4), and FSL-1 (Pam2CGDPKHPKSF, a synthetic diacylated lipopeptide), Muramyl dipeptide (MDP), Blisticidin, ZeociTM, HEK-BlueTM Selection, and QUANTI-BlueTM were from InvivoGen (San Diego, CA, USA). Cell Proliferation Reagent WST-1 was obtained from Roche Applied Science (Penzberg, Germany).

**Cell culture**

The HEK-Blue\textsuperscript{TM}-2 and HEK-Blue\textsuperscript{TM}-4 cells were maintained in DMEM high glucose supplemented with 10% FCS, 1% penicillin/streptomycin/glutamine, and HEK-Blue\textsuperscript{TM} Selection. The NOD2 WT cells were maintained in DMEM supplemented with 10% FCS, 1% penicillin/streptomycin, 0.06% Blisticidin, and 0.1% ZeociTM.

**Anti-inflammatory assay**

The HEK-Blue\textsuperscript{TM}-2 and HEK-Blue\textsuperscript{TM}-4 cells were seeded at 1 × 10\(^5\) cells/mL and the NOD2 WT cells at 5 × 10\(^5\) cells/mL, into 96-well plates, and incubated for 24 hours at 37°C, 5% CO\(_2\). The honey extracts, at a suitable dose range (5.3%–14.3%) as determined by preliminary half maximal inhibitory concentration (IC\(_{50}\)) data, and the controls (75 mM Ibuprofen, 1 mg/mL PMA, and a solvent control) were added to the plate and further incubated for 24 hours. The appropriate ligand for each cell line was added (3.125 μg/mL LPS, 10 ng/mL Pam3CSK4, 10 ng/mL FSL-1, and 22.73 μg/mL MDP), and all were incubated for 24 hours. The secreted embryonic alkaline phosphatase (SEAP) production was measured, using QUANTI-Blue, every 10 minutes for 50 minutes. Cell viability was determined by WST-1 after 60-, 90-, and 120-minute incubations. The results were normalized for cell viability and against a solvent control.
Statistical analysis

A generalized linear model was fitted to test the anti-inflammatory effect of eight honey extracts at five different concentrations (% honey) compared with untreated cells, in the presence of the specific ligand corresponding to the cell line. The means with standard error and estimates with 95% confidence interval along with \( P \)-value were calculated.

Table 1 provides the anti-inflammatory effect of the honey extracts at five different concentrations (% honey) compared with untreated cells, in the HEK-Blue™-4 cell line using the LPS ligand.

Table 2 provides the anti-inflammatory effect of the honey extracts at five different concentrations compared with untreated cells, in the HEK-Blue™-2 cell line using the FSL-1 ligand.

Table 3 provides the anti-inflammatory effect of the honey extracts at five different concentrations compared with untreated cells, in the HEK-Blue™-2 cell line using the Pam3CSK4 ligand.

Table 4 provides the anti-inflammatory effect of the honey extracts at five different concentrations compared with untreated cells, in the NOD2-WT cell line using the MDP ligand. Figure 1 provides the materials, preparation, and observation process flow. Figure 2 provides the anti-inflammatory effect of the four honey extracts when differentiated by honey phenolic and crude honey extract. The units were expressed as SEAP relative to the control (%). The higher the value, the higher was the level of SEAP, resulting in a lower anti-inflammatory effect. A \( P \)-value of less than 0.05 indicated that a significant anti-inflammatory effect was observed in those cells treated with honey compared with those cells that were not. All analyses were carried out using SAS® 9.3 (SAS Institute, Cary, NC, USA).

Results

Effect of honey extracts on inflammation in HEK-Blue™-4 cells

The honey extracts were analyzed in the HEK-Blue™-4 cell line in the presence of LPS. Table 1 details their
Table 1 The anti-inflammatory effect of honey extracts at five different concentrations (% honey) compared with untreated cells, in the HEK-Blue™-4 cell line using the LPS ligand

| Honey treatment                      | Concentration (%) | Mean (SE)     | Estimate (95% CI)             | P-value    |
|--------------------------------------|-------------------|---------------|------------------------------|------------|
| Phenolic manuka                      | 0.0               | 1.04 (0.01)   | 0.0                          |            |
|                                      | 5.3               | 1.00 (0.05)   | -0.039 (-0.111 to 0.034)     | 0.2713     |
|                                      | 7.1               | 0.94 (0.01)   | -0.096 (-0.168 to -0.024)    | 0.0131     |
|                                      | 10.7              | 0.99 (0.04)   | -0.050 (-0.123 to 0.022)     | 0.1579     |
|                                      | 12.5              | 0.98 (0.03)   | -0.053 (-0.126 to 0.019)     | 0.1371     |
|                                      | 14.3              | 0.91 (0.06)   | -0.125 (-0.198 to -0.053)    | 0.0024     |
| Phenolic kanuka                      | 0.0               | 1.06 (0.03)   | 0.0                          |            |
|                                      | 5.3               | 1.03 (0.02)   | -0.036 (-0.113 to 0.041)     | 0.3310     |
|                                      | 7.1               | 0.95 (0.02)   | -0.112 (-0.189 to -0.035)    | 0.0077     |
|                                      | 10.7              | 0.97 (0.02)   | -0.097 (-0.174 to -0.020)    | 0.0173     |
|                                      | 12.5              | 0.98 (0.07)   | -0.085 (-0.162 to -0.007)    | 0.0340     |
|                                      | 14.3              | 0.98 (0.05)   | -0.087 (-0.164 to -0.010)    | 0.0300     |
| Phenolic manuka/kanuka               | 0.0               | 0.98 (0.04)   | 0.0                          |            |
|                                      | 5.3               | 1.01 (0.05)   | 0.029 (-0.042 to 0.099)       | 0.3982     |
|                                      | 7.1               | 0.97 (0.02)   | -0.008 (-0.078 to 0.063)     | 0.8218     |
|                                      | 10.7              | 0.99 (0.05)   | 0.010 (-0.060 to 0.080)       | 0.7716     |
|                                      | 12.5              | 0.98 (0.05)   | 0.0 (-0.070 to 0.071)         | 0.9889     |
|                                      | 14.3              | 0.97 (0.05)   | -0.003 (-0.073 to 0.068)      | 0.9402     |
| Phenolic clover                      | 0.0               | 0.97 (0.06)   | 0.0                          |            |
|                                      | 5.3               | 1.02 (0.02)   | 0.050 (-0.037 to 0.136)       | 0.2393     |
|                                      | 7.1               | 1.02 (0.03)   | 0.054 (-0.032 to 0.141)       | 0.1983     |
|                                      | 10.7              | 1.05 (0.05)   | 0.078 (-0.008 to 0.165)       | 0.0731     |
|                                      | 12.5              | 1.06 (0.04)   | 0.093 (0.007 to 0.180)        | 0.0360     |
|                                      | 14.3              | 1.04 (0.06)   | 0.071 (-0.015 to 0.158)       | 0.0988     |
| Crude manuka                         | 0.0               | 1.00 (0.06)   | 0.0                          |            |
|                                      | 5.3               | 0.99 (0.04)   | -0.009 (-0.084 to 0.065)      | 0.7934     |
|                                      | 7.1               | 1.01 (0.04)   | 0.003 (-0.072 to 0.077)       | 0.9411     |
|                                      | 10.7              | 1.00 (0.02)   | -0.001 (-0.075 to 0.074)      | 0.9853     |
|                                      | 12.5              | 1.03 (0.04)   | 0.024 (-0.051 to 0.098)       | 0.5072     |
|                                      | 14.3              | 0.98 (0.05)   | -0.025 (-0.099 to 0.050)      | 0.4916     |
| Crude kanuka                         | 0.0               | 0.95 (0.07)   | 0.0                          |            |
|                                      | 5.3               | 1.00 (0.01)   | 0.057 (-0.011 to 0.125)       | 0.0956     |
|                                      | 7.1               | 1.01 (0.03)   | 0.063 (-0.006 to 0.131)       | 0.0691     |
|                                      | 10.7              | 1.02 (0.04)   | 0.074 (0.005 to 0.142)        | 0.0362     |
|                                      | 12.5              | 1.03 (0.03)   | 0.084 (0.016 to 0.152)        | 0.0193     |
|                                      | 14.3              | 1.02 (0.04)   | 0.069 (0.001 to 0.137)        | 0.0485     |
| Crude manuka/kanuka                  | 0.0               | 0.98 (0.05)   | 0.0                          |            |
|                                      | 5.3               | 0.99 (0.04)   | 0.011 (-0.055 to 0.078)       | 0.7170     |
|                                      | 7.1               | 0.96 (0.04)   | -0.019 (-0.085 to 0.047)      | 0.5478     |
|                                      | 10.7              | 0.96 (0.02)   | -0.011 (-0.077 to 0.055)      | 0.7235     |
|                                      | 12.5              | 1.01 (0.02)   | 0.039 (-0.027 to 0.105)       | 0.2302     |
|                                      | 14.3              | 1.00 (0.01)   | 0.026 (-0.040 to 0.092)       | 0.4069     |
| Crude clover                         | 0.0               | 1.00 (0.05)   | 0.0                          |            |
|                                      | 5.3               | 1.00 (0.01)   | -0.006 (-0.056 to 0.044)      | 0.7903     |
|                                      | 7.1               | 1.03 (0.01)   | 0.027 (-0.023 to 0.077)       | 0.2631     |
|                                      | 10.7              | 1.04 (0.03)   | 0.038 (-0.013 to 0.088)       | 0.1299     |
|                                      | 12.5              | 1.05 (0.03)   | 0.042 (-0.008 to 0.092)       | 0.0919     |
|                                      | 14.3              | 1.03 (0.01)   | 0.030 (-0.020 to 0.080)       | 0.2211     |

Abbreviations: CI, confidence interval; LPS, lipopolysaccharide; SE, standard error.

Anti-inflammatory effect. No substantive effect was observed with treatment by any of the extracts. Following an increase in honey concentration, a noticeable anti-inflammatory effect was observed with the higher concentrations of the phenolic and crude kanuka extracts. The manuka phenolic extract produced a significant effect at the 7.1% (P=0.0131) and 14.3% (P=0.0024) concentrations. No significant difference was observed between both the manuka/kanuka blend and clover honeys and untreated cells at any concentration, with the exception of the 12.5% concentration of the phenolic clover extract (P=0.0360).
Table 2 The anti-inflammatory effect of honey extracts at five different concentrations (% honey) compared with untreated cells, in the HEK-Blue™-2 cell line using the FSL-1 ligand

| Honey treatment          | Concentration (%) | Mean (SE)  | Estimate (95% CI)     | P-value |
|--------------------------|------------------|-----------|-----------------------|---------|
| Phenolic manuka          | 0.0              | 1.10 (0.12)| 0.0                   | 0.5833  |
|                          | 5.3              | 1.14 (0.05)| 0.039 (–0.121 to 0.198)| 0.5324  |
|                          | 7.1              | 1.12 (0.01)| 0.016 (–0.143 to 0.176)| 0.6635  |
|                          | 10.7             | 1.06 (0.06)| –0.044 (–0.204 to 0.115)| 0.1109  |
|                          | 12.5             | 1.14 (0.06)| 0.031 (–0.129 to 0.190)| 0.1109  |
|                          | 14.3             | 0.98 (0.09)| –0.123 (–0.282 to 0.036)| 0.0390  |
| Phenolic manuka          | 0.0              | 1.09 (0.19)| 0.0                   | 0.8068  |
|                          | 5.3              | 1.13 (0.12)| 0.032 (–0.262 to 0.326)| 0.2915  |
|                          | 7.1              | 1.24 (0.15)| 0.142 (–0.152 to 0.436)| 0.3772  |
|                          | 10.7             | 0.98 (0.06)| –0.117 (–0.411 to 0.177)| 0.1862  |
|                          | 12.5             | 0.91 (0.13)| –0.182 (–0.476 to 0.112)| 0.0390  |
|                          | 14.3             | 0.78 (0.08)| –0.315 (–0.609 to –0.021)| 0.016  |
| Phenolic manuka/kanuka   | 0.0              | 1.17 (0.08)| 0.0                   | 0.6884  |
|                          | 5.3              | 1.23 (0.05)| 0.058 (–0.271 to 0.387)| 0.0668  |
|                          | 7.1              | 1.45 (0.31)| 0.277 (–0.052 to 0.606)| 0.8596  |
|                          | 10.7             | 1.20 (0.04)| 0.026 (–0.304 to 0.355)| 0.1044  |
|                          | 12.5             | 1.08 (0.13)| –0.097 (–0.426 to 0.233)| 0.2209  |
|                          | 14.3             | 0.99 (0.07)| –0.187 (–0.516 to 0.142)| 0.3739  |
| Phenolic clover          | 0.0              | 1.11 (0.19)| 0.0                   | 0.0462  |
|                          | 5.3              | 1.27 (0.20)| 0.161 (–0.240 to 0.562)| 0.4308  |
|                          | 7.1              | 1.49 (0.25)| 0.380 (–0.021 to 0.781)| 0.0600  |
|                          | 10.7             | 1.24 (0.08)| 0.130 (–0.271 to 0.531)| 0.4675  |
|                          | 12.5             | 1.24 (0.01)| 0.132 (–0.269 to 0.533)| 0.4613  |
|                          | 14.3             | 0.97 (0.13)| –0.142 (–0.543 to 0.259)| 0.4308  |
| Crude manuka             | 0.0              | 1.08 (0.02)| 0.0                   | 0.4756  |
|                          | 5.3              | 0.99 (0.00)| –0.091 (–0.376 to 0.194)| 0.8645  |
|                          | 7.1              | 1.06 (0.02)| –0.021 (–0.306 to 0.264)| 0.1364  |
|                          | 10.7             | 0.88 (0.10)| –0.203 (–0.488 to 0.082)| 0.4613  |
|                          | 12.5             | 0.79 (0.06)| –0.292 (–0.577 to –0.006)| 0.4308  |
|                          | 14.3             | 1.09 (0.12)| 0.014 (–0.271 to 0.299)| 0.9099  |
| Crude kanuka             | 0.0              | 1.06 (0.08)| 0.0                   | 0.6202  |
|                          | 5.3              | 1.15 (0.05)| 0.085 (–0.302 to 0.472)| 0.4836  |
|                          | 7.1              | 1.19 (0.13)| 0.121 (–0.266 to 0.508)| 0.8003  |
|                          | 10.7             | 1.11 (0.04)| 0.043 (–0.344 to 0.430)| 0.5983  |
|                          | 12.5             | 0.97 (0.25)| –0.090 (–0.477 to 0.297)| 0.2849  |
|                          | 14.3             | 1.31 (0.10)| 0.244 (–0.144 to 0.631)| 0.1804  |
| Crude manuka/kanuka      | 0.0              | 1.15 (0.10)| 0.0                   | 0.7439  |
|                          | 5.3              | 1.11 (0.11)| –0.041 (–0.324 to 0.242)| 0.8150  |
|                          | 7.1              | 1.18 (0.07)| 0.029 (–0.254 to 0.312)| 0.0649  |
|                          | 10.7             | 0.88 (0.15)| –0.262 (–0.545 to 0.021)| 0.9437  |
|                          | 12.5             | 1.16 (0.08)| 0.009 (–0.274 to 0.292)| 0.2849  |
|                          | 14.3             | 1.29 (0.06)| 0.139 (–0.145 to 0.422)| 0.1936  |
| Crude clover             | 0.0              | 1.08 (0.09)| 0.0                   | 0.8902  |
|                          | 5.3              | 1.10 (0.03)| 0.013 (–0.209 to 0.236)| 0.6250  |
|                          | 7.1              | 1.13 (0.04)| 0.048 (–0.174 to 0.270)| 0.1986  |
|                          | 10.7             | 0.95 (0.04)| –0.133 (–0.355 to 0.089)| 0.7733  |
|                          | 12.5             | 1.11 (0.11)| 0.028 (–0.194 to 0.250)| 0.9733  |
|                          | 14.3             | 1.22 (0.02)| 0.135 (–0.087 to 0.357)| 0.9733  |

Abbreviations: CI, confidence interval; SE, standard error; FSL-1, Pam2CGDPKHPKS.

Effect of honey extracts on inflammation in HEK-Blue™-2 cells

Honey treatment was investigated in HEK-Blue™-2 cells in the presence of two ligands, FSL-1 and Pam3CSK4. FSL-1 is specific to TLR2 and TLR1. Tables 2 and 3 detail the effect of the honey extracts on the inflammatory response in the HEK-Blue™-2 cells.

By examining honey treatment using two ligands, the specific pathway through which honey might act could
Table 3 The anti-inflammatory effect of honey extracts at five different concentrations (% honey) compared with untreated cells, in the HEK-Blue™-2 cell line using the Pam3CSK4 ligand

| Honey treatment               | Concentration (%) | Mean (SE) | Estimate (95% CI)               | P-value |
|-------------------------------|-------------------|-----------|---------------------------------|---------|
| Phenolic manuka               | 0.0               | 1.24 (0.14)| 0.0                             | 0.0     |
|                              | 5.3               | 1.05 (0.09)| -0.194 (-0.396 to -0.008)       | 0.0593  |
|                              | 7.1               | 0.99 (0.03)| -0.248 (-0.450 to -0.046)       | 0.0184  |
|                              | 10.7              | 0.95 (0.14)| -0.294 (-0.496 to -0.092)       | 0.0064  |
|                              | 12.5              | 0.97 (0.17)| -0.274 (-0.476 to -0.072)       | 0.0102  |
|                              | 14.3              | 0.87 (0.17)| -0.374 (-0.576 to -0.172)       | 9.27E-04|
| Phenolic kanuka               | 0.0               | 1.15 (0.10)| 0.0                             | 0.0     |
|                              | 5.3               | 0.98 (0.07)| -0.172 (-0.298 to -0.047)       | 0.0095  |
|                              | 7.1               | 0.91 (0.04)| -0.238 (-0.363 to -0.112)       | 7.54E-04|
|                              | 10.7              | 0.77 (0.08)| -0.378 (-0.504 to -0.253)       | 3.28E-06|
|                              | 12.5              | 0.69 (0.09)| -0.460 (-0.585 to -0.334)       | 1.82E-07|
|                              | 14.3              | 0.63 (0.07)| -0.518 (-0.643 to -0.392)       | 2.67E-08|
| Phenolic manuka/kanuka        | 0.0               | 1.09 (0.08)| 0.0                             | 0.0     |
|                              | 5.3               | 1.04 (0.13)| -0.050 (-0.230 to 0.130)        | 0.5669  |
|                              | 7.1               | 1.03 (0.10)| -0.065 (-0.245 to 0.115)        | 0.4561  |
|                              | 10.7              | 0.94 (0.10)| -0.152 (-0.332 to 0.028)        | 0.0933  |
|                              | 12.5              | 0.93 (0.15)| -0.165 (-0.345 to 0.015)        | 0.0694  |
|                              | 14.3              | 0.93 (0.16)| -0.168 (-0.348 to 0.012)        | 0.0658  |
| Phenolic clover               | 0.0               | 1.20 (0.11)| 0.0                             | 0.0     |
|                              | 5.3               | 1.13 (0.12)| -0.076 (-0.289 to 0.138)        | 0.4698  |
|                              | 7.1               | 1.10 (0.05)| -0.099 (-0.312 to 0.115)        | 0.3469  |
|                              | 10.7              | 1.08 (0.17)| -0.125 (-0.338 to 0.089)        | 0.2374  |
|                              | 12.5              | 1.03 (0.19)| -0.173 (-0.387 to 0.040)        | 0.1064  |
|                              | 14.3              | 0.98 (0.17)| -0.22 (-0.434 to -0.007)        | 0.0437  |
| Crude manuka                  | 0.0               | 1.01 (0.12)| 0.0                             | 0.0     |
|                              | 5.3               | 1.01 (0.04)| -0.006 (-0.138 to 0.125)        | 0.9216  |
|                              | 7.1               | 0.96 (0.07)| -0.052 (-0.184 to 0.079)        | 0.4086  |
|                              | 10.7              | 0.95 (0.08)| -0.064 (-0.195 to 0.068)        | 0.3158  |
|                              | 12.5              | 0.99 (0.05)| -0.024 (-0.156 to 0.108)        | 0.7012  |
|                              | 14.3              | 0.91 (0.07)| -0.105 (-0.236 to 0.027)        | 0.1096  |
| Crude kanuka                  | 0.0               | 1.03 (0.08)| 0.0                             | 0.0     |
|                              | 5.3               | 1.06 (0.07)| 0.023 (-0.119 to 0.165)         | 0.7337  |
|                              | 7.1               | 1.08 (0.07)| 0.048 (-0.094 to 0.190)         | 0.4824  |
|                              | 10.7              | 1.03 (0.09)| -0.003 (-0.146 to 0.139)        | 0.9600  |
|                              | 12.5              | 1.07 (0.05)| 0.038 (-0.104 to 0.181)         | 0.5710  |
|                              | 14.3              | 0.97 (0.08)| -0.066 (-0.208 to 0.076)        | 0.3378  |
| Crude manuka/kanuka           | 0.0               | 1.02 (0.08)| 0.0                             | 0.0     |
|                              | 5.3               | 1.10 (0.01)| 0.082 (-0.054 to 0.219)         | 0.2169  |
|                              | 7.1               | 1.13 (0.11)| 0.112 (-0.025 to 0.248)         | 0.1006  |
|                              | 10.7              | 1.05 (0.11)| 0.030 (-0.106 to 0.167)         | 0.6423  |
|                              | 12.5              | 1.03 (0.05)| 0.009 (-0.127 to 0.146)         | 0.8864  |
|                              | 14.3              | 0.95 (0.09)| -0.068 (-0.205 to 0.068)        | 0.3015  |
| Crude clover                 | 0.0               | 1.02 (0.05)| 0.0                             | 0.0     |
|                              | 5.3               | 1.13 (0.08)| 0.110 (-0.051 to 0.271)         | 0.1642  |
|                              | 7.1               | 1.13 (0.17)| 0.104 (-0.056 to 0.265)         | 0.1850  |
|                              | 10.7              | 1.06 (0.06)| 0.034 (-0.126 to 0.195)         | 0.6530  |
|                              | 12.5              | 1.08 (0.06)| 0.056 (-0.105 to 0.216)         | 0.4700  |
|                              | 14.3              | 0.95 (0.04)| -0.075 (-0.235 to 0.086)        | 0.3351  |

Abbreviations: CI, confidence interval; SE, standard error; Pam3CSK4, Pam3CysSerLys4.

be determined. Honey treatment at all concentrations had little impact, as compared with that in untreated cells with FSL-1, except for the 14.3% concentration of the phenolic kanuka extract (P=0.0390) and the 12.5% concentration of the crude manuka extract (P=0.0462). Stronger anti-inflammatory effects were observed in the presence of the Pam3CSK4 ligand, where the manuka and kanuka phenolics were particularly effective. At the highest concentrations, manuka honey significantly decreased the inflammatory response. With kanuka honey, all five concentrations significantly
Table 4 The anti-inflammatory effect of honey extracts at five different concentrations (% honey) compared with untreated cells, in the NOD2-WT cell line using the MDP ligand

| Honey treatment                  | Concentration (%) | Mean (SE)      | Estimate (95% CI) | P-value |
|----------------------------------|-------------------|----------------|-------------------|---------|
| Phenolic manuka                  | 0.0               | 0.91 (0.06)    | 0.0               |         |
|                                  | 5.3               | 1.02 (0.17)    | 0.108 (-0.274 to 0.490) | 0.5154 |
|                                  | 7.1               | 1.01 (0.20)    | 0.092 (-0.290 to 0.474) | 0.5766 |
|                                  | 10.7              | 1.09 (0.13)    | 0.172 (-0.210 to 0.554) | 0.3127 |
|                                  | 12.5              | 0.94 (0.15)    | 0.027 (-0.355 to 0.408) | 0.8707 |
|                                  | 14.3              | 0.88 (0.0)     | -0.031 (-0.498 to 0.437) | 0.8778 |
| Phenolic kanuka                  | 0.0               | 1.00 (0.08)    | 0.0               |         |
|                                  | 5.3               | 1.01 (0.13)    | 0.006 (-0.536 to 0.548) | 0.9807 |
|                                  | 7.1               | 0.97 (0.19)    | -0.032 (-0.574 to 0.510) | 0.8888 |
|                                  | 10.7              | 0.90 (0.18)    | -0.100 (-0.642 to 0.442) | 0.6681 |
|                                  | 12.5              | 0.76 (0.34)    | -0.240 (-0.782 to 0.302) | 0.3195 |
|                                  | 14.3              | 0.72 (0.0)     | -0.283 (-0.947 to 0.381) | 0.3372 |
| Phenolic manuka/kanuka           | 0.0               | 0.94 (0.07)    | 0.0               |         |
|                                  | 5.3               | 1.04 (0.06)    | 0.096 (-0.666 to 0.259) | 0.2029 |
|                                  | 7.1               | 0.94 (0.07)    | -0.007 (-0.169 to 0.155) | 0.9218 |
|                                  | 10.7              | 0.98 (0.06)    | 0.032 (-0.131 to 0.194) | 0.6583 |
|                                  | 12.5              | 1.00 (0.03)    | 0.059 (-0.103 to 0.221) | 0.4180 |
|                                  | 14.3              | 0.89 (0.12)    | -0.051 (-0.214 to 0.111) | 0.4779 |
| Phenolic clover                  | 0.0               | 0.94 (0.05)    | 0.0               |         |
|                                  | 5.3               | 1.00 (0.02)    | 0.068 (-0.198 to 0.334) | 0.5647 |
|                                  | 7.1               | 0.97 (0.01)    | 0.031 (-0.235 to 0.297) | 0.7931 |
|                                  | 10.7              | 1.13 (0.24)    | 0.193 (-0.073 to 0.459) | 0.1304 |
|                                  | 12.5              | 0.94 (0.07)    | 0.005 (-0.261 to 0.271) | 0.9683 |
|                                  | 14.3              | 1.00 (0.08)    | 0.063 (-0.203 to 0.329) | 0.5922 |
| Crude manuka                     | 0.0               | 1.11 (0.30)    | 0.0               |         |
|                                  | 5.3               | 0.99 (0.01)    | -0.113 (-0.488 to 0.261) | 0.4970 |
|                                  | 7.1               | 1.11 (0.18)    | 0.006 (-0.368 to 0.380) | 0.9717 |
|                                  | 10.7              | 1.03 (0.10)    | -0.072 (-0.446 to 0.302) | 0.6621 |
|                                  | 12.5              | 0.91 (0.01)    | -0.192 (-0.566 to 0.182) | 0.2641 |
|                                  | 14.3              | 0.97 (0.21)    | -0.134 (-0.509 to 0.240) | 0.4239 |
| Crude kanuka                     | 0.0               | 1.25 (0.28)    | 0.0               |         |
|                                  | 5.3               | 1.13 (0.24)    | -0.117 (-0.511 to 0.276) | 0.5033 |
|                                  | 7.1               | 1.11 (0.06)    | -0.133 (-0.527 to 0.260) | 0.4499 |
|                                  | 10.7              | 0.98 (0.12)    | -0.264 (-0.657 to 0.130) | 0.1567 |
|                                  | 12.5              | 1.00 (0.04)    | -0.242 (-0.636 to 0.151) | 0.1891 |
|                                  | 14.3              | 1.01 (0.19)    | -0.232 (-0.625 to 0.161) | 0.2059 |
| Crude manuka/kanuka              | 0.0               | 1.23 (0.34)    | 0.0               |         |
|                                  | 5.3               | 1.09 (0.11)    | -0.142 (-0.531 to 0.247) | 0.4170 |
|                                  | 7.1               | 1.14 (0.06)    | -0.093 (-0.481 to 0.296) | 0.5913 |
|                                  | 10.7              | 1.10 (0.11)    | -0.126 (-0.515 to 0.263) | 0.4700 |
|                                  | 12.5              | 0.99 (0.04)    | -0.233 (-0.622 to 0.156) | 0.1990 |
|                                  | 14.3              | 0.90 (0.17)    | -0.330 (-0.719 to 0.059) | 0.0850 |
| Crude clover                     | 0.0               | 1.19 (0.23)    | 0.0               |         |
|                                  | 5.3               | 1.07 (0.08)    | -0.122 (-0.426 to 0.183) | 0.3762 |
|                                  | 7.1               | 1.12 (0.13)    | -0.073 (-0.377 to 0.231) | 0.5874 |
|                                  | 10.7              | 1.07 (0.02)    | -0.125 (-0.429 to 0.179) | 0.3646 |
|                                  | 12.5              | 0.88 (0.09)    | -0.315 (-0.619 to 0.011) | 0.0442 |
|                                  | 14.3              | 0.91 (0.13)    | -0.285 (-0.589 to 0.019) | 0.0621 |

**Abbreviations:** CI, confidence interval; NOD2-WT, nucleotide oligomerization domain 2-Wild Type; MDP, muramyl dipeptide; SE, standard error.

reduced the level of inflammation compared with that of no treatment. Treatment with the manuka/kanuka blend, the clover honey phenolics, and the crude extracts from all four honeys had no significant impact on the inflammatory response.

Figure 2 illustrates the anti-inflammatory effect observed using the HEK-Blue<sup>™</sup>-2 cell line when treated with the highest concentration (14.3%) of honey phenolics and crude extracts and stimulated with Pam3CSK4. A significant decrease in inflammation was observed for each
A variety of honeys. This research, however, demonstrated that the honeys examined did not produce a significant anti-inflammatory effect via either the TLR4 or NLR signaling pathway, observing a noticeable but not significant anti-inflammatory activity with honey treatment.

Anti-inflammatory activity with honey treatment was observed in the HEK-Blue™-2 cell line and most significantly with kanuka honey. The kanuka phenolic extract was highly anti-inflammatory and had a greater effect than did the crude extract, indicating that a higher phenolic content correlates with its elevated anti-inflammatory activity. The manuka honey phenolics also had an anti-inflammatory effect in the HEK-Blue™-2 cells, although to a lesser extent than for the kanuka honey phenolics, with the highest concentrations producing a significant difference as compared with no treatment, thereby supporting the importance of polyphenols in the anti-inflammatory activity of honey. The anti-inflammatory effect by the kanuka and manuka honeys was strongest in the presence of the Pam3CSK4 ligand, indicating that the honeys act through the TLR1/TLR2 signaling pathway. The anti-inflammatory activity of kanuka and manuka honeys is therefore pathway-specific. No significant effect was observed with honey treatment, at any concentration, in the NOD2-WT cell line, supporting the anti-inflammatory activity of honey being pathway-specific. A hypothesis for the means by which kanuka honey exhibits anti-inflammatory activity is through the downregulation of proinflammatory mediators, such as IL-1β and NF-KB.

In wound healing, the inflammatory response is one phase of repair that is fundamental for normal healing. An elevated or prolonged inflammatory response is associated with a delay in wound repair, an increase in tissue damage, and the development of nonhealing, chronic wounds. By demonstrating significant anti-inflammatory activity, kanuka honey has the potential to be an effective treatment in preventing chronic wounds. International studies have shown that honey has a significant effect on the inflammatory response and support the use of honey in wound healing.18,24,31

Research has also shown a causal association between inflammatory diseases and treatment with honey.27,29,32 This study sought to investigate and further advance these findings. Anti-inflammatory assays were conducted using HEK-Blue™-2, HEK-Blue™-4, and NOD2-WT cell lines, acting through different signaling pathways. The results demonstrate that kanuka honey exhibits anti-inflammatory effects in a pathway-specific manner. Further investigation would help to discover the exact mechanisms of action by which honeys act.
HEK-Blue™-2 is a more sensitive cell line than is HEK-Blue™-4. The noticeable but not significant anti-inflammatory effect observed by the honey phenolics in the HEK-Blue™-4 cells contrasts with the significant impact in the HEK-Blue™-2 cells, suggesting a reduced sensitivity rather than no anti-inflammatory activity. Further investigation using larger volumes of honey would be required to determine whether more significant results could be obtained.

Conclusion
New Zealand honeys have a well-established anti-inflammatory effect in topical wound healing. However, less was known of their effect in vitro and of the signaling pathways through which they act. Treatment with kanuka and manuka honeys resulted in powerful anti-inflammatory effects in HEK-Blue™-2 cells, but not in the HEK-Blue™-4 or NOD2-WT cells. Specifically, the anti-inflammatory effect occurred via the TLR1/TLR2 signaling pathway. The effects suggest a correlation with the phenolic content of the honeys, with a higher phenolic content producing an elevated anti-inflammatory effect. Kanuka and manuka honeys therefore can have a positive impact on the inflammatory response associated with wound healing. Subsequent investigation is needed to determine the specific compounds present in the honeys that are agents responsible for their anti-inflammatory activity.

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