Antibacterial activity of Western Australian honey

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Foreword

Health attributes of honey are linked to the pureness of the product, and hence have become a consumer preference when purchasing honey. Whilst most of the honey is sugar, in the process of the honey bee transforming the flower nectar in an energy-loaded honey, many other special attributes are retained from the plant nectar and added to by the honey bee. Thus, if you compare a sugar solution to a honey, there can be a 100-fold difference in antibacterial activity depending on the floral source and health of the honey bee colony.

In south-west of WA, most of the honey comes from wild, native forest and the honey produced is some of the purest that is on offer world-wide. Even the honey bee husbandry, with so few diseases, makes this honey uniquely pure. This combination of ancient forest with healthy honey bees produces many honeys with particularly high levels of antibacterial activity.

Exciting was the development of the link between the nectar source and the antibacterial activity, and discovery of new plant sources that can produce high bioactivity. The new antibacterial test broadening the range of bacteria responding to honey has highlighted honey activity differences, with certain honeys possibly better to treat certain medical conditions than others. These discoveries will lead to a better understanding and characterisation of this antibacterial activity in honey, opening therapeutic opportunities.

Any alternative to the use of antibiotics is becoming more favoured, and so this interest in the using these newly discovered special honeys as a therapeutic is certainly the next stage for the WA industry.

Dr Liz Barbour
CEO

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About the Authors

Dr Kate Hammer is a Senior Lecturer and Researcher at The University of Western Australia. She teaches both undergraduate and postgraduate students, and supervises Honours, Masters and PhD students. Dr Hammer has been investigating the antimicrobial effects of natural products for more than 20 years. Her current research focuses on the antimicrobial activity of honey, particularly honeys from Western Australia. Previous research has included studying the impact of tea tree oil on microbial adhesion and biofilm development, investigation of potential bacterial stress or adaptive responses to tea tree oil and exploring the potential for bacterial resistance to arise against tea tree oil. Dr Hammer has more than 80 publications in natural products and antimicrobial activity.

Kathryn Green joined the Hammer Research group as a Research Officer mid-2017 at the beginning of this project after completing a Master of Clinical Pathology in 2016. She has been responsible for generating almost all the antibacterial activity and physicochemical data within this report and played an important role in compiling and interpreting the data.

Robbie Haines joined the group in 2021 after completing a Master of Infectious Diseases that same year. He has assisted in data generation and interpretation, as well as manuscript preparation in the closing stages of the project.

Apis mellifera and *Banksia sessilis*

Acknowledgments

The authors thank all Western Australian beekeepers for providing many of the honeys examined in this study and thank those within the honeybee industry for sharing their knowledge and expertise with use along the way.

Many students contributed to generating data during this project. These included Honours students Moses van Bawi Chawn, Zach Jones, Anna Takahashi and Allouette Bena, and Master of Infectious Diseases Research Project students Azhar Sindi, Magda Escorcia, Brayden Gray, Ayushi Chhawchharia and Shuihui Xi.

This project was conducted in close collaboration with the research group of Dr Connie Locher, School of Allied Health, UWA. The PhD students Md Khairul Islam and Ivan Lawag, who are both co-supervised by Dr Locher and Dr Hammer, generated data that was utilised across both CRC Projects 12 and 13.

We thank the laboratory of Professor Peter Brooks at University of the Sunshine Coast for determining levels of methylglyoxal in *Leptospermum* honeys.

The authors acknowledge the collaboration with Ken Dods at Chem Centre that enabled the development of the new protocol for determining the antibacterial activity of honey, which was funded by the Department of Primary Industry and Regional Development's (DPIRD) Grower Group Research and Development Program (GGRD 2015-0028-AGSC; https://dpird.wa.gov.au/), and CRC for Honeybee Products (Grant no. 015; http://www.crchoneybeeproducts.com/).

The authors wish to acknowledge the support of AgriFutures Australia, who through their support of the CRC for Honey Bee Products contributed to this research.

The authors acknowledge the Whadjuk people of the Noongar nation, who are the traditional custodians of the land on which this research was conducted. We also acknowledge the traditional custodians of the lands from which the honeys used in this study were collected, including the Noongar, Yawuru, and Yamatji people, and pay respects to their elders of past, present, and future.

Abbreviations

AAV	Antibacterial activity value
ATCC	American Type Culture collection
DHA	Dihydroxyacetone
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
GMP	Good manufacturing process
h	Hours
HPTLC	High performance thin layer chromatography
MGO	Methylglyoxal
µM	Micromolar
MIC	Minimum inhibitory concentration
n	Number
NPA	Non-peroxide activity
PE	Phenol equivalence
TA	Total activity
TE	Trolox equivalent
TGA	Therapeutic Goods Administration
WA	Western Australia
w/v	Weight for volume

Executive Summary

Aims

The major aim of this research was to investigate and measure the antibacterial activity of a wide range of different types of honeys from across Western Australia. The purpose of this was to gain an in-depth understanding of the characteristics of each honey type in terms of the level of antibacterial activity and chemical characteristics.

Methods

Over 430 honeys samples were obtained from more than 40 unique, Western Australian floral sources. Honeys were obtained from individual beekeepers or purchased from retailers.

Antibacterial activity was quantified using several different methods. The first was the phenol equivalence assay, which is an agar-diffusion based test that generates a 'Total Activity' or TA value. The second was the broth microdilution assay, which is a broth-based assay that generates a minimum inhibitory concentration (MIC). The third assay was an adaptation of the broth microdilution assay, in which the endpoint is determined using optical density and this assay generates an Antibacterial Activity Value (AAV) for each honey, based on the levels of bacterial growth inhibition. This last assay was developed in conjunction with ChemCentre WA, with funding provided by Department of Primary Industry and Regional Development's (DPIRD) Grower Group Research and Development Program and the CRC for Honey Bee Products.

A selection of physicochemical parameters was determined, including refractive index, Brix, pH, colour and the generation of hydrogen peroxide. Additional characteristics, namely total phenolics content and antioxidant activity were determined by students within Dr Connie Locher's research group, as was the High-Performance Thin Layer Chromatography (HPTLC) non-sugar signatures, which were instrumental in confirming the floral sources of individual honey samples.

Results

Analysis of antibacterial activity data showed that the honeys that were generally the highest in antibacterial activity were derived from Jarrah (*Eucalyptus marginata*), Marri (*Corymbia calophylla*), Forest Blackbutt (*Eucalyptus patens*), Red Bell (*Calothamnus*), Karri (*Eucalyptus diversicolor*) and Coastal Blackbutt (*Eucalyptus todtiana*). Moderate antibacterial activity was observed for most honeys derived from *Leptospermum* species, Wandoo (*Eucalyptus wandoo*), Powderbark (*Eucalyptus astringens*), *Melaleuca* species and Parrot bush (*Banksia sessilis*). Relatively low activity was seen for most honeys from Peppermint (*Agonis flexuosa*), Yate (*Eucalyptus occidentalis*), Moort (*Eucalyptus platypus*) and *Banksia menziesii*, in addition to mallee, spring and wildflower honeys.

For many of the more unusual or rare honeys, only a few samples were obtained, which meant that no generalisations about the antibacterial activity of that floral source could be made.

Since the honeys were derived from across the south-west of Western Australia, honeys were grouped according to three biogeographical regions: Coastal areas, Inland areas including Esperance, and the Southwest forest region. Publications have been prepared describing the honeys derived from each of these unique biogeographical regions.

In addition to the data described above, several other, specific projects were undertaken as part of this research project.

In the first subproject, we tested the activity of a small selection of honeys against bacteria that the skin infection known as “school sores” or impetigo is caused by. The purpose of this was to assess whether honey may be a possible treatment option for mild cases of this skin infection. The results were positive and indicated that the pathogenic bacteria are indeed susceptible to honey. The next stage of investigation would be to conduct clinical studies with patients to see if honey works in a real-world situation.

In the second sub-project we tested a selection of honeys against a range of yeasts, again to see whether honey can inhibit the growth or kill yeasts. Results were positive, indicating that honey does inhibit and kill yeasts, although higher concentrations are required compared to the amount required to inhibit the growth of bacteria.

In a third subproject we investigated whether honey could be combined with standard antiseptics or with essential oils to see what effect this had on antibacterial activity. Sometimes when antibacterial agents are combined, chemical interactions between the two different agents can lead to unexpected changes in antibacterial activity, such as lower or higher than expected activity. In many cases, combining two agents leads to no changes, which is mostly what we observed when honey was combined with either antiseptics or essential oils. However, when honey was combined with chlorhexidine antiseptic, we observed antagonism, meaning that the overall antibacterial activity was in fact lower than expected. The reasons for this are not yet known but indicate that honey should probably not be combined with chlorhexidine in pharmaceutical products.

In subproject 4, we purchased several commercial manuka honey samples, and tested them for levels of methylglyoxal (MGO), which is an important contributor to antibacterial activity. We also measured antibacterial activity using a broth microdilution assay, and then correlated antibacterial activity with levels of MGO. For some bacterial species the correlation was reasonably strong, whereby higher MGO was associated with higher antibacterial activity but for other bacterial species the relationship was relatively weak, and MGO levels had little impact on antibacterial activity. This indicates that bacteria differ in their responses to manuka honey and MGO and highlights the importance of testing more than one bacterial species when determining susceptibility.

Implications and Recommendations

Many important discoveries have been made as part of this research, including a comprehensive dataset describing the antibacterial activity and physicochemical characteristics of WA honeys, the potential suitability of honey for treating the mild skin infection impetigo, the activity of honeys against yeasts, interactions between honey and antiseptics or essential oils, and an investigation of the antibacterial activity of commercial manuka honeys. Importantly, activity was determined using the broth microdilution method and AAV methodology, meaning that activity could be accurately quantified in all honeys. This contrasts with the phenol equivalence assay, which is not sensitive enough to detect moderate to low levels of activity and is also subject to inter-laboratory variation. All these discoveries and insights provide valuable information to the Western Australian beekeeping industry and may enhance the profitability of beekeeping in Western Australia.

Academic outputs

The following manuscripts are in preparation, submitted or have been published, because of this research project. The major findings and implications from each of these outputs is described in a subsequent section of this report.

OUTPUT (Manuscript title)	STATUS
1. "Anti-biofilm effects and characterisation of the hydrogen peroxide activity of a range of Western Australian honeys compared to Manuka and multifloral honeys"	**Published**
2. "Honeys derived from plants of the coastal sandplains of Western Australia: antibacterial and antioxidant activity, and other characteristics."	**Published**
3. "Antibacterial interactions between two monofloral honeys and several topical antiseptics, including essential oils."	**Published**
4. "Activity of honeys on bacteria associated with the skin infection impetigo"	**Published**
5. "Antibacterial activity of Manuka and Leptospermum honeys"	**Published**
6. "Effect of different storage conditions on the antibacterial activity of honeys"	In preparation
7. "Activity of Western Australian honeys against clinically important yeasts"	In preparation
8. "Insights into the mechanism of antibacterial action of some Western Australian honeys"	In preparation
9. "Antibacterial and antioxidant activities, and phytochemical characteristics of honeys derived from plants in the Goldfields-Esperance and Wheatbelt Regions of Western Australia"	In preparation
10. "Antibacterial and antioxidant activities, and phytochemical characteristics of honeys derived from the southwest forests of Western Australia"	In preparation

Industry outputs

1) CRC Case Study: “Measuring the antibacterial activity of honey”

This case report describes the most common methods used for quantifying the antibacterial activity of honeys. It compares the phenol equivalence method, determination of minimum inhibitory concentrations (MICs) and the antibacterial activity value (AAV).

It also describes results of a “ring-test” conducted across different laboratories to assess the AAV methodology.

In addition to publication as a CRC Case Study, in June 2022 this was submitted for publication to The Australasian Beekeeper.

2) Bibliography of published, industry and grey data describing characteristics of WA honeys

This is a compilation of all publications that describe any characteristics of any honeys or other bee products derived from WA endemic plants. It is presented in a subsequent section of this report.

Introduction

Western Australia is home to eight of Australia's 15 biodiversity hotspots and has unique endemic flora. Honey bees (*Apis mellifera*) forage on many of these unique species, collecting nectar and/or pollen, which they turn into honey. Where they have collected nectar from predominantly a single floral source, a monofloral or varietal honey will be produced, whereas if they forage from multiple nectar sources a multifloral honey results. Different monofloral honeys vary in a range of characteristics, such as colour, viscosity and flavour, and in antibacterial activity.

Many iconic honeys are produced in WA, including Jarrah (from *Eucalyptus marginata*), Marri (from *Corymbia calophylla*), Karri (from *Eucalyptus diversicolor*), Red Bell (from *Calothamnus* spp.) and Peppermint (from *Agonis flexuosa*), to name a few. Some are renowned for their medicinal properties, such as antibacterial and antioxidant activity, however, very little scientific data have been published to support this.

For example, at the beginning of this study in 2017, searches in the medical and scientific literature databases PubMed and ScienceDirect using the phrase "Jarrah honey", which is probably the best-known WA honey, found only one publication per database (Irish et al., 2011; Alquarni et al 2014). When this search was repeated in June 2022 this had increased to six publications in each database, of which only one overlapped, meaning that 11 publications were now found, compared to two. Although this is by no means a comprehensive search of all relevant published and unpublished literature, it illustrates that whilst very little data has been published, it is a major marketing narrative in marketing campaigns for honey produced from Australian flora.

The antibacterial of honey is complex, as it occurs via a combination of antibacterial factors, several of which change depending on the concentration of honey being examined [1-3]. For example, at high honey concentrations, the high concentration of sugars exerts a substantial osmotic effect and draws water from inside the bacterial cells to the outside, effectively dehydrating them. At lower honey concentrations, this effect may not occur as the sugars are too dilute, in which case additional antibacterial factors, such as the relatively low pH and hydrogen peroxide, may exert antibacterial effects. In addition to the osmotic, pH and hydrogen peroxide effects, antibacterial activity may be attributed to a range of minor components such as organic acids, nectar-derived phytochemicals, and bee-derived defensins [4-6]. Whilst the osmotic activity and pH are found across all honeys, hydrogen peroxide and the remaining minor components may vary substantially between floral sources.

Manuka honey has been particularly well-studied for both antibacterial mechanisms and other characteristics. Manuka honeys contains the antibacterial compound methylglyoxal (MGO), which is derived from the precursor molecule dihydroxyacetone (DHA) that is present in the pollen and nectar that the bees collect from specific *Leptospermum* species [7, 8]. MGO contributes to the non-peroxide activity (NPA) of these honeys [9], which is antibacterial activity that is due to factors other than hydrogen peroxide. Historically, this type of activity has been deemed highly desirable as it is stable over time, but there is no reason to believe that honeys with peroxide activity, such as Jarrah and Marri, do not also have beneficial antibacterial activity and therapeutic potential.

Robust scientific data are required to support claims made about our unique Western Australian honey, whether this relates to antibacterial activity, antioxidant activity, floral source, health claims, characteristic flavour or other claims. This project has built on foundational work by Rob Manning, and others at the Department of Agriculture, analysing the antibacterial activity of Western Australian honeys. The data

described in this report and resulting publications, provides a valuable resource and wealth of information for both current WA Honeybee industry participants and those joining the industry in the future.

Objective

The objective of this project, as per the Project Agreement, is as follows:

The overall aim of this research project is to establish a comprehensive database on the spectrum of antimicrobial activity and chemical composition of WA honeys.

This aim is further expanded under each of the key activities below.

Key activities

The key activities of this project, as per the Project Agreement, are as follows:

1. **Collect a variety of WA honeys from a range of biogeographical areas at different time points and confirm the identity of the floral source.**

The collection of honeys will be done with the assistance of a local beekeeper, who will facilitate the collection of unique monofloral honeys from members of the local beekeeping community. All honeys will be mapped according to the biogeographical region from which they were obtained, to ensure that a range of honeys representing each of the different zones are obtained. Honeys from specific floral sources may also be targeted to ensure that the spectrum of monofloral honeys are investigated.

2. **Conduct a thorough investigation of the antimicrobial activity of these honeys.**

The antimicrobial activity of honey has historically been determined using a Phenol Equivalence Assay [10]. This assay, whilst well-known in the honey industry, is not without problems [11] as results can vary both between test repeats and between different laboratories. In addition, results produced by the method may not be representative of the "true" activity of the honey, as the assay uses only one test isolate and relies on the diffusion of honey through agar, which is a potential source of variability [12]. Given these significant shortcomings, several different assays will be used to investigate antimicrobial activity, including liquid -based assays, which ensure a more even dispersal of honey in solution. Also, honeys will be tested for activity against a range of relevant microorganisms, rather than just a single species. This ensures that all aspects of bioactivity are covered.

3. **Obtain a High-Performance Thin Layer Chromatography (HPTLC) non-sugar signature of each honey as point of reference and as a visual analysis tool for future authentication and quality control.**

Very recently, HPTLC signatures have been developed as a tool for assessing the floral source of honeys, based on the unique banding patterns produced by the non-sugar HPTLC analysis. However, only a few honeys have been investigated by this technique so this will be expanded to ensure that unique signatures are achievable for all honeys and to identify bands unique to each honey type.

4. Characterise the physicochemical properties of each honey.

Standard characteristics of each honey will be considered, including water activity, pH, colour, hydrogen peroxide production, total phenolic and total flavonoids. Investigation of these parameters will enable subsequent analyses investigating significant relationships between parameters to be performed.

5. Monitor bioactivity and chemical composition over time when exposed to different storage and processing conditions

Honeys that possess hydrogen peroxide activity are known in the honey industry to lose activity over time. This part of the project will systematically investigate levels of activity and chemical composition over time and in relation to specific storage conditions.

6. Construct a database containing all relevant honey parameters

A database will be constructed that will contain all the relevant honey parameters. This will enable database analyses to be undertaken, which may identify key phytochemical and bioactivity characteristics of honeys by bioregion and mono-floral honey opportunities. The database will be used to analyse relationships between key compositional components, evaluate trends in floral origin of honey analysed, analyse relationships between bioactivity and chemical characteristics, determine key characteristics that contribute to high antibacterial activity, identify important relationships between bioregion and honey bioactivity and to correlate key honey characteristics with other relevant features or events such as unusual weather patterns or flowering seasons.

7. Evaluate the therapeutic potential of WA honeys.

The data generated in relation to bioactivity and chemical characteristics will be evaluated in the context of determining the suitability of honeys for therapeutic purposes. One of the best-known uses of medicinal honey is in the treatment of chronic wounds or ulcers, and data will be evaluated to determine whether this is also a suitable application for the honeys investigated in this study. In addition, alternative therapeutic options will be investigated to determine if further opportunities may be appropriate.

Impacts

The impacts of this project, as per the Project Agreement are as follows:

1. The obtained information on these honeys can be used for marketing and valuing honey bee products from different regions of WA. New, unique bioactive honeys may be identified.
2. The suitability of HPTLC as a simple, real time analysis tool for beekeepers and packers in honey quality control can be assessed.

Outputs

The outputs of this project, as per the Project Agreement are as follows:

1. Database analysis undertaken to report on key phytochemical and bioactivity characteristics of honeys by bioregion and mono-floral honey opportunities
2. In-depth antimicrobial analysis and bacteria assessment of selected honeys of interest. Correlation to chemical analysis undertaken.

To maximise the dissemination of the project results, data have been organised into the following manuscripts for publication. The table below shows how each of the outputs above are related to each manuscript.

An additional output not explicitly stated in the Project Agreement was to compile a bibliography of published, industry and grey data describing characteristics of WA honeys.

Manuscript	Relevant outputs	MANUSCRIPT STATUS
1. "Anti-biofilm effects and characterisation of the hydrogen peroxide activity of a range of Western Australian honeys compared to Manuka and multifloral honeys"	**2**	**Published**
2. "Honeys derived from plants of the coastal sandplains of Western Australia: antibacterial and antioxidant activity, and other characteristics."	**1, 2**	**Published**
3. "Antibacterial interactions between two monofloral honeys and several topical antiseptics, including essential oils."	2	**Published**
4. "Activity of honeys on bacteria associated with the skin infection impetigo"	2	**Published**
5. "Antibacterial activity of Manuka and Leptospermum honeys"	2	**Published**
6. "Effect of different storage conditions on the antibacterial activity of honeys"	2	In preparation
7. "Activity of Western Australian honeys against clinically important yeasts"	2	In preparation
8. "Insights into the mechanism of antibacterial action of some Western Australian honeys"	1, 2	In preparation
9. "Antibacterial and antioxidant activities, and phytochemical characteristics of honeys derived from plants in the Goldfields-Esperance and Wheat belt Regions of Western Australia"	**1, 2**	In preparation
10. "Antibacterial and antioxidant activities, and phytochemical characteristics of honeys derived from the southwest forests of Western Australia"	**1, 2**	In preparation

1. Anti-biofilm effects and characterisation of the hydrogen peroxide activity of a range of Western Australian honeys compared to Manuka and multifloral honeys

This paper was published in the journal **Scientific Reports** in 2019.
https://www.nature.com/articles/s41598-019-54217-8

2. Honeys derived from plants of the coastal sandplains of Western Australia: antibacterial and antioxidant activity, and other characteristics.

This paper was published in the **Journal of Apicultural Research** in March 2022.

https://www.tandfonline.com/doi/full/10.1080/00218839.2022.2073953

Summary: This paper is the first of three papers describing the characteristics of Western Australian honeys derived from specific bioregions. This paper describes the antibacterial activity and physicochemical characteristics of coastal area honeys such as Banksia, Red Bell, Coastal Blackbutt, spring and wildflower. It also includes some honeys from the Broome area of Western Australia.

3. Antibacterial interactions between two monofloral honeys and several topical antiseptics, including essential oils.

Authors: Brayden H. Gray, Kathryn J. Green, Robbie R. Haines, Katherine A. Hammer.

This paper was published in the journal **BMC Complementary Medicine and Therapies**. Dec 2022, 22, 1, 10 p., 228.

https://bmccomplementmedtherapies.biomedcentral.com/articles/10.1186/s12906-022-03695-x

Summary: This paper investigates interactions between two honeys (Marri and Manuka) with antiseptics (chlorhexidine digluconate, silver nitrate and benzalkonium chloride) or essential oils (tea tree oil and eucalyptus oil). The two test bacteria were *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The interactions between honey and other agents were largely additive or indifferent (and did not indicate synergy). The exception was chlorhexidine digluconate combined with either marri or manuka, which was shown to be antagonistic, whereby the antibacterial activity was reduced when the two agents were combined.

4. Activity of honeys on bacteria associated with the skin infection impetigo

This manuscript has been published in the journal **Complementary Therapies in Clinical Practice**. Volume 49, November 2022, 101640.

https://www.sciencedirect.com/science/article/pii/S1744388122001086?via%3Dihub

Summary: In this paper, 10 clinical isolates each of *Staphylococcus aureus*, coagulase negative staphylococci (normal skin flora) and *Streptococcus pyogenes* were tested for their susceptibility to honey and hydrogen peroxide. Six types of honey were investigated including Jarrah, Marri, Banksia sessilis, Red Bell, Wandoo, multifloral, Manuka and artificial honey. All staphylococci were susceptible to the honeys

whereas the streptococci were less susceptible. The streptococci require additional protein in the culture medium to grow properly, and it was discovered that the additional of protein reduced the antibacterial activity of honey, suggesting antagonism. Although these data are encouraging, the true usefulness of honey in treating mild skin infections can only be established by conducting a clinical trial.

5. Antibacterial activity of Manuka and Leptospermum honeys

This manuscript was published in **PLoS One,** volume 17 (July), e0272376.

https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0272376

Summary: This manuscript examined the antibacterial activity of 29 manuka/Leptospermum honeys from New Zealand and Australia. Levels of MGO were determined by Dr Peter Brooks at the University of the Sunshine Coast.

6. Effect of different storage conditions on the antibacterial activity of honeys

This manuscript will be finalised by June 2022. The current plan is to submit this paper to the research topic "Honey Research in Ecological and Human Sustenance", within **Frontiers in Nutrition**.

Summary: Ten honeys (six Jarrah and four Marri) were subjected to different storage temperatures and times. Antibacterial activity was quantified, as was hydrogen peroxide generation, colour and water content (see Output 6). Storage temperatures investigated were ~23°C (room temperature), 4°C, -20°C, -80°C, 37°C and 45°C. Honeys were storage for up to 32 weeks and were re-tested at regular intervals for antibacterial activity. In addition, a collection of honeys that had been stored for months to years were re-tested for antibacterial activity after the extended storage periods.

7. Activity of Western Australian honeys against clinically important yeasts

This manuscript will be finalised by August 2022. The plan is to submit it to the journal **Medical Mycology**.

Summary: This study investigated the susceptibility of 10 yeast isolates to honeys. Six types of honey were investigated including Jarrah, Marri, Banksia sessilis, Red Bell, Wandoo, multifloral, Manuka and artificial honey. MICs were determined and time-kill data were obtained, which illustrated that the honeys exerted a relatively slow rate of killing against the yeasts. The MIC data demonstrated that yeasts are killed by honeys, but at higher concentrations than those required for bacteria. This may be a result of the major differences in cell wall architecture of yeasts and bacteria, and/or the substantially higher osmo-tolerance of yeasts compared to bacteria.

8. Insights into the mechanism of antibacterial action of some Western Australian honeys

This work is ongoing. This manuscript will be finalised by December 2022.

Summary: This work will describe various studies conducted investigating the mechanism of action of Western Australian honeys.

9. Antibacterial and antioxidant activities, and phytochemical characteristics of honeys derived from plants in the Goldfields-Esperance and Wheatbelt Regions of Western Australia

This manuscript is currently being prepared. The journal has not yet been selected.

Summary: This paper is the second of three papers describing the characteristics of Western Australian honeys derived from specific bioregions. This paper describes the antibacterial activity and physicochemical characteristics of honeys harvested from inland areas such as the wheatbelt and goldfields, as well as the southern bioregions of Mallee and Esperance. This includes honeys such as Mallee, Yate, *Eremophila*, Moort and Mallet.

10. Antibacterial and antioxidant activities, and phytochemical characteristics of honeys derived from the southwest forests of Western Australia

This manuscript will be finalised by July 2022. The journal has not yet been selected.

Summary: This paper is the last of three papers describing the characteristics of Western Australian honeys derived from specific bioregions. This paper describes the antibacterial activity and physicochemical characteristics of honeys harvested from the Jarrah forest and Warren bioregions of Western Australia. It includes Jarrah, Marri, Wandoo/White gum, Karri and Forest Blackbutt.

Bibliography of published, industry and grey data describing characteristics of WA honeys

Since there is very little data published on the properties of WA honeys, and it may sometimes be 'hidden' within a publication, meaning that it is not immediately obvious that the data is even there, a bibliography of literature describing WA hive products has been provided below.

The list includes only publications that mention WA hive products (honey, pollen, propolis) and either bioactivity (antimicrobial, antioxidant) or composition. The collection is sorted from most recent to oldest and brief notes have been added to denote what relevant data the publication contains.

In total, there are 30 papers/reports listed below.

2022 - 2020
Al-kafaween MA, Al-Jamal HAN. A comparative study of antibacterial and anti-virulence activities of four selected honeys to Manuka honey. Iran J Microbiol. 2022;14(2):238-51. Honeys: Jarrah Data: Antibacterial (zones of inhibition, MICs, Time-kill assays, anti-biofilm)
Green KJ, Islam MK, Lawag I, Locher C, Hammer KA. Honeys derived from plants of the coastal sandplains of Western Australia: antibacterial and antioxidant activity, and other characteristics. Journal of Apicultural Research. 2022:1-14. doi: 10.1080/00218839.2022.2073953. Honeys: Banksia, Red Bell, Peppermint, Coastal Blackbutt, Parrot bush Data: Antibacterial (MICs, AAV, TA), physicochemical (pH, colour, Brix, hydrogen peroxide, total phenolics), Antioxidant activity, HPTLC profiles
Islam MK, Lawag IL, Green KJ, Sostaric T, Hammer KA, Lim LY, et al. An investigation of the suitability of melissopalynology to authenticate Jarrah honey. Curr Res Food Sci. 2022;5:506-14. doi: 10.1016/j.crfs.2022.02.014. Honeys: Jarrah, Blackbutt Data: HPTLC organic extract profiles, pollen analysis
Islam MK, Sostaric T, Lim LY, Hammer K, Locher C. Sugar Profiling of Honeys for Authentication and Detection of Adulterants Using High-Performance Thin Layer Chromatography. Molecules. 2020;25(22). doi: 10.3390/molecules25225289. Honeys: Jarrah, Marri, Karri, Peppermint, Wandoo Data: Sugar content

Islam MK, Sostaric T, Lim LY, Hammer K, Locher C. Development of an HPTLC-based dynamic reference standard for the analysis of complex natural products using Jarrah honey as test sample. Plos One. 2021;16(7). doi: ARTN e0254857. 10.1371/journal.pone.0254857. Honeys: Jarrah Data: HPTLC organic extract profiles
Islam MK, Sostaric T, Lim LY, Hammer K, Locher C. Antioxidant HPTLC-DPPH Fingerprinting of Honeys and Tracking of Antioxidant Constituents upon Thermal Exposure. Foods. 2021;10(2). doi: ARTN 357. 10.3390/foods10020357. Honeys: Marri, Leptospermum Data: Antioxidant activity
Islam MK, Vinsen K, Sostaric T, Lim LY, Locher C. Detection of syrup adulterants in manuka and jarrah honey using HPTLC-multivariate data analysis. Peerj. 2021;9. doi: ARTN e12186 10.7717/peerj.12186. Honeys: Jarrah Data: HPTLC sugar and organic extract profiles
Lawag IL, Yoo O, Lim LY, Hammer K, Locher C. Optimisation of Bee Pollen Extraction to Maximise Extractable Antioxidant Constituents. Antioxidants (Basel). 2021;10(7). doi: 10.3390/antiox10071113. Honeys: no honeys: Jarrah and Marri pollen Data: Antioxidant activity
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Honeys: unspecified WA honeys

Data: Chemical residues, microbiological contamination

Manning R. Pollen Analysis of Eucalypts in Western Australia. A report for the Rural Industries Research and Development Corporation. RIRDC Publication No 01/53. 2001.

Honeys: no honeys, pollen

Data: Chemical composition

pre-2000

Chandler BV, Fenwick D, Orlova T, Reynolds T. Composition of Australian Honeys. Division of Food Research Technical Paper No 38; Commonwealth Scientific and Industrial·Research Organisation, Australia. 1974;(Division of Food Research Technical Paper No. 38).

Honeys: Yorrell (*E. gracilis*), Jarrah, Karri, Marri, Forest Blackbutt, Gimlet, Banksia, Grass tree

Data: Moisture, crystallinity, ash, pH, acidity, sugars, diastase, HMF, colour

Smith FG. The Sucrose Content of Western Australian Honey. J Apicult Res. 1965;4(3):177-84. doi: 10.1080/00218839.1965.11100120.

Honeys: Banksia

Data: Sucrose content

Project activities and results

The project activities of this project, as per the Project Agreement are as follows:

Collect a variety of WA honeys from a range of biogeographical areas at different time points and confirm the identity of the floral source.

More than 430 honeys were collected between June 2017 and May 2022. Within each year, honey collection time points reflected the various flowering seasons over spring and summer. For example, multifloral, spring, banksia and red bell were largely collected at the start of the honey season in spring, whereas Marri and Jarrah followed later in summer.

Honeys were obtained from several biogeographical regions in Western Australia, including the Swan coastal plain, Geraldton sandplains, Dampierland, Avon Wheatbelt, Coolgardie, Mallee, Esperance, Jarrah Forest and Warren bioregions, and are summarised in the table below.

Table 1: Collection of honeys obtained during CRC Project 12.

Grouping for publication*	Bioregion	Number of honeys	Major floral sources
Coastal region	Dampierland	21	Bloodwood, Grevillea, Melaleuca, Watermelon
	Geraldton sandplains	17	Banksia, Bottlebrush, Coastal, Wildflower
	Swan coastal plain	110	Banksia, Parrot bush, Red Bell, Peppermint, Coastal Blackbutt
Inland/Esperance region	Avon wheatbelt	18	Mallee, Marri, Powderbark, Wandoo, York gum
	Coolgardie	19	Mallee, Merrit, “Goldfields”, Gimlet
	Esperance	33	Mallee, Moort, Yate
	Mallee	6	Mallee, Yate
	Murchison	3	Eucalypt
	Yalgoo	1	Wildflower
Forest region	Jarrah forest	126	Jarrah, Marri, Powderbark, Wandoo, Forest Blackbutt
	Warren	26	Karri
	No data	59	
	TOTAL	439	

* Honeys were grouped into three major bioregion clusters for publication.

Regarding “confirming floral source”, this is not as straightforward as it may sound. The floral source was provided by the beekeeper and was assumed to be correct. It was usually provided as a common name, examples of which include Parrot bush, Red Bell, Yate, Jarrah, Marri and Mallee. Where beekeepers also provided harvest location the floral source could be cross-checked with FloraBase to check whether the floral source was found at the location specified.

Scientific techniques used to establish floral source include pollen counting, quantification of specific biomarker molecules and characterisation of phenolics profiles. The HPTLC technique developed by Dr Connie Locher for CRC Project 13 was used to investigate the non-sugar signature (which includes the phenolics) of honeys from specific floral sources collected for this project. Where enough honey samples were obtained that showed a consistent HPTLC signature, a “typical” signature was established. Any new honeys obtained could then be checked against the reference signature to confirm the floral source. Typical non-sugar signatures were established for several, but not all, floral sources during this project.

Conduct a thorough investigation of the antimicrobial activity of these honeys.

The antibacterial activity of all honeys was investigated by determining three values for each honey, as follows:

1) Phenol equivalence value (or Total Activity) using *S. aureus* ATCC 25923
2) Minimum inhibitory concentrations (MICs) again *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853.
3) AAVs (antibacterial activity values) [12]

Additional investigations were conducted on a subset of honeys as part of specific smaller projects conducted throughout the research project.

Results of these investigations are described in detail in the manuscripts arising from this research. A summary of antibacterial data is provided below. In the table, the floral sources are those that were provided by the beekeepers. Some have been verified by HPTLC fingerprinting whereas for others, too few samples were obtained thus no “typical” fingerprint as established.

For MIC data, note that the maximum concentration of honey tested was 30%, and sometimes bacterial growth was evident even at this maximum concentration. In these instances, a value of 32 was ascribed so that analyses could still be performed, despite off-scale results.

Overall, *S. aureus* showed the greatest range of susceptibility, with MICs as low as less than 2% honey, but also MICs of greater than 30%. The two Gram negative organisms (*E. coli* and *P. aeruginosa*) were almost always inhibited below 30%, presumably due to their susceptibility to osmotic activity, which is a function of the cell envelope structure and in particular, the outer membrane. The outer membrane is presumably susceptible to the osmotic activity, whereas in contrast the Gram-positive cell wall offers more protection against osmotic activity.

Also, for reference, typical values obtained for artificial honey, which contains sugars only, are MICs of >30% for *S. aureus*, *E. faecalis*, and *E. coli*, and 28% for *P. aeruginosa*. The AAV was 194 and the TA was <7.

Table 2: Summary of antibacterial activity data obtained for the more commonly harvested Western Australian honeys

Honey type	n	Mean MIC (% honey)				Mean AAV (range)	Mean TA (range)
		S. aure	*E. faeca*	*E. coli*	*P. aeru*		
Agonis flexuosa **(Peppermint)**	11	27	31	30	22	292 (223 - 373)	<7 (<7-22)
Banksia menziesii	10	26	32	28	24	232 (120-390)	<7 (<7-12)
Banksia sessilis **(Parrot bush)**	12	18	32	24	19	324 (158-492)	10 (<7-30)
Calothamnus species **(Red Bell)**	12	7	28	14	12	501 (318-588)	23 (10-31)
Corymbia calophylla **(Marri)**	39	8	21	16	12	527 (185-647)	25 (<7-45)
Eucalyptus accedens **(Powderbark)**	15	15	29	23	17	411 (228-608)	13 (<7 -37)
Eucalyptus diversicolor **(Karri)**	24	7	27	17	14	493 (384-597)	24 (<7-40)
Eucalyptus marginata **(Jarrah)**	58	6	19	13	10	571 (197-655)	30 (<7-53)
Eucalyptus occidentalis **(Yate)**	13	24	32	27	21	314 (182-575)	9 (<7-30)
Eucalyptus patens **(Forest Blackbutt)**	14	4	15	11	9	619 (563-651)	35 (26-52)
Eucalyptus wandoo **(Wandoo/White gum)**	28	10	29	20	15	442 (157-623)	21 (<7-54)
Melaleuca species	14	10	30	22	14	442 (285-643)	20 (<7-42)
Multifloral	29	26	31	28	22	281 (127-580)	8 (<7-35)
Eucalyptus platypus **(Moort)**	9	20	32	27	20	319 (208-437)	9 (<7-25)
Eucalyptus todtiana **(Coastal Blackbutt)**	9	9	24	19	13	474 (209-648)	24 (<7-48)

Obtain a HPTLC signature of each honey as point of reference and as a visual analysis tool for future authentication and quality control

The HPTLC analysis is reported separately under CRC Project 13 and is mentioned above under Project activity 1. These data are also reported in the PhD thesis of Md Khairul Islam and associated publications [13-17].

Characterise the physicochemical properties of each honey

The following parameters were quantified for all honeys: pH, water content, colour, hydrogen peroxide production, total phenolics content and antioxidant activity.

Methods used for each of these are described in several publications [12, 18] and are therefore not described in this report.

Results of these investigations are described in the manuscripts published [18] or being prepared from this research project .

Monitor bioactivity and chemical composition over time when exposed to different storage and processing conditions

Ten honeys (six Jarrah and four Marri) were subjected to different storage temperatures (-80, -20, 4, 23, 37 and 45°C) and times (0 - 16 weeks). Antibacterial activity was quantified at each time point, as was hydrogen peroxide generation, colour and water content (see Output 6 above).

In addition, several honeys were identified that had been stored for months to years at ambient temperature and these were re-tested for antibacterial activity to see if activity had changed overtime.

In the short-term study (16 weeks) it was observed that only relatively minor changes in antibacterial activity occurred over the period, with the largest changes occurring during storage at 45°C. MICs decreased by about 5 (e.g., from 5% honey to 10% honey) at week 12, and decreased further at 16 weeks (e.g., from 12% honey to 22% honey). These are substantial changes in antibacterial activity.

For honeys stored longer term (months to years) at ambient temperature, activity remained relatively stable up until about two years, but for honeys stored for longer (5 years), antibacterial activity decreased in some samples but also increased in several samples. This indicates that very long-term storage at ambient temperature may lead to unpredictable changes in antibacterial activity.

Analysing the changes to the composition and chemistry of the honeys than underpin these changes in antibacterial activity was beyond the scope of this project. However, it was noted that after storage at temperatures greater than ambient (37 and 45°C) there was a loss of hydrogen peroxide activity and an increase in the colour of the honey samples. Many honeys stored for months to years were also darker after storage compared to baseline values.

Results of this investigation are described in a manuscript arising from this research, which is in preparation as of June 2022.

Table 3: Summary of physicochemical characteristics of the more commonly harvested Western Australian honeys

Honey type	n	pH	Refract-ive index	Brix	Colour		Phenolics[a]	Anti-oxidant activity[b]		Hydrogen peroxide (µM)				
					no filtra-tion	filtra-tion		Fe^{2+}	TE	1h	2h	4h	6h	24h
Agonis flexuosa (Peppermint)	11	4.76	1.497	82.3	438	258	31.8	3.94	1410	3	3	1	1	1
Banksia menziesii	10	4.70	1.498	82.9	303	163	25.5	2.84	1009	20	28	40	55	30
Banksia sessilis (Parrot bush)	12	4.86	1.498	82.7	391	224	30.0	3.50	1262	34	46	68	131	176
Calothamnus sp. (Red Bell)	12	4.46	1.498	82.8	697	476	56.1	8.98	3750	2	6	18	37	261
Corymbia calophylla (Marri)	39	4.49	1.497	82.5	363	194	31.1	4.67	2051	78	121	240	337	252
Eucalyptus accedens (Powderbark)	15	4.31	1.498	82.9	359	158	28.5	3.54	1660	45	56	61	67	5
Eucalyptus diversicolor (Karri)	24	4.60	1.498	82.7	275	143	25.9	3.77	1450	85	162	266	338	278
Eucalyptus marginata (Jarrah)	58	5.30	1.498	82.7	554	379	36.1	5.58	2124	74	130	282	500	805
Eucalyptus occidentalis (Yate)	13	4.20	1.498	82.7	318	146	24.9	4.44	2140	4	3	1	1	3
Eucalyptus patens (Forest Blackbutt)	14	4.92	1.499	83.0	574	394	38.6	6.44	2788	69	136	295	429	667
Eucalyptus wandoo (Wandoo/Whitegum)	28	4.39	1.497	82.5	228	116	19.1	2.98	1286	43	33	12	4	1
Melaleuca	14	4.84	1.498	82.6	514	334	31.6	3.63	1328	102	231	375	440	155
Multifloral	29	4.53	1.498	82.9	342	175	29.0	3.78	1558	67	91	97	95	87
Eucalyptus platypus (Moort)	9	4.16	1.493	81.0	389	219	24.6	3.58	1349	55	82	88	105	94
Eucalyptus todtiana (Coastal Blackbutt)	9	4.47	1.497	82.5	426	210	29.3	3.78	1599	20	24	25	27	8

[a] Expressed as Gallic acid equivalents (GAE) /100g honey. [b] Expressed as Fe^{2+} mmol/kg and Trolox Equivalents (TE) µmol/kg

Construct a database containing all relevant honey parameters

All bioactivity and physicochemical parameters data are collated within an Excel spreadsheet. A chemistry database has been developed by Prof Sharon Purchase in Project 39 and this data will be made available to the industry via B-QUAL Australia.

Evaluate the therapeutic potential of WA honeys.

The therapeutic potential of honeys may relate directly to the bioactivity of honey, or to other beneficial properties of honeys. About bioactivity, the major characteristics investigated in this project were antibacterial activity, and antioxidant activity.

Antibacterial activity has historically been one of the main reasons that honey has been used therapeutically. Many studies over the years have demonstrated that honey assists in the healing of chronic wounds such as leg ulcers [19-27], presumably by reducing the microbial load within the wound environment. In our study, differences in antibacterial activity between honeys were apparent *in vitro*, with honeys showing a range of activity from relatively low to relatively high. Whilst it is tempting to assume that a honey with high antibacterial activity will have a better therapeutic effect than a low activity honey, this has not been investigated in human clinical trials.

To date there are no published studies that correlate levels of activity established *in vitro* with clinical outcomes for conditions or infections treated with honey, which means that the relevance of differences *in vitro* to treatment outcomes remain unknown. Since this relationship has not been established, it is impossible to know whether specific WA honeys have potential as therapeutic agents. One would assume that honeys with relatively high bioactivity, such as Jarrah and Marri, would be excellent therapeutic candidates, but this remains to be assessed clinically.

Another way to investigate the therapeutic potential of WA honey is to determine what the requirements are for a “therapeutic honey”. Here we consider what is required for making honey into a pharmaceutical product [28]. It is relevant to point out that terms such as “therapeutic grade”, “medical grade” and “medicinal honey” are not well-defined and are largely used as marketing terms.

For honey to be made into a therapeutic product, it would need to be packaged appropriately under Good Manufacturing Process (GMP) conditions and sterilised. There are no criteria for a minimum level of antibacterial activity required for a therapeutic honey, which means that any honey that is packaged appropriately constitutes a therapeutic honey.

Sterilisation by gamma irradiation has been shown previously to have no effect [29] or only a minor impact [30] on the antibacterial activity of honeys. Other “sterilisation” techniques, such as pasteurisation, do not achieve complete sterility of the product and also involve heating which is detrimental to the antibacterial activity and quality of honey [31].

There are other criteria to consider, such as whether the honey would be packaged pure, or combined with other excipients in a formulation. Formulation may potentially delay undesirable changes such as crystallisation, or may improve the product, for example, by decreasing the stickiness or amending the texture.

Given that there are currently no criteria for defining what level of antibacterial activity corresponds to a “medical grade” honey, any WA honey could be developed as a therapeutic product. This would obviously

require significant investment and support from industry but would be a beneficial approach for value-adding to an already valuable industry.

Implications

Whilst all honeys have similarities due to their sugar composition, honeys from individual floral sources are also unique, and can be differentiated based on characteristics such as colour and flavour profile. Furthermore, honeys can be distinguished by bioactive characteristics determined *in vitro*, such as antibacterial and antioxidant activity.

Antibacterial activity has been used for decades as a point of difference between honeys, particular in sales and marketing, with honeys with higher antibacterial activity attracting a higher sale price. This was achieved despite the somewhat fragmented scientific data supporting the claims, and antibacterial activity data that was obtained using methods now largely considered to be unsuitable for many honeys (i.e., the phenol equivalence method). The current study has provided a comprehensive evaluation of the antibacterial activity of honeys from range of floral sources, coupled with a verification of nectar source by HPTLC. As such, it provides an up to date, relevant and timely assessment of the bioactivity and characteristics of WA honeys.

These foundational data may be used to support further investigation of the therapeutic potential of WA honeys. The antibacterial activity of some WA honeys may make them suitable candidates for the topical treatment of superficial skin infections, or as skin infection prophylaxis, however, clinical data are required to evaluate their suitability and efficacy.

Recommendations

These data have demonstrated clear differences in the antibacterial activity of WA honeys from different floral sources. However, most honey is used as a foodstuff (i.e., it is consumed), as is not actually used for its antibacterial activity. Furthermore, ingesting honey with high antibacterial activity could potentially harm the gut microbiome if large enough quantities of honey are consumed and the antibacterial factors within honey are active in the gut for any length of time.

The first recommendation is, therefore, that the marketing of honeys that are consumed as foods are transitioned away from an antibacterial activity emphasis, and focuses on other unique characteristics, such as flavour profiles, the “clean and green” image and the sheer uniqueness of WA and its botanical flora. This would enable the differentiation of products either as unique foods or therapeutic products.

The second recommendation is that the therapeutic potential of one or more WA monofloral honeys be investigated clinically. This would be done with a view to developing a therapeutic product that would be listed by the Therapeutic Goods Administration (TGA) Australia. Currently in Australia, manuka honey is the only described type of honey available in commercial, therapeutic products. Developing additional medicinal honey products from Australian honeys, and manufactured in Australia, would be of substantial benefit.

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