CHARACTERISING THE HEALING ACTIVITY OF HONEY

June 2022

CRCHBP
FOR HONEY BEE PRODUCTS

ISBN
ISSN
TITLE: Characterising the healing activity of honey
Publication No.
Project: Program 2 No. 16

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Electronically published by CRC for Honey Bee Products at www.CRChoneybeeproducts.com

Forward

Understanding how honey attributes contribute to health outcomes is the next step in providing solid proof to the consumer of the health benefits of honey. The contribution of the natural antioxidant activity in honey to regenerative and anti-inflammatory activities during the healing process brings a new focus on honeys that have high antioxidant activity. Combining this with antibacterial activity makes for a strong, natural healing agent.

Evidence of this chemical measurement of honey health benefits to cellular performance is showing a promising link. The next step is going to human trials to see if what has been found on the benchtop is translated into real health benefits. When chronic wounds remain a significant health burden, with >430,000 Australians afflicted by non-healing wounds, and an associated $3.5 billion imposition on the annual healthcare budget (McCosker et al., 2019), honeys identified as bioactive in this Project, could be selected for randomized, placebo-controlled trials in patients with chronic wounds.

What we have always known about honey will take time to unravel, and this project opened a new pathway for Australian honeys into the health care market.

Dr Liz Barbour
CEO

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About the Authors

Dr Fraser Russell

Dr. Fraser Russell is an Associate Professor in Biochemical Pharmacology at the University of the Sunshine Coast (USC), Queensland. Since joining USC (2005-), he has developed a research program in natural product therapies, with a focus on providing new ideas for the management of patients with abdominal aortic aneurysm and aberrant wound healing responses, where there are limited pharmacological options, or where patients are refractory to drug treatment. An example of this research includes the discovery of a small molecule, present in Australian stingless bee cerumen, that has wound-healing potential. Cerumen is collected by native bees from the resin of trees and is used by the bees to protect the hive against pathogens. Dr. Russell is the lead investigator of a CRC project investigating the antioxidant and anti-inflammatory potential of Australian honeys.

Dr Soheila Beiranvand

Soheila accepted the CRC for Honey Bee Products scholarship and undertook a PhD to support the outcomes of this project.

Acknowledgements

This project was supported by the Cooperative Research Centres (CRC) Program for Honey Bee Products, Australia. The authors acknowledge the support of the CRC, and in particular the CEO, Dr. Liz Barbour, and the CRC Executive Board. *Eucalyptus* honey samples were supplied by Capilano Honey Ltd. Australian non-*Eucalyptus* honeys were supplied by Capilano Honey Ltd., and Blue Hills Honey. New Zealand manuka honey was supplied by Global Proficiency, NZ. The authors acknowledge the support provided by the University of the Sunshine Coast, and the work carried out by past and present research candidates, Soheila Beiranvand, Ashley Williams, Symsia Long, Jeanne Visagie and Jamie Noll.

Executive Summary

This Project examined a kinetic model for antioxidant activity, called the radical scavenging activity. We found that honeys that had a high radical scavenging capacity (RSC), were also potent antioxidants, as determined by measurement of the EC_{50} values. Grey ironbark, river red gum and messmate honeys had the most favorable antioxidant profile of the 16 different varieties of *Eucalyptus* honeys that were tested in this study. NZ manuka, grey ironbark and river red gum honeys all had high gallic acid equivalents which correlated well with the high radical scavenging activity. This is also consistent with other studies that identified strong correlations between phenolic content and antioxidant activity. However, not all honeys behaved as predicted. Overall, there was a weak correlation between the RSC of *Eucalyptus* honeys and their phenolic acid content. There are several plausible reasons for this disparity, including potential antioxidant activity on other, non-phenolic constituents of honeys, or different distributions of phenolic compounds within honey samples of differing botanical origin.

There is growing interest in the medicinal benefits of honey, with a wide range of honey-impregnated bandages on the market for facilitating wound healing. This Project investigated the wound-healing potential of honey by examining its effect on immune responses. Interleukin-6 (IL-6) is a pleiotropic cytokine that stimulates either inflammatory or anti-inflammatory effects, depending on whether the cytokine binds to soluble IL-6 receptors (pro-inflammatory) or to membrane bound IL-6 receptors (regenerative and anti-inflammatory). The project found that honey contains lipopolysaccharide (LPS), an endotoxin, that can stimulate secretion of IL-6 by fibroblasts. An implication of this Project is that honey may provide important immunomodulatory effects that can be harnessed to manage inflammation that occurs in conditions such as autoimmune disease and sepsis.

Objectives

The main objective of this Project was to provide information for the beekeeping industry about the potential wound healing properties of Australian honeys by reporting on their antioxidant and anti-inflammatory activities.

Specific aims were to:

- Develop a method for the analysis of the radical scavenging capacity of Australian honeys using a kinetic free-radical scavenging assay and to compare this to single time-point measures of potency.

- To examine the immunomodulatory effect of Australian honeys using cells that are known to be important to wound healing responses.

Key activities

1. Fractionation of individual phenolic compounds from Australian honey samples, with identification using HPLC.

2. Evaluation of honey that can be used as a universal measure of antioxidant activity. Initial studies focused on total antioxidant activity of honey phenolics using a free radical scavenging assay, and the oxygen radical absorbance capacity (ORAC) method. This study used cell-free assays to assess antioxidant activity.

3. Examine bioactivities of crude honey extracts and individual chemical constituents of the honeys for anti-inflammatory activity. This was achieved using cell-free and cell-based (macrophages, fibroblasts) to assess anti-inflammatory activity.

Figure 1: (Behind) Dr Fraser Russell (front) Dr Soheila Beiranvand in the University of Sunshine Coast laboratories

Introduction

As part of the CRC, this Project explored the antioxidant activity of Australian honeys. Reactive oxygen species (ROS) are primarily produced by the mitochondria of the cells. Free radicals have very important roles in mediating cell differentiation, cell proliferation and cell signaling. They also help to eradicate harmful pathogens from the body. So, for example, if we have an infection, our immune cells become activated and these cells kill pathogens by generating ROS.

The amount of ROS that is produced in the cells is kept in check by the expression of enzymes (biological catalysts) that convert the reactive oxygen species to less harmful intermediates. For example, superoxide dismutase is an enzyme that converts superoxide to hydrogen peroxide, and catalase is an enzyme that converts hydrogen peroxide to water and oxygen.

Problems arise when the amount of antioxidant enzyme being produced is insufficiently high to control levels of ROS. When this happens, ROS oxidise key biomolecules such as lipids, proteins and nucleic acids leading to cellular dysfunction and tissue damage (Figure 2).

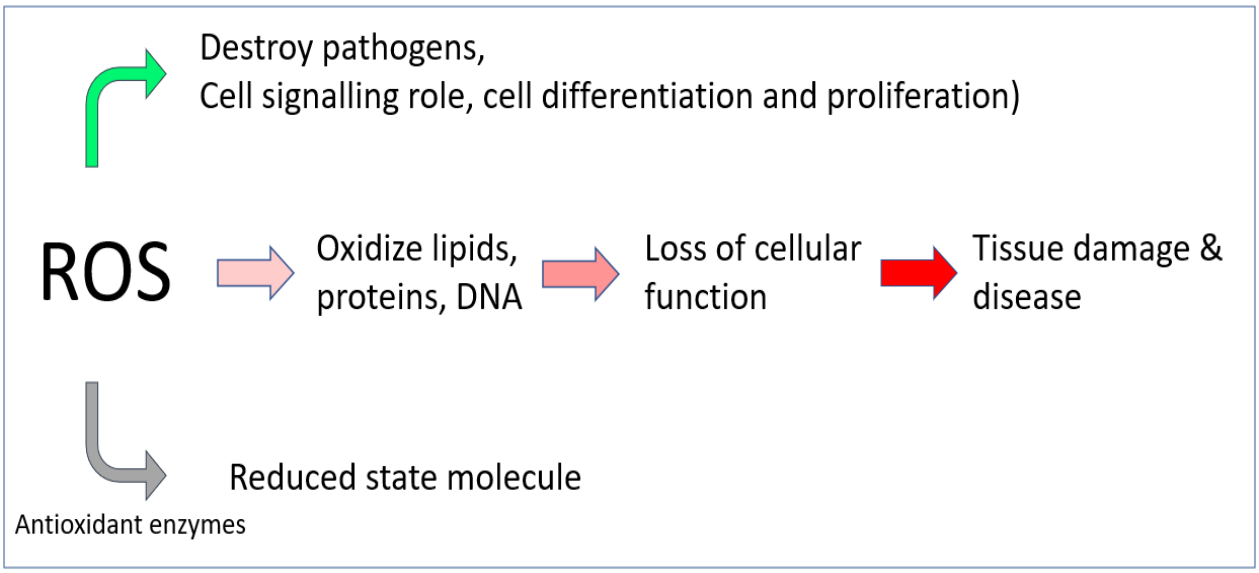

Figure 2: Reactive oxygen species is a crucial regulator of cell signaling but can also cause cell damage if levels are too high.

Honey is a complex mixture of natural substances that are derived from the nectar of plants and from bee secretions. Sugars make up about 81 or 82% of honey and comprise primarily fructose and glucose, but also other sugars such as maltose. Water makes up about 17 or 18% of honey. The antibacterial properties of honey are attributed in part to high osmotic stress caused by the high concentration of sugars. Minor constituents within honeys include enzymes, phenolic acids and flavonoids, vitamins such as vitamin C, minerals, amino acids and proteins. While these minor constituents account for only 4% of the total honey composition, they are responsible for many of the therapeutic properties of honeys. The bioactivity of the honeys is very much dependent on which minor constituents are present. For example, manuka honey is obtained from bees that forage on Leptospermum scoparium (a species of tea tree). These honeys contain methylglyoxal, a reasonably

non-polar substance that has anti-bacterial properties. Other honeys contain hydrogen peroxide that can also contribute to antibacterial effects.

Currently, the influence of botanical and geographical origin of honeys on antioxidant activity of Australian honeys is yet to be established. A limited number of studies have examined the potency of honeys for antioxidant activity, that is, the concentration required to produce a half-maximal antioxidant effect. Most of these studies have examined international sources of honey. To date, no studies have examined the time-course for antioxidant activity (the radical scavenging capacity of honeys).

STUDY 1: Antioxidant activity of Australian honeys

For this study, 53 samples from 16 varieties of Australian *Eucalyptus* honeys were collected by Capilano and local beekeepers. Honey samples were sourced from the South-East corner of Queensland, from the Northern Rivers region of NSW, from the South-east corner of NSW and from southern regions of South Australia. We also used 4 samples of NZ Manuka honey for comparison. A sugar mixture replicating the approximate sugar content of honeys was used as a control.

This study used the model, stable free radical, DPPH, to examine antioxidant effects of the honeys. DPPH is a chemical that when in solution has a purple colour. The solution absorbs light maximally at 518 nm wavelength, and this can be quantitated using a plate reader. The magnitude of light absorption is directly proportional to the amount of DPPH that is present.

Key findings: i) NZ manuka honey had excellent radical scavenging capacity (Figure 3). ii) Not all Australian *Eucalyptus* honeys were the same in terms of radical scavenging capacity. Honeys with particularly high activity were grey ironbark, river red gum and messmate honeys. iii) The sugar, whilst the main constituent of honey, did not contribute to the antioxidant activity. For a complete list of activities, readers are referred to Beiranvand *et al.*, Food Chemistry 2021;342:128332. The absolute rate of antioxidant activity was at least 10-fold higher at this early time-point that at the 150-minute mark. The order of activity for the different honeys, whilst slightly different, was largely the same at the early time-point compared to the later time-point. Honeys that performed best were the NZ manuka, grey ironbark, river red gum and messmate honeys.

Several previous studies have noted a correlation between antioxidant activity and the phenolic and flavonoid content of the honeys. We hypothesized that the high activity of the NZ manuka and Eucalyptus honeys might also correlate to the total phenolic content. Total phenolic content was determined using a reverse phase HPLC method.

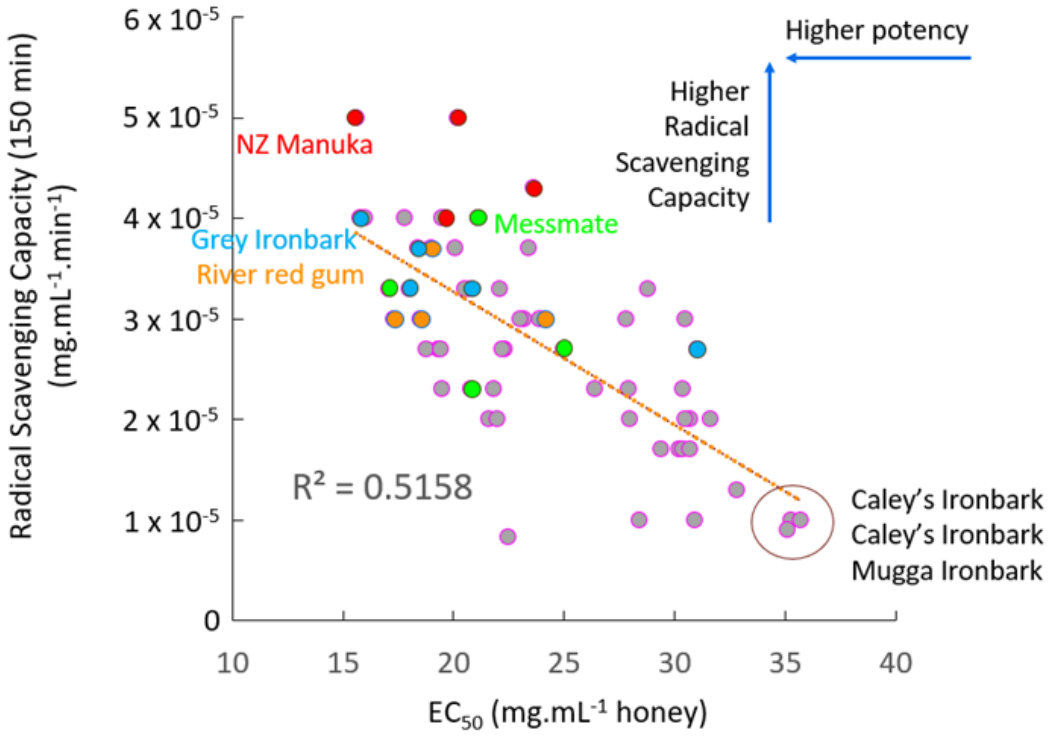

Figure 3: Correlation between radical scavenging capacity and potency (EC_{50} value) for Australian *Eucalyptus* honeys and NZ manuka honey.

Key findings:

i) NZ manuka, which had the highest radical scavenging activity, also had the highest total phenolic content.

ii) Of the *Eucalyptus honeys*, grey ironbark honey had the highest radical scavenging activity, and it also had the highest total phenolic content. There were some surprises, however. For example, peppermint honey had one of the lower radical scavenging capacities but had a relatively high total phenolic content.

iii) Overall, there was a weak correlation between the radical scavenging capacity of the *Eucalyptus* honeys and total phenolic content.

The types of phenolic compounds present in honeys was determined by retention time in the HPLC trace and UV spectra with comparison to phenolic standards. River red gum honey contained *p*-hydroxybenzoic acid (1.1 mg/kg), 3-phenyllactic acid (9.9 mg/kg) and benzoic acid (1.3 mg/kg) while grey ironbark honey contained *p*-hydroxybenzoic acid (0.8 mg/kg) and benzoic acid (2.1 mg/kg).

Published paper

Beiranvand, S., Williams, A., Long, S., Brooks, P., **Russell, F.D.** (2021). Use of kinetic data to model potential antioxidant activity: Radical Scavenging Capacity of Australian Eucalyptus honeys. *Food Chemistry*, 342; Article 128332.

ABSTRACT

Antioxidant activity of honeys may be beneficial in wound healing processes by protecting cells against lipid oxidation. The DPPH assay assesses the efficacy of antioxidant molecules to reduce DPPH$^{\bullet}$ to DPPHH. Studies determining EC_{50} are limited by single time-point determinations of antioxidant effect and can miss vital information about the rate of antioxidant response. Acquisition of kinetic data allows determination of the radical scavenging capacity (RSC) of honeys. The purpose of this study was to determine the RSC of 53 honeys from 16 species of Australian *Eucalyptus* trees and four samples of New Zealand manuka (*Leptospermum scoparium*) honey. Whereas honeys could not be differentiated based on EC_{50} values, significant differences were observed for RSC, supporting collection of kinetic data for honey analysis. The greatest RSC was observed for New Zealand manuka ($4.6\pm0.3\times10^{-5}$ mg.mL^{-1}.min^{-1}), grey ironbark (*E. paniculate*; $3.4\pm0.2\times10^{-5}$ mg.mL^{-1}.min^{-1}) and river red gum honeys (*E. camaldulensis*; $3.2\pm0.2\times10^{-5}$ mg.mL^{-1}.min^{-1}).

STUDY 2: Investigation of immune cell response to Australian honeys.

Several Australian honey varieties were assessed for their potential stimulatory or inhibitory effect on IL-6 secretion from fibroblasts, including river red gum, grey ironbark, messmate, yellow box, peppermint, cheeseberry and Tasmanian manuka honey. Samples were compared to a sample of New Zealand manuka and to a sugar control. Honeys were obtained by beekeepers from Queensland, New South Wales, and Tasmania, and supplied by Hive and Wellness Australia Pty Ltd. The New Zealand manuka was supplied by Global Proficiency, Ltd.

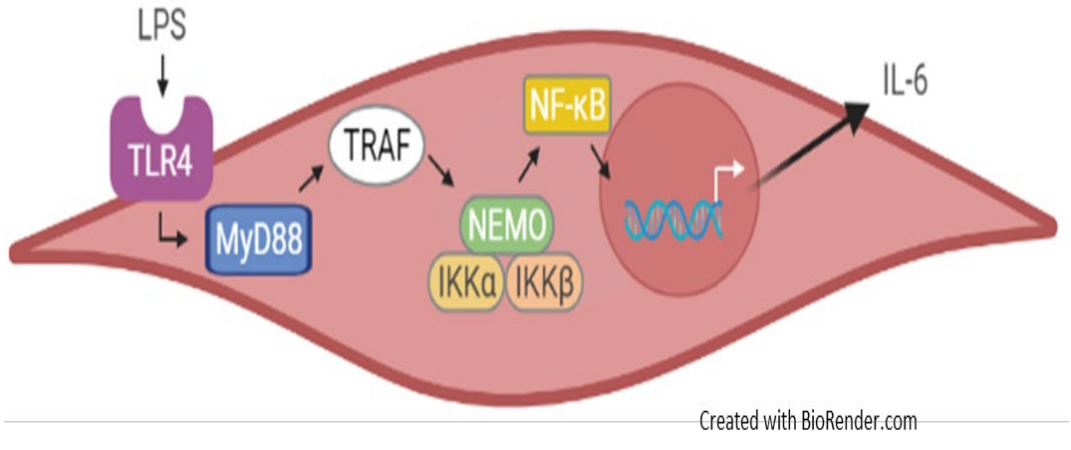

Figure 4: Lipopolysaccharide (LPS) activates toll like receptor 4 (TLR4) on fibroblasts, leading to secretion of interleukin-6 (IL-6).

We examined whether the addition of honey samples to cell culture media might modulate the secretion of IL-6 by cultured fibroblasts when the cells are exposed to the pro-inflammatory mediator, lipopolysaccharide (LPS). LPS activates a toll like receptor 4 (TLR4) signalling pathway that leads to secretion of cytokines, including interleukin-6 (IL-6) (Figure 4).

In fibroblasts, we observed an expected increase in IL-6 secretion after a 24-hour incubation of cells with 1.0 μg/mL LPS. However, it was not known whether honey might be able to interfere with the LPS response, and potentially reduce the secretion of IL-6. To test this, we incubated cells with a variety of honey samples, with and without LPS. We found that the different honey samples all stimulated an increase in IL-6 secretion. The magnitude of the secretory response was not uniform, with some honeys producing a moderate increase, while others stimulated a marked increase in IL-6 secretion.

Key findings:

1. Grey ironbark honey produced the largest secretory response of all the honeys tested, and this was also significantly greater than the response produced by the LPS. Interestingly, when cells were co-incubated with LPS and the honey, the response to honey was suppressed, and was no greater than that observed to LPS alone.

   This finding raised two hypotheses:
   a. that LPS was blocking a binding site that was otherwise occupied by the honey sample, or
   b. that chronic exposure of the cells to LPS led to the development of "endotoxin tolerance", a phenomenon in which cells become refractory to continued stimulus with the LPS.

   The first hypothesis is unlikely because the honey alone stimulated a cytokine secretory response. The second hypothesis is possible. Endotoxin tolerance is evident where there is a hyporesponsive secretion of inflammatory cytokines and is important clinically in several inflammatory conditions including neonatal encephalopathy (O'Dea et al. 2021) and sepsis (Leligdowicz et al., 2022).

   If endotoxin tolerance was the cause of the hypo-responsiveness to honey, we would expect to see a suppressed response to LPS with increasing concentrations of LPS. We therefore examined the secretion of IL-6 from cultured fibroblasts that were incubated with 0.05 – 1.00 μg/mL LPS. Consistent with the development of tolerance, the secretory effect of 1.0 μg/mL LPS was about half that of 0.1 μg/mL LPS. To exclude the possibility that the reduced secretory response to the higher concentration of LPS was due to a cytotoxic effect, we examined cell viability using an MTT assay and examined cell morphology using phase contrast microscopy. The findings revealed no cytotoxic effect of LPS at concentrations up to 1.00 μg/mL, and this was confirmed by examination of the cells using the microscopy.

2. The greatest secretion of IL-6 was observed to grey ironbark honey, for all honeys tested. We wanted to know whether this was a consistent finding for this variety of honey, and so tested the Il-6 secretory response in four samples collected from Queensland and NSW. The findings showed that the secretory response was significantly different between the four samples, suggesting that this response was not primarily associated with floral source, but rather another yet unidentified factor. The main constituent of honey is sugar, and this does

not vary greatly amongst samples. Nonetheless, the high sugar content imparts high osmotic stress and could conceivably stimulate secretion of cytokines from the cells. To test this possibility, we exposed cultured fibroblasts to an "artificial honey", that comprised the same types of sugars at the same concentrations that have been identified in genuine honey samples. The findings showed that sugar alone at concentrations up to 20 mg/mL had no stimulatory effect on IL-6 secretion, and that it didn't interfere with the secretory response to LPS.

We next asked, "what is the identity of the molecules in honey that stimulates a cytokine secretory response in cells"? To answer this, we examined the literature pertaining to honeybees and their association with bacterial contaminants (noting that Gram negative bacterial express LPS on their surface). It has been reported previously that bacteria exist within nectar, honey, and beebread (a mixture of pollen and nectar or honey), and that bacteria (primarily phylum Proteobacteria – alpha 2.1, alpha 2.2, and alpha 1; and the phylum Firmicutes – *Lactobacillus kunkeei*) reside within the foregut (specifically, the crop) of honeybees (Figure 5). The bacteria can be passed from forager honeybees after their return to the hive via process called trophallaxis (mouth-to-mouth exchange of nectar) (Wainselboim and Farina, 2003). We used an endotoxin detection assay kit to provide definitive evidence for the presence of LPS in the honey sample. In a 125 µg/mL sample of grey ironbark honey, LPS was detected at a level of about 0.88 µg/mL. To confirm the presence of LPS, the honey sample was filtered using a 10-kDa molecular weight cut off filter. Since the molecular weight of LPS is between 10-20 kDa in size, depending on the nature of the oligosaccharide chain, filtration would be expected to remove LPS from solution (Petsch and Anspach, 2000). When the ultrafiltered eluant was tested, no endotoxin was detected using the assay kit. To confirm this hypothesis, the ultrafiltrate of grey ironbark honey, and the ultrafiltrate of LPS, produced no stimulatory effect on IL-6 secretion in cultured fibroblasts, in marked contrast to the large secretion of IL-6 by non-filtered honey and non-filtered LPS.

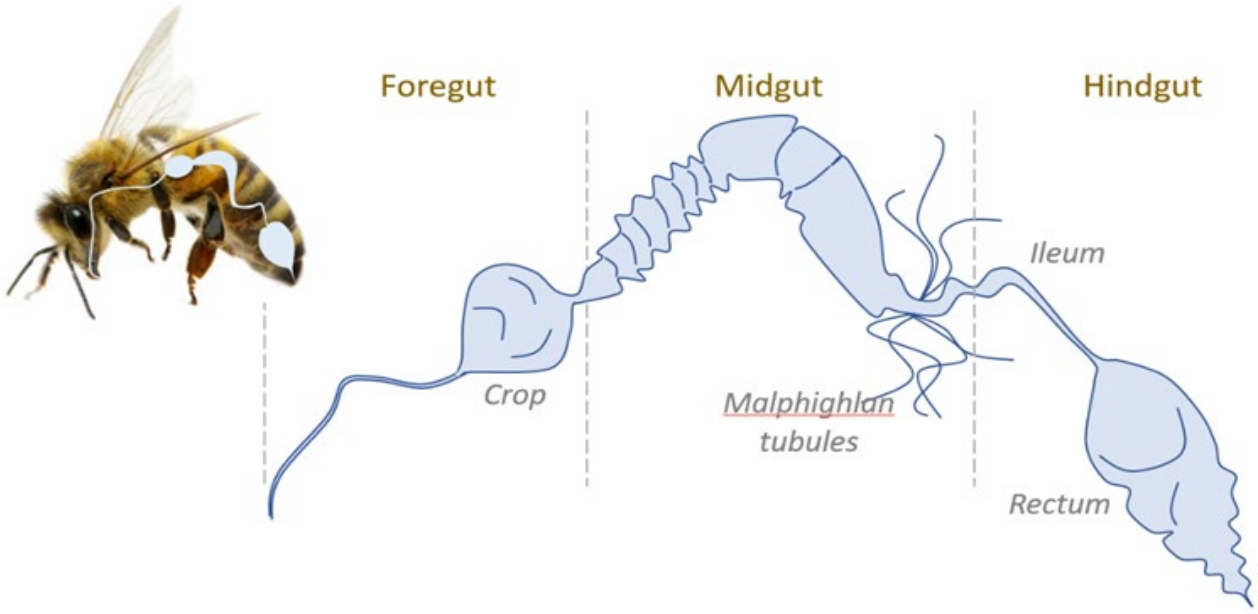

Figure 5: Bacteria reside in the crop of forager honeybees and can be transmitted to other bees via a process called traphallaxis (mouth-to-mouth exchange of nectar)

Gram negative bacteria are known to shed LPS (Wu et al., 2021). We therefore investigated whether the source of LPS in honey was associated with bacteria or with LPS that is shed from bacteria. To do this, the honey sample was first filtered through a 0.22 μm syringe filter, a process that would remove bacteria but allow the flux of shed LPS. Filtration of the honey in this manner had no significant effect on IL-6 secretion by cultured fibroblasts, indicating that the LPS is primarily shed from the bacteria rather than associated with the bacteria.

Honey contains several proteins, and so we examined the possibility that a honey protein might contribute to the IL-6 secretory response. To test this, the honey was boiled for 15 minutes; a process that would lead to protein denaturation and loss of function. The findings revealed that boiled and non-boiled honey samples were equally effective at stimulating secretion of IL-6 from fibroblasts, indicating that proteins within honey do not contribute to the response. Finally, low pH is a known stimulator of IL-6 secretion from cells (Rafiee et al., 2009). It was possible that addition of honey may have reduced the pH of the culture medium, which could have stimulated the secretion of IL-6. The honey was being used at a low concentration (i.e., diluted) and was being added to a buffered culture medium, and so we hypothesised that this addition of honey would have a negligible impact on pH. Consistent with this hypothesis, pH was not significantly altered, thus excluding altered pH as a contributor to the observed IL-6 response.

Having identified LPS in honey, we hypothesised that honey should therefore be able to activate the TLR4 signalling pathway (recall that LPS activates TLR4 receptors). To test this, we obtained a cell line (murine RAW264.7 macrophages) that express TLR4 on their cell surface. The cells contained a knock-in gene for secreted embryonic alkaline phosphatase (SEAP). In these cells, when TLR4 is activated, SEAP activity increases allowing it to catalyse the conversion of a SEAP substrate to a blue coloured product. Formation of this product, detected using a plate reader, is therefore evidence of TLR4 activation (Figure 6). In this novel cell type, exposure of the cells led to marked product formation, consistent with the activation of TLR4 signalling by honey (LPS). The response was abolished by incubation of the honey with polymyxin B, an antibiotic that chelates LPS. As expected, the artificial honey (sugar mixture) was without effect on the TLR4 signalling pathway. Definitive evidence of TLR4 activation was provided in cells that express SEAP but have knockout of the TLR4 gene. In these cells, honey did not stimulate blue coloured product formation.

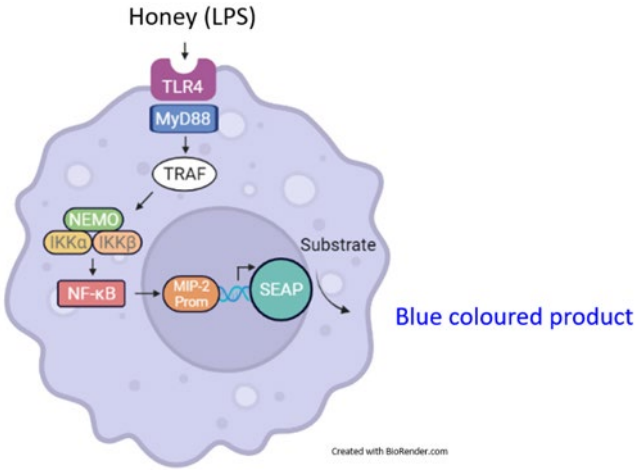

Figure 6: RAW264.7 cells used to investigate the effect of honey on TLR4 signalling.

Published paper

Russell, F.D., Visagie, J.C., Noll, J.L. Secretion of IL-6 by fibroblasts exposed to Australian honeys involves lipopolysaccharide and is independent of floral source. Scientific Reports submitted 31.08.2022.

ABSTRACT

Honey stimulates cellular secretion of cytokines, which has been attributed to activation of lipopolysaccharide (LPS)-dependent and LPS-independent pathways. The objective of this study was to identify whether LPS is present in Australian honey samples at levels that can stimulate interleukin-6 (IL-6) secretion by fibroblasts and whether it can transduce cell signaling by activating toll-like receptor 4 (TLR4). IL-6 was measured in culture media of fibroblasts exposed to honey for 24 h. LPS was detected in Australian *Eucalyptus* (grey ironbark) honey (0.61±0.05 μg/g honey) using an endotoxin assay. TLR4 signaling was observed in RAW264.7 macrophages that were exposed to honey and this activation was prevented by preincubating honey with the LPS-neutralising agent, polymyxin B. Australian *Eucalyptus*, *Leptospermum* and *Cyathode* honeys stimulated IL-6 secretion in cultured human dermal fibroblasts. To examine whether the response was dependent on floral source, fibroblasts were exposed to four different samples of grey ironbark honey obtained from Queensland and New South Wales. The magnitude of the cytokine response to these honeys was highly varied. We conclude that Australian honeys contain endotoxin at levels that can stimulate IL-6 secretion by fibroblasts and that signaling in macrophages involves TLR4 activation. The IL-6 secretory response was independent of floral source.

Impacts

This Project was most aligned with capability enhancement as it relates to the potential to value-add to commercial honey suppliers and beekeepers. The Project was instrumental in identifying methods for quantifying the antioxidant activity of Australian honeys. This information can be used by beekeepers to select honey varieties that provide greatest potential for antioxidant activity (Study 1) or immunomodulatory effect (Study 2).

An SOP was commissioned by the CRC for Honey Bee Products to compare two commercially available kits for the FRAP assay (Abcam and Invitrogen kits). Conditions for each kit were established for optimal measurement of antioxidant activity (temperature, duration, wavelength), and honey processing requirements (use of non-filtered honey). This project delivered a 9-page SOP that can be used by the honey industry for the measurement of antioxidant activity.

Outputs

The key educational outputs, including details of any courses developed.

Associate Professor devised a laboratory class for third year students enrolled in the Bachelor of Biomedical Science (BIM341 Biochemical Pharmacology). The laboratory class examined the effect of Australian honey samples on cell viability using the MTT cytotoxicity assay. The course is well received by students.

PhD Thesis: Ms. Soheila Beiranvand. Title of thesis: "Modulatory effects of Australian honeys on inflammation, oxidative stress and wound healing". The PhD candidate is currently responding to reviewers' comments, and anticipates submission of this response, together with revised thesis in July 2022. The final PhD oral presentation is scheduled for June 2022.

BSc (Honours) Thesis: Ms. Ashley Williams. Title of thesis: "Antioxidant enzyme activity of honey constituents in human macrophages." Ashley was awarded a First-Class Honours.

Special Research Project (SRP) Report: Ms. Jeanne Visagie. Title of project: "Effects of honey on IL-6 production in LPS-stimulated myofibroblasts using ELISA and confocal microscopy."

Special Research Project (SRP) Report: Ms. Jamie Noll: Title of project: "Inflammatory effect of LPS and honey on IL-6 expression in dermal fibroblasts."

Special Research Project (SRP) Report: Ms Symsia Long: Title of project: "The correlation of colour and pH of Australian honeys with antioxidant activity."

Peer-reviewed paper: Beiranvand, S., Williams, A., Long, S., Brooks, P., Russell, F.D. (2021). Use of kinetic data to model potential antioxidant activity: Radical Scavenging Capacity of Australian Eucalyptus honeys. *Food Chemistry*, 342; Article 128332. [Impact Factor 6.306; Q1, Analytical Chemistry; Q1, Food Science; Q1, Medicine (miscellaneous)]

This article has a Field Weighted Citation Index (FWCI) of 1.42, meaning that it has achieved 1.42-fold the expected impact relative to the world literature.

Peer-reviewed paper: Russell, F.D., Visagie, J.C., Noll, J.L. (2022). Secretion of IL-6 by fibroblasts exposed to Australian honeys involves lipopolysaccharide and is independent of floral source. Scientific Reports. *Submitted 31.08.2022.*

Presentations

This Project was also used to disseminate information about the potential medicinal benefits of Australian honeys. New knowledge about Australian honeys was reported via presentations to the local community and to Australian scientific conferences:

Russell, F.D., Beiranvand, S., Williams, A., Pappalardo, L., Askew, C.D., Brooks, P.R. Phenolic and flavonoid content of Australian honeys and their antioxidant potential. Australasian Honey Bee Research Conference, Oral presentation, 1st July 2021.

Russell, F.D., Beiranvand, S., Williams, A., Long, S., Brooks, P.R. Modelling the kinetics of antioxidant activity for Australian *Eucalyptus* honeys. Australasian Honey Bee Research Conference Oral presentation, 1st July 2021.

Russell, F.D. Antioxidant and anti-inflammatory activity in Australian honey. South Australian Apiarists' Association Conference, Oral presentation, Adelaide, 10th June 2019.

Russell, F.D. Speaker at "Moreton Minds", Caboolture, 13th Sept. 2019 "Healing Honeys".

Soheila Beiranvand, 3MT Competition, Genecology Showcase "Modulatory effects of Australian honeys on inflammation, oxidative stress and wound-healing" – 2nd Place Award.

Implications and Recommendations

This Project has established Australian honeys as a valuable and marketable asset for prevention of oxidative stress and aberrant immune responses. It was identified that some Australian honeys are more efficacious than others for producing these effects. Three *Eucalyptus* honeys had superior activity amongst several *Eucalyptus* varieties that were tested, and these were grey ironbark, river red gum and messmate honeys. Manuka honeys from Tasmania and New Zealand were also identified as having superior antioxidant activity. It is a recommendation of this Project that these honeys be investigated further in an *in vivo* setting to examine their wound-healing potential.

A main aim of the Project was to use a kinetic-based model to examine the radical scavenging capacity of Australian honeys over an extended time-course (150 minutes) and to test multiple concentration of honey. We found that this approach could be used to complement traditional methods of measuring antioxidant activity, which often entailed use of a single concentration of honey at a single time-point. The Project revealed that honeys that had a high radical scavenging capacity (kinetic model) were often also potent antioxidants, as determined by the measurement of EC_{50} values. The project showed that the botanical origin of the nectar that is collected by bees is a more important determinant of antioxidant activity than the honey's geographical origin. An implication of this is that beekeepers can be confident that if right type of honey is sourced, based on flora rather than geographical origin, a desired antioxidant characteristic can be attained.

This Project also studied the immunomodulatory effects of Australian honeys. For our studies, we focused on a non-immune cell type (fibroblast) because of its central role in wound healing responses. We found that these cells responded to honey samples with the secretion of interleukin-6 (IL-6), a pleiotropic cytokine capable of stimulating inflammatory, regenerative, and anti-inflammatory responses. The identification of lipopolysaccharide as a key mediator of the stimulatory response has implications in terms of how honey is used in a wound-healing setting. Our study supports current medical practice of sterilization of honey samples prior to application to open wounds. We emphasize that whilst honey might contain LPS, this does not present a health issue for ingestion of honey because of the excellent protective barrier afforded by the gastrointestinal tract against bacteria and LPS (Wassenaar et al., 2018).

Future research opportunities

This Project did not investigate honey samples in an *in vivo* (in the live animal) setting. Future research opportunities exist to determine whether Australian honey samples that were identified as having high antioxidant and immunomodulatory activity, can be used to to suppress immune responses in macrophages, to regulate autoimmune disease, infection, and inflammation. Chronic wounds remain a significant health burden, with >430,000 Australians afflicted by non-healing wounds, and an associated $3.5 billion imposition on the annual healthcare budget (McCosker et al., 2019). The investigation of honeys identified as bioactive in this Project, could be selected for randomized, placebo-controlled trials in patients with chronic wounds.

Further research is needed to provide a more thorough investigation of the immunomodulatory capacity of the Australian honeys. The Project investigated the effect of honeys on a single cytokine (IL-6). Expansion of this work to examine the effect on cell secretion of both proinflammatory (e.g., tumour necrosis factor-alpha) and anti-inflammatory cytokines (e.g., interleukin-10) is warranted. The Project has commenced these studies as well as an investigation of the effect of honey samples on cell migration and proliferation. For cell-based studies, there is also an opportunity to investigate cell-to-cell interactions, an expansion on the current use of monocultures. This could be achieved in a variety of ways, including the use of 3-D cell culture models and multi-chamber systems (e.g., Quasi-Vivo technology).

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