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Potential pathway of anti-inflammatory effect by New Zealand honeys

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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 Pam3Cys-SerLys4 (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-α (TNF-α).[8,17] These proinflammatory cytokines stimulate nitric oxide production, an important mediator of inflammation. Nitric oxide production

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and NF-KB activation are inhibited by the flavonoids present in honey.[18] When healing is impaired, chronic wounds develop, characterized by proinflammatory cytokines and reactive oxygen species.[8,19–22]

Honey's effectiveness in wound care has been hypothesized to be largely due to its anti-inflammatory action.[8] The specific compounds and the mechanisms involved are largely undetermined.[23] However, it has been suggested that specific polyphenols, the flavonoids, and caffeic acid phenethyl ester, are important factors.[24–26] The antioxidants found in honey are considered to be important determinants of its anti-inflammatory activity.[2] An elevated inflammatory response results from hydrogen peroxide oxygen radicals present at the wound site, triggering NF-KB to enhance the inflammatory response.[2,8] New Zealand honeys have been suggested to display significant anti-inflammatory activity, particularly kanuka and manuka honeys, by reducing neutrophil superoxide production.[23] Manuka honey has been shown to specifically decrease the inflammatory response associated with ulcerative colitis, an inflammatory bowel disease characterized by an over-expression of inflammatory cells, possibly by increasing antioxidant activity.[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 Comvita 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™-2, HEK-Blue™-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 lipoprotein), Muramyl dipeptide (MDP), Blasticidin, Zeocin™, HEK-Blue™ Selection, and QUANTI-Blue™ 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™-2 and HEK-Blue™-4 cells were maintained in DMEM high glucose supplemented with 10% FCS, 1% penicillin/streptomycin/glutamine, and HEK-Blue™ Selection. The NOD2 WT cells were maintained in DMEM supplemented with 10% FCS, 1% penicillin/streptomycin, 0.06% Blasticidin, and 0.1% Zeocin.

Anti-inflammatory assay

The HEK-Blue™-2 and HEK-Blue™-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.

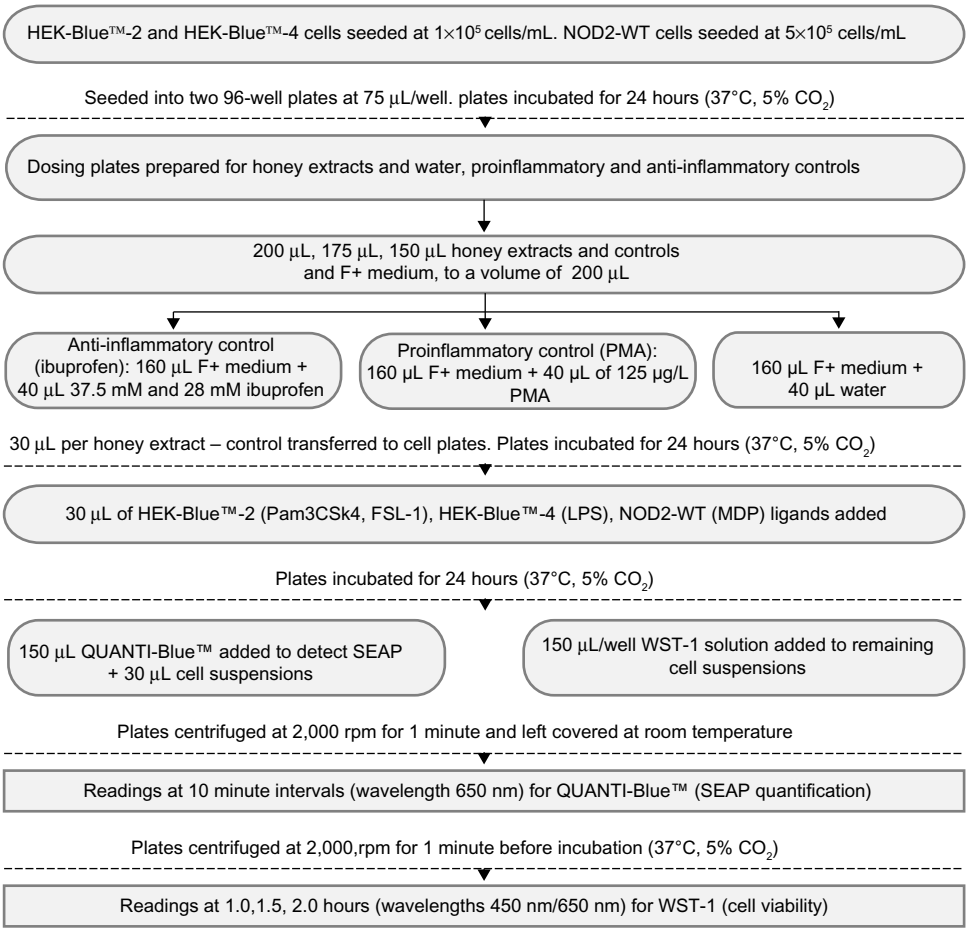

Figure 1 Materials, preparation, and observation process flow chart.
Note: HEK-Bluet™-2 and QUANTI-Blue™ (InvivoGen, San Diego, CA, USA).
Abbreviations: LPS, lipopolysaccharide; MDP, muramyl dipeptide; NOD2-WT, nucleotide oligomerization domain 2-Wild Type; PMA, phorbol 12-myristate 13-acetate; SEAP, secreted alkaline phosphatase; Pam3CSK4, Pam3CysSerLys4; FSL-1, Pam2CGDPKHPKSF.

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	
	5.3	1.14 (0.05)	0.039 (–0.121 to 0.198)	0.5833
	7.1	1.12 (0.01)	0.016 (–0.143 to 0.176)	0.8137
	10.7	1.06 (0.06)	–0.044 (–0.204 to 0.115)	0.5324
	12.5	1.14 (0.06)	0.031 (–0.129 to 0.190)	0.6635
	14.3	0.98 (0.09)	–0.123 (–0.282 to 0.036)	0.1109
Phenolic kanuka	0.0	1.09 (0.19)	0.0	
	5.3	1.13 (0.12)	0.032 (–0.262 to 0.326)	0.8068
	7.1	1.24 (0.15)	0.142 (–0.152 to 0.436)	0.2915
	10.7	0.98 (0.06)	–0.117 (–0.411 to 0.177)	0.3772
	12.5	0.91 (0.13)	–0.182 (–0.476 to 0.112)	0.1862
	14.3	0.78 (0.08)	–0.315 (–0.609 to –0.021)	0.0390
Phenolic manuka/kanuka	0.0	1.17 (0.08)	0.0	
	5.3	1.23 (0.05)	0.058 (–0.271 to 0.387)	0.6884
	7.1	1.45 (0.31)	0.277 (–0.052 to 0.606)	0.0868
	10.7	1.20 (0.04)	0.026 (–0.304 to 0.355)	0.8596
	12.5	1.08 (0.13)	–0.097 (–0.426 to 0.233)	0.5104
	14.3	0.99 (0.07)	–0.187 (–0.516 to 0.142)	0.2209
Phenolic clover	0.0	1.11 (0.19)	0.0	
	5.3	1.27 (0.20)	0.161 (–0.240 to 0.562)	0.3739
	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	
	5.3	0.99 (0.00)	–0.091 (–0.376 to 0.194)	0.4756
	7.1	1.06 (0.02)	–0.021 (–0.306 to 0.264)	0.8645
	10.7	0.88 (0.10)	–0.203 (–0.488 to 0.082)	0.1364
	12.5	0.79 (0.06)	–0.292 (–0.577 to –0.006)	0.0462
	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	
	5.3	1.15 (0.05)	0.085 (–0.302 to 0.472)	0.6202
	7.1	1.19 (0.13)	0.121 (–0.266 to 0.508)	0.4836
	10.7	1.11 (0.04)	0.043 (–0.344 to 0.430)	0.8003
	12.5	0.97 (0.25)	–0.090 (–0.477 to 0.297)	0.5983
	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	
	5.3	1.11 (0.11)	–0.041 (–0.324 to 0.242)	0.7439
	7.1	1.18 (0.07)	0.029 (–0.254 to 0.312)	0.8150
	10.7	0.88 (0.15)	–0.262 (–0.545 to 0.021)	0.0649
	12.5	1.16 (0.08)	0.009 (–0.274 to 0.292)	0.9437
	14.3	1.29 (0.06)	0.139 (–0.145 to 0.422)	0.2849
Crude clover	0.0	1.08 (0.09)	0.0	
	5.3	1.10 (0.03)	0.013 (–0.209 to 0.236)	0.8902
	7.1	1.13 (0.04)	0.048 (–0.174 to 0.270)	0.6250
	10.7	0.95 (0.04)	–0.133 (–0.355 to 0.089)	0.1986
	12.5	1.11 (0.11)	0.028 (–0.194 to 0.250)	0.7733
	14.3	1.22 (0.02)	0.135 (–0.087 to 0.357)	0.1936

Abbreviations: CI, confidence interval; SE, standard error; FSL-1, Pam2CGDPKHPKSF.

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 TLR6, and Pam3CSK4 is recognized by 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	
	5.3	1.05 (0.09)	–0.194 (–0.396–0.008)	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	
	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	
	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	
	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	
	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	
	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	
	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	
	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 (–0.066 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™-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

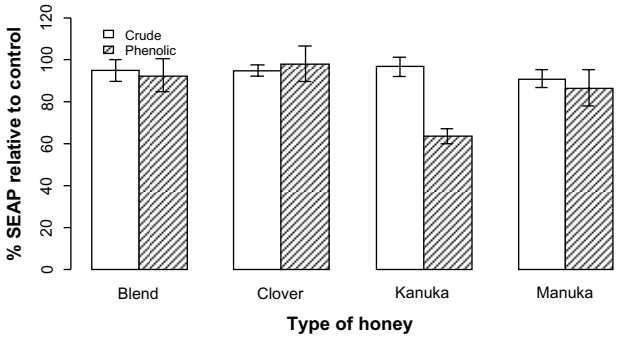

Figure 2 The anti-inflammatory effect of the four honey extracts when differentiated by honey phenolics and crude honey extracts.
Notes: Results are expressed as % SEAP relative to control. The lower the value, the lower the level of SEAP, resulting in a lower anti-inflammatory effect. Blend is a mixture of crude manuka and kanuka honey.
Abbreviation: SEAP, secreted alkaline phosphatase.

extract compared with PMA (data not shown). Treatment with the phenolic and crude manuka/kanuka blend and clover extracts did not differ markedly from treatment with solvent. Treatment with the kanuka phenolic extract differed considerably from the solvent and the crude kanuka extract. The kanuka phenolic extract produced a comparable anti-inflammatory effect to that of ibuprofen (results not shown).

Effect of honey extracts on inflammation in NOD2-WT cells

Table 4 details the anti-inflammatory effect of honey treatment in NOD2-WT cells. There was no significant effect by the honeys, at any concentration, on the inflammatory response when compared with no treatment, with the exception of the 12.5% concentration of the crude clover extract, where a significant anti-inflammatory effect was observed (P=0.0442).

Discussion

An inflammatory response was induced in HEK-Blue™-2, HEK-Blue™-4, and NOD2-WT cell lines using different ligands, to illustrate whether treatment with four honeys could produce an anti-inflammatory effect. The cell lines act through different signaling pathways (TLR2, TLR4, and NOD-like receptor [NLR] respectively). Thus, by being able to observe in which cell line(s) the honey treatment was effective, the pathway through which it produced an anti-inflammatory response could be demonstrated. The NOD2-WT cell line acts through the NLR signaling pathway and recognizes the MDP ligand. A substantial body of research exists to support the anti-inflammatory activity of 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.[30] 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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References

1. Lee DS, Sinno S, Khachemoune A. Honey and wound healing: an overview. *Am J Clin Dermatol*. 2011;12(3):181–190.
2. Molan PC. Using honey in wound care. *IJCA*. 2006;3(2):21−24.
3. Molan PC. The role of honey in the management of wounds. *J Wound Care*. 1999;8(8):415–418.
4. Mathews KA, Binnington AG. Wound management using honey. *Compendium for the Continuing Education of the Practicing Veterinarian*. 2002;24(1):53−60.
5. Subrahmanyam M. Honey dressing versus boiled potato peel in the treatment of burns: a prospective randomized study. *Burns*. 1996;22(6):491–493.
6. Bogdanov S, Jurendic T, Sieber R, Gallmann P. Honey for nutrition and health: a review. *J Am Coll Nutr*. 2008;27(6):677–689.
7. Khan FR, Ul Abadin Z, Rauf N. Honey: nutritional and medicinal value. *Int J Clin Pract*. 2007;61(10):1705–1707.
8. Molan PC. Re-introducing honey in the management of wounds and ulcers – theory and practice. *Ostomy Wound Manage*. 2002;48(11):28–40.
9. Tonks AJ, Dudley E, Porter NG, et al. A 5.8-kDa component of manuka honey stimulates immune cells via TLR4. *J Leukoc Biol*. 2007;82(5):1147−1155.
10. Mwipatayi BP, Angel D, Norrish J, Hamilton MJ, Scott A, Sieunarine K. The use of honey in chronic leg ulcers: a literature review. *Primary Intention*. 2004;12(3):107−108, 110−112.
11. Steinhorn G, Sims IM, Carnachan SM, Carr AJ, Schlothauer R. Isolation and characterisation of arabinogalactan-proteins from New Zealand kanuka honey. *Food Chemistry*. 2011;128(4):949−956.
12. Lund LR, Romer J, Bugge TH, et al. Functional overlap between two classes of matrix-degrading proteases in wound healing. *EMBO J*. 1999;18(17):4645–4656.
13. Pilcher BK, Dumin JA, Sudbeck BD, Krane SM, Welgus HG, Parks WC. The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix. *J Cell Biol*. 1997;137(6):1445–1457.
14. Majtan J, Kumar P, Majtan T, Walls AF, Klaudiny J. Effect of honey and its major royal jelly protein 1 on cytokine and MMP-9 mRNA transcripts in human keratinocytes. *Exp Dermatol*. 2010;19(8):e73–e79.
15. Schultz GS, Mast BA. Molecular analysis of the environments of healing and chronic wounds: cytokines, proteases and growth factors. *Primary Intention*. 1999;7(1):7−14.
16. Lee Y, Kim H, Kim S, Kim KH, Chung JH. Activation of toll-like receptors 2, 3 or 5 induces matrix metalloproteinase-1 and -9 expression with the involvement of MAPKs and NF-kappaB in human epidermal keratinocytes. *Exp Dermatol*. 2010;19(8):e44–e49.
17. Chung TW, Moon SK, Chang YC, et al. Novel and therapeutic effect of caffeic acid and caffeic acid phenyl ester on hepatocarcinoma cells: complete regression of hepatoma growth and metastasis by dual mechanism. *FASEB J*. 2004;18(14):1670–1681.
18. Hämäläinen M, Nieminen R, Vuorela P, Heinonen M, Moilanen E. Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-kappaB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-kappaB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. *Mediators Inflamm*. 2007;2007:45673.
19. Brandner JM, Zacheja S, Houdek P, Moll I, Lobmann R. Expression of matrix metalloproteinases, cytokines, and connexins in diabetic and nondiabetic human keratinocytes before and after transplantation into an ex vivo wound-healing model. *Diabetes Care*. 2008;31(1):114–120.
20. Trengove NJ, Stacey MC, MacAuley S, et al. Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors. *Wound Repair Regen*. 1999;7(6):442–452.
21. Diegelmann RF, Evans MC. Wound healing: an overview of acute, fibrotic and delayed healing. *Front Biosci*. 2004;9:283–289.
22. van den Berg AJ, van den Worm E, van Ufford HC, Halkes SB, Hoekstra MJ, Beukelman CJ. An in vitro examination of the antioxidant and anti-inflammatory properties of buckwheat honey. *J Wound Care*. 2008;17(4):172–174, 176.
23. Leong AG, Herst PM, Harper JL. Indigenous New Zealand honeys exhibit multiple anti-inflammatory activities. *Innate Immun*. 2012;18(3):459–466.

24. Kassim M, Achoui M, Mustafa MR, Mohd MA, Yusoff KM. Ellagic acid, phenolic acids, and flavonoids in Malaysian honey extracts demonstrate in vitro anti-inflammatory activity. *Nutr Res*. 2010;30(9):650–659.
25. Khalil M, Sulaiman SA, Boukraa L. Antioxidant properties of honey and its role in preventing health disorder. *The Open Nutraceuticals Journal*. 2010;3(1):6−16.
26. Pyrzynska K, Biesaga M. Analysis of phenolic acids and flavonoids in honey. *TrAC*. 2009;28(7):893−902.
27. Mahgoub AA, el-Medany AH, Hagar HH, Sabah DM. Protective effect of natural honey against acetic acid-induced colitis in rats. *Trop Gastroenterol*. 2002;23(2):82–87.
28. Medhi B, Prakash A, Avti PK, Saikia UN, Pandhi P, Khanduja KL. Effect of Manuka honey and sulfasalazine in combination to promote antioxidant defense system in experimentally induced ulcerative colitis model in rats. *Indian J Exp Biol*. 2008;46(8):583–590.
29. Prakash A, Medhi B, Avti PK, Saikia UN, Pandhi P, Khanduja KL. Effect of different doses of Manuka honey in experimentally induced inflammatory bowel disease in rats. *Phytother Res*. 2008;22(11):1511–1519.
30. Uhlen M, Oksvold P, Fagerberg L, et al. Towards a knowledge-based Human Protein Atlas. *Nat Biotechnol*. 2010;28(12):1248–1250.
31. Al-Waili NS, Boni NS. Natural honey lowers plasma prostaglandin concentrations in normal individuals. *J Med Food*. 2003;6(2):129–133.
32. Bilsel Y, Bugra D, Yamaner S, Bulut T, Cevikbas U, Turkoglu U. Could honey have a place in colitis therapy? Effects of honey, prednisolone, and disulfiram on inflammation, nitric oxide, and free radical formation. *Dig Surg*. 2002;19(4):306–311; discussion 311–312.