Antioxidant enzyme activity of honey constituents in human macrophages

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Thesis

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Abstract

Intake of exogenous antioxidants assist with protecting cells form oxidative damage. Phenolic acids and flavonoids in honey are attributed to the antioxidant properties. The presence and quantities of active compounds is important in the understanding of how the floral and geographical origin of honey influences antioxidant activity.

Analytical RP-HPLC was used to determine the phenolic content of a range of Australian mono-floral honey samples. DPPH and ORAC assays were used to examine the free radical scavenging activity of honey samples. The phenolic and flavonoid compounds were extracted from a selection of honeys and their antioxidant activity analysed by DDPH and ORAC. The phenolic extract from Cheeseberry honey was used to treat blood-derived human macrophages and measure the effects on glutathione peroxidase (GPx), 8-isoprostane and pro-inflammatory cytokines *in vitro*. Preparative HPLC allowed individual compounds to be isolated and tested.

Total phenolic content varied between samples of different botanical and geographical origin. *Leptospermum*, Jarrah and Cheeseberry honey samples showed high antioxidant activity in ORAC and DPPH assays. The total phenolic content was found to be correlated to ORAC activity (r=0.5318). The properties of honey were retained in the phenolic extracts, which reduced 8-isoprostane in LPS-stimulated macrophages.

Australian *L. scoparium, L. whitei*, *L. poligalifolium*, Jarrah and Cheeseberry were identified as species of mono-floral honey with high antioxidant activity. The geographical origin of samples was shown to affect the composition. Phenolic extracts of honey reduced oxidative stress in human macrophages, the antioxidant effects were attributed to several phenolic compounds. Leptosperin and methyl syringate were identified as highly active antioxidant compounds in *Leptospermum* honey. Australian honey may be suitable as a medicinal substance, due to rich antioxidant activity attributed to the presence of phenolic compounds, obtained from the botanical and geographical origin.

Table of Contents

List of Figures vi
List of Tables vii
Acknowledgments viii
Statement of Originality viii
1. Introduction and Aims 1
2. Literature review 3
2.1 Pathogenesis of oxidative stress 3
2.2 Antioxidant defence mechanisms 4
2.3 Honey – an overview 5
2.4 Antioxidant Properties of honey 7
2.5 Antioxidant constituents in honey 9
2.6 Measuring antioxidant capacity of honey 10
2.7 Medical applications for antioxidant properties of honey 13
2.8 Bioavailability of antioxidants in honey 16
2.9 Properties of Australian honey 17
2.10 Future directions 20
3. Materials and Methods 22
3.1 Honey samples 22
3.2 Analysis of unfractionated honey samples 23
Reverse Phase-High Performance Liquid Chromatography of phenolics and flavonoids 23
Reverse Phase-High Performance Liquid Chromatography of Leptosperin and lepteridine 24
2,2-diphenyl-1-picrylhydrazyl (DPPH) of unfractionated honey 25
Oxygen Radical Absorbance Capacity (ORAC) of unfractionated honey 26
Free radical initiator 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) 27
3.3 Isolation of active compounds from whole honey 29
Extraction of phenolic compound from honey 29
Analysis of phenolic extracts 29
3.4 Cell-based assays 30
Glutathione peroxidase (GPx) enzyme assay 30
TNF-α and IL-6 assays 31
8-isoprostane Assay 31
3.5 Isolation of antioxidant compounds from phenolic extracts 32
Preparative HPLC 32
Analysis of phenolic fractions of honey 32

4. Results 34
4.1 Whole honey 34
RP-HPLC of phenolics and flavonoids 34
Leptosperin and lepteridine analysis 36
Inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) by unfractionated honey samples 38
Determination of Oxygen Radical Absorbance Capacity in unfractionated honey samples 40
Antioxidant activity of honey constituents on 2,2'-azobis-(2-amidinopropane dihydrochloride) 45
4.2 Phenolic extracts 50
Analytical RP-HPLC 50
Determination of Oxygen Radical Absorbance Capacity (ORAC) in phenolic extracts of honey 51
Inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) by phenolic extract of honey samples 51
4.3 Cell based assays 52
Glutathione peroxidase (GPx) 53
Levels of 8-isoprostane detected in blood-derived macrophages 54
Cytokine assays 55
4.4 Isolated fractions from phenolic extracts of honey 56
RP-HPLC of isolated phenolic constituents 56
Inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) by fractionated compound of honey 57
Oxygen Radical Absorbance Capacity (ORAC) of fractionated honey compounds 58
5. Discussion 60
5.1 RP-HPLC analysis of unfractionated honey 60
5.2 Antioxidant activity of unfractionated honey 61
5.3 Correlation of phenolic content to antioxidant activity 64
5.4 Justification for selection of samples for further analysis 64
5.5 Analysis of phenolic extracts of honey 64
5.6 Cell-based assays 65
5.7 Evaluation of fractionated phenolic extracts of honey 66
6. Conclusion 68
7. References 69
8. Appendix 76

List of Figures

Figure 4.1: Example of chromatogram of RP-HPLC results……..34

Figure 4.2: Detection of leptosperin in Tasmanian Manuka……..36

Figure 4.3: Detection of lepteridine in Tasmanian Manuka……..36

Figure 4.4: Dose-response curve of DPPH inhibition (%) in Tasmanian Manuka……38

Figure 4.5: Standard curve for inhibition of DPPH with gallic acid……38

Figure 4.6: Determination of ORAC of Trolox and honey samples……41

Figure 4.7: Antioxidant kinetics of Trolox standards and Tasmanian Manuka……43

Figure 4.8: Antioxidant reaction kinetics……44

Figure 4.9: Lepteridine and leptosperine chromatogram/UV spectra……48

Figure 4.10: Total phenolic content vs antioxidant activity (ORAC) unfractionated honey….49

Figure 4.11: Total phenolic content vs antioxidant activity (DPPH) unfractionated honey….49

Figure 4.12: RP-HPLC of unfractionated Tasmanian Manuka……..50

Figure 4.13: RP-HPLC of Tasmanian Manuka phenolic extract……50

Figure 4.14: Percentage DPPH inhibition of the phenolic extracts of Cheeseberry honey…..51

Figure 4.15: Phase contrast microscopy of blood derived monocytes……53

Figure 4.16: Standard curve for the Lowry protein assay……54

Figure 4.17: UV spectra RPO-HPLC for 4-MethoxyphenylLactic Acid……57

Figure 4.18: DPPH analysis of 5 fractions of Tasmanian Manuka……58

Figure 4.19: Antioxidant reaction kinetics for Cheeseberry phenolics……58

List of Tables

Table 3.1: Honey samples selected for analysis……..22

Table 3.2: Honey samples selected for the ORAC assay…….26

Table 3.3: Honey samples selected for AAPH analysis……28

Table 3.4: Honey samples selected for phenolic extraction……29

Table 3.5: Fractions of phenolic extracts……...32

Table 4.1: The combined peak areas for honey samples……35

Table 4.2: Lepteridine and leptosperin in *Leptospermum* honey samples……37

Table 4.3: DPPH results for honey samples……..39

Table 4.4: Samples selected for ORAC……42

Table 4.5: ORAC activity of sugar solution. ……44

Table 4.6: ORAC activity for Trolox 30µmole TE/L and sugar solution……44

Table 4.7: ORAC activity for honeys diluted 1:100……45

Table 4.8: HPLC analysis of phenolics and flavonoids with AAPH……46

Table 4.9: ORAC activity of phenolic extracts……51

Table 4.10: DPPH results for phenolic extracts……..52

Table 4.11: GPx activity in cells in human macrophages……54

Table 4.12: Levels of 8-isoprostane in human macrophages……55

Table 4.13: Pro-inflammatory cytokines in human macrophages……55

Table 4.14: Analytical RP-HPLC for phenolic extracts of honey……56

Table 4.15: ORAC activity of individual honey constituents……59

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Statement of Originality

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made.

Ashley Williams

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1. Introduction and Aims

Reactive oxygen species are important for essential cellular functions, however an accumulation of free radicals can cause harmful effects when antioxidant defences are overwhelmed. An imbalance to the cellular redox state can result in oxidative stress, linked to the pathogenesis of many diseases, disorders, conditions and injuries. Treatment options targeting oxidative stress and related conditions are limited, or involve medication which provokes adverse side effects in patients. Current treatments for neurological disorders associated with oxidative stress are only able to assist with reducing severity of symptoms and not disease progression. Therefore, there is a demand for effective and safe treatment options that are aimed at the inhibition of oxidative stress.

Intake of exogenous antioxidants can assist in maintaining a healthy balance of cellular oxidation and reduction, maintaining the redox system in living organisms. The use of naturally-derived antioxidants could be potentially valuable in the treatment of many disease states. Antioxidant compounds including polyphenols, derived from local flora, can be transferred to subsidiary products such as honey. Honey is natural source of antioxidants, capable of demonstrating protection from oxidative injury.

A widescale analysis of a range of honey types would allow identification of distinct mono-floral species which possess high antioxidant properties. The climate, soil composition and species of bees found in different locations are among many geographical-specific factors that can influence the phenolic composition of samples. An analysis of antioxidant activity between different mono-floral honey species would present an opportunity to compare the influence of geographical origin on the antioxidant activity of samples derived from the same botanical origin. The proposed analysis could allow the discovery of unique Australian mono-floral honey species with potent antioxidant activity. Furthermore, comparisons between mono-floral honey samples could assist with the identification of regions in Australian that are suitable for producing honey, rich in antioxidant activity.

It is not yet known which specific compounds are responsible for antioxidant bioactivity in Australian honeys. Individual constituents need to be isolated from honey samples and tested for their antioxidant activity. Variation in antioxidant activity between individual compounds could challenge the understanding that total phenolic content is an indication of the antioxidant potential of a

sample. Hence, comparisons between phenolic content and antioxidant activity are required to determine if a direct correlation exists.

Methods used to successfully identify total antioxidant activity of whole honey and its bioactive constituents could be used in future studies to create a standard for antioxidant testing in the bee industry. The identification of botanical and geographical origins which produce potent antioxidant honey may additionally increase the value of the medical honey industry in Australia.

The aims for the work described in this thesis are as follows:

1) To compare the antioxidant activity of Australian mono-floral honey from a range of botanical origins and determine which mono-floral species have greatest therapeutic potential for treatment of pro-oxidant disease states.

2) To determine the influence of geographical origin on the antioxidant activity of samples from the same botanical origin.

3) To compare total antioxidant activity and total phenolic content of honey samples and determine if samples with high phenolic content will have greater antioxidant activity than those with lower phenolic content.

4) To isolate chemical constituents of Australian honey samples and test their individual antioxidant bioactivities.

2. Literature review

2.1 Pathogenesis of oxidative stress

The prevalence of chronic and degenerative diseases contributes to a large proportion of mortality worldwide. Heart disease, cancer, respiratory disease, Alzheimer's disease, diabetes, kidney disease and atherosclerosis are among the leading causes of death (1). Oxidative stress is a common factor implicated in the pathogenesis of a multitude of disease states, injuries and disorders. The presence of oxidative stress has been linked to diabetes, cancer, neurological disorders, multiple sclerosis (2) and hypertension in addition to a number of cardiovascular and respiratory disorders (3). It has not yet been established if oxidative stress is the cause, or occurs as a consequence of the related disease states (4).

Oxidative stress is characterized by an imbalance in the redox reaction involving complementary oxidation and reduction of chemical species in biological systems. Free radicals are generated from nitrogen, sulphur or oxygen, producing reactive nitrogen species (RNS) and reactive sulphur species, (RSS) or reactive oxygen species (ROS). ROS include superoxide radical ($O_2^{-\bullet}$), hydroxyl radical ($^{\bullet}OH$) and peroxyl radical ($ROO^{\bullet}$), hydrogen peroxide and singlet oxygen ($O_2^{\bullet}$). The unstable nature of free radicals, due to an unpaired electron, initiates chemical reactions with other molecules (5). For example, the dissociation of hydroperoxyl radical forms the highly reactive superoxide anion (5). ROS are initiated through oxidation. Initiation of auto-oxidation by ROS leads to a chain reaction, where free radical intermediate products are repetitively generated, propagating the reaction. The damaging free radical chain reaction is only terminated when two radicals quench one another. Production of ROS occurs endogenously through normal physiological processes such as cellular respiration and is important for regulatory functions including cell proliferation and inflammatory response processes. ROS can also be initiated exogenously through exposure to ultra violet rays, smoking, hyperoxia, ionizing radiation, ozone exposure (3), pollutants or toxic chemicals (6).

The ability of endogenous antioxidant systems to reduce ROS formation can be overwhelmed, resulting in oxidative stress. Accumulation of free radicals leads to the damage of DNA, protein and lipid cellular components. Free radicals have deleterious effects on DNA and can interfere with chromosomal arrangement (5). Alterations to protein cellular components can occur through free radical mediated peptide cleavage, protein cross-linkage or amino acid modification (7). Free radical induced lipid peroxidation compromises the integrity of cellular membranes, causing alterations to

fluidity (8). The involvement of mitochondria in the production of ROS increases the susceptibility of mitochondrial DNA to damage (4), resulting in mitochondrial dysfunction, often associated with disease states involving oxidative stress.

2.2 Antioxidant defence mechanisms

The ability for cellular protection from harmful accumulation of ROS is dependent on antioxidant defence mechanisms. Direct antioxidants are either preventative, inhibiting the initiation process, or chain breaking, which compete with propagation reactions. Indirect antioxidants such as enzymes work by increasing the levels of endogenous antioxidants (9). Antioxidant enzymes include catalase, glutathione peroxidase and superoxide dismutase which convert oxidized molecules to their reduced states, allowing free radical scavenging to continue. Antioxidants are recycled endogenously, however can also be obtained exogenously through micronutrients and other small molecules found in certain foods. Many links have been found between diets high in antioxidants and reduced risk of disease (10). Elevated oxidant levels and reduced antioxidant levels in individuals have been correlated with an increased susceptibility to damage caused by oxidative stress (1, 3, 10). Supplementation of antioxidants may therefore benefit individuals susceptible to oxidative stress-related disease states.

Antioxidants in the treatment of oxidative stress

Antioxidant treatment enhances cellular reprogramming in induced pluripotent stem cells (iPSC) by alleviating cell senescence (11). A clinical trial demonstrated that administration of 2000 IU/day of alpha-tocopherol (vitamin E) reduced cognitive decline in patients with mild to moderate Alzheimer's disease (12). Studies using antioxidant supplementation including ascorbic acid (vitamin C) and alpha-tocopherol have proven mostly beneficial (5) however some conflicting results exist (5, 13), particularly in studies involving neurological diseases (5). Antioxidant supplementation may result in formation of pro-oxidants which have scavenged free radicals (1), or may be unable to penetrate the blood brain barrier (BBB) to reduce oxidative stress in neurological disorders. Therefore, the selection of an antioxidant compound and its delivery method are important considerations for therapeutic use. Synthetic antioxidants such as BHT (butylated hydroxytoluene), BHA (butylated hydroxyanisole) and NDGA (Nordihydroguaiaretic acid) are commonly used as food preservatives. Synthetic antioxidants are becoming increasingly unpopular with emerging evidence linking NDGA and renal cystic disease, along with reports contradicting the safe levels of BHT and

BHA for human consumption (5). The popularity of naturally-derived dietary antioxidants has therefore driven renewed interest in research of honey as a natural therapeutic.

2.3 Honey – an overview

Synthesis and composition

Honey is a super-saturated sugar solution produced by honey bees (1). The nectar or secretions of plants is collected by honeybees, assembled, dehydrated and left to ripen (14). This natural substance is classified by the botanical source (15). Honeydew honey is derived from the secretion of sap-sucking insects while floral honey is sourced from the nectar of plants (16). Mono-floral honey is derived predominantly from the nectar and pollen of one principal plant species (17), when honey is composed of nectar from a blend of multiple plant species it is classified as poly-floral. Mono-floral honey contains bioactive constituents originating from the botanical source, which are transferred into the honey (18). The composition of honey consists mainly of a mixture of complex carbohydrates (19), predominantly glucose and fructose, in addition to maltose, sucrose and other disaccharides (20). The carbohydrate profile and water content (19) are responsible for the consistency of honey and is a critical component for the fermentation process. The water content of honey can be influenced by the environment or manipulated by beekeepers (21). High moisture content can lead to an undesirable sour taste caused by the formation of acetic acid and alcohol, produced by osmotolerant yeasts (22). Low moisture content such as that found in Manuka honey (22), improves storage life and inhibits microbial growth (22). A maximum of 20g of water per 100g honey is recommended to prevent yeast fermentation (16) and granulation (19), with international guidelines specifying the total proportion of water must remain under 20% (19). Low moisture content is therefore used as an indicator for high product quality (22). Honey may contain 180 compounds in total (23), of which vitamins, minerals, amino acids, proteins, enzymes, and polyphenols (24) represent a small proportion of its total composition. Bioactive compounds in honey synergistically contribute to its therapeutic potential.

Applications as a therapeutic substance

The use of honey dates back to the stone ages (20), evidence referencing the use of honey as a medicine can be traced to 2100-2000 BC (25). The ancient Egyptians utilized honey in the embalming process (20), demonstrating the first use of honey in preventing oxidation. Honey has been widely used as a therapeutic substance for centuries. The utilization of honey as a remedy for wound healing dates back to Ancient Greek and Chinese cultures (25) and has been mentioned in a number

of therapeutic remedies over history for the treatment of wounds, eye sight and respiratory ailments (20). Research into the therapeutic properties of bioactive constituents in honey has been renewed in more recent years (1).

Evidence supports findings on the wide range of beneficial health effects of honey. Anti-fungal, anti-viral and anti-bacterial properties reduce the risk of infection by evasive pathogens. Fungal infections from *Aspergillus*, *Penicillium*, dermatophytes, *Candida albicans* and certain strains of mycoses have been indicated as susceptible to topical application of honey (20). Similar application has demonstrated inhibitory effects on the symptoms of hepatitis C and rubella virus infections (20).

Honey has been effectively used to aid wound healing (26), with antibacterial actions a major contributing factor. The presence of glucose oxidase and glucose promotes formation of hydrogen peroxide (25), which along with non-peroxide activity of polyphenolics and methylglyoxal and polyphenolics, contributes to the antimicrobial properties of honey. Methylglyoxal is a phytochemical produced endogenously as a side product of metabolic pathways. Advanced glycosylated end products derived from methylglyoxal are linked to neuroglial and microvascular degenerative pathology, diabetic retinopathy (27) and increased expression of COX-2 (28). This highly reactive glycolytic metabolite is present in high levels in Manuka honey and although it is associated with increased levels of intracellular oxidative stress (29), it has potent antibacterial actions. Antibacterial actions of honey against *Escherichia coli, Enterobacter aerogenes, Salmonella typhimurium, Staphylococcus aureus,* ß-haemolytic *streptococci* have been observed. Honey has shown promise in inhibiting the highly pathogenic *Helicobacter pylori* and several antibiotic resistant strains, such as methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococci* (25). The lack of bacterial resistance to honey (25) indicates that it may be useful as a substitute for antibiotics in an age of increasing antibiotic-resistance in bacterial species. When honey is administered in combination with antibiotics, it has shown to improve outcomes of methicillin-resistant *S. aureus* infections through synergistic enhancement of common antibiotics (30).

The therapeutic applications of honey extend beyond protection from infections, with further indications including hepatoprotective, gastroprotective, anti-inflammatory, anti-hypertensive (31), anti-tumour and antioxidant properties. Several combined mechanisms may be responsible for synergistically contributing to its therapeutic benefits, such as combined anti-inflammatory and antioxidant mechanisms (20).

2.4 Antioxidant Properties of honey

The antioxidants present in honey prevent oxidation and spoilage, it is therefore it is used as a natural food preservative. Storage and processing conditions as well as moisture content can affect the antioxidant activity of honey (32, 33). Storage time and temperature affects hydrogen peroxide-dependent antibacterial activity in honey, however has very little negative impact on non-peroxide antibacterial activity (34). The interaction between reducing sugars with amino acids and proteins, followed by dehydration and fragmentation, generates intermediate Maillard reaction products (35). The non-enzymatic browning of these intermediate products forms melanoidins (16), which effectively absorb UV-light (35), contributing to a darker colour. These high molecular weight polymers are potent antioxidants, supporting correlations found between honey with darker colour and greater antioxidant potential (33). Although Maillard reaction products are formed during thermal treatment of honey (35), the geographical origin and harvest season (16) have proven to have greater impact on the antioxidant properties than thermal treatment and processing. Although many factors contribute to the antioxidant activity of honey, the most influential is the botanical origin (22), related to the presence and quantity of active compounds (15, 36).

Phenolic compounds including benzoic acid, caffeic acid and p-coumaric acid are present in most mono-floral honeys (37). Mono-floral honeys known for high antioxidant activity such as Manuka honey, derived from *Leptospermum scoparium,* are often used as the gold standard for comparing antioxidant activity of other honeys (26) as they contain an abundance of phenolic compounds. Manuka honey is known to contain characteristic phenolic acids such as methyl syringate and syringic acid (37) 4-methoxyphenyllactic acid, phenyl lactic, 2-methoxybenzoic acid (38), 4-hydroxybenzoic acid, caffeic acid, gallic acid, trans-ferulic acid and p-coumaric acid (26). Phenolic acids account for approximately 2.36mg/kg of the total weight of Manuka honey. Gallic acid is the most predominant of these phenolic acids (26). Flavonoids have been observed in proportions of around 0.8mg/kg Manuka honey (26) and these include kaempferol, apigenin, luteolin, isorhamnetin and the most abundant of which is quercetin (26). The presence of syringic acid, phenyllactic acid, dehydrovomifoliol, phenylacetic acid, benzoic acid, and lumichrome are used to distinguish Manuka honey (38) from other honeys. Further studies (39) have suggested leptosperin, acetyl-2-hydroxy-4-(2-methoxyphenyl)-4- oxobutanate, 3-hydroxy-1-(2-methoxyphenyl)-penta-1,4-dione, kojic acid, 5-methyl-3-furancarboxylic acid, 2-Methylbenzofuran, 2'-hydroxyacetophenone, and 2'-methoxyacetophenone to be used as unique floral markers of Manuka honey. *Leptospermum polygalifolium* contains higher concentrations of 2-methoxybenzoic acid cis-linalool oxide, 3,4,5-

trimethylphenol and lower quantities of 2'-methoxyacetophenone than Manuka (39). Although both species belong to the *Leptospermum* family, the honeys have markedly different chemical composition.

Light microscopy was originally used for authentication of botanical origin of honey, however, this method experienced difficulty discriminating between pollens from the same subfamily (40). Accurate classification of mono-floral honey types has been achieved by obtaining the infrared spectra and relative frequencies of pollen grains with the use of infrared spectroscopy and attenuated total reflection techniques (41). Recently, molecular sequencing and DNA barcoding has been developed as a method to identify mixed pollen species, which has previously been a time-consuming task with unreliable results (40). Analytical methods such as high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) are frequently used to determine botanical and geographical origin of food products including honey. HPLC allows the identification and quantification of compounds through polarity-based separation. The high level of sensitivity and accuracy capabilities of LC-MS is ideal for analysis of phenolic constituents (42). Volatile compounds or physicochemical parameters distinctive to honey from specific botanical source (43) can therefore be identified. The phenolic profiles of honeys has been frequently used to authenticate the floral source origin of samples and can be used as a marker to determine the geographical origin (17). As the composition of honey is influenced by environmental characteristics, the antioxidant activity can vary between samples originating from the same botanical species (37).

The antioxidant activity of honey varies depending on geographical location (44). Honeys derived from the same botanical source produce unique compositions relating to their origin (17, 37). Weather and soil conditions dictate areas within which a species of plants may grow, such as the moist, low-nutrient soils *Leptospermum* has commonly been found to flourish in. Rainfall, climate, specific soil composition, regional endemic flora (17), altitude, meteorological conditions (45) and seasonal production are some contributing factors (21, 36) causing variations to phenolic composition. Higher phenolic content has been found in honeys sourced from arid regions compared to non-arid sourced counterparts (46), with increased presence of free amino acids and carotenoids observed (21).

2.5 Antioxidant constituents in honey

Medicinal applications of honey are largely due to the presence of bioactive compounds such as phenolics and flavonoids, present in honey. Phenolic acids and flavonoids are the main compounds responsible for antioxidant effects observed in honey. Benzoic acids and cinnamic acids are the two major subclasses of phenolic acids (47). Phenolic acids exhibit antioxidant mechanisms including hydrogen donation, free radical scavenging and chelation of metal ions which catalyse lipid peroxidation (48). Caffeic acid, p-coumaric acid, p-OH benzoic acid (37), ellagic acid, gallic acid, vallinic acid, syringic acid, chlorogenic acid and 4- dimethylaminobenzoic acid are those most commonly reported in honey (1, 24). Flavanols, flavones and flavanones are the three major subclasses of flavonoids (47). Quercetin, kaempferol, myricetin and chrysin are some of the most common flavonoids found in honey. Flavonoids display high hydrogen peroxide scavenging ability and are oxidised by radicals, producing more stabilized and less-reactive radicals. Flavonoids use a similar mechanism to phenolics to prevent ROS generation through metal ion chelation, preventing iron-dependent accumulation of lethal lipid ROS. Flavonoids are also able to activate antioxidant enzymes and inhibit oxidases (5).

Many other compounds work in combination with phenolics and flavonoids, contributing to the antioxidant capacity of honey through synergistic antioxidant mechanisms. Maillard reaction products such as melanoidins (35) neutralize ROS in a process of free radical scavenging. Small molecule antioxidants including carotenoids, ascorbic acid, tocopherols and glutathione, similarly exhibit free radical scavenging activity. Carotenoids participate in singlet oxygen quenching and scavenge peroxyl radicals more efficiently than other ROS, protecting cell membranes and lipoproteins. Water-soluble ascorbic acid (vitamin C) and lipid soluble tocopherols (vitamin E) are free radical scavengers and contribute to the termination of lipid peroxidation chain reactions. Glutathione is a tripeptide found within the cell (49) which can convert tocopherols and ascorbic acid to their active forms, serving as an electron donor and reducing hydrogen peroxide into oxygen and water (3).

Honey contains enzymatic antioxidants such as such as glutathione peroxidase, superoxide dismutase, glucose oxidase and catalase (1). Glutathione peroxidase reduces hydrogen peroxide (49) with the assistance of glutathione reductase. NADPH is the electron donor in the reduction of oxidised glutathione to active form by glutathione reductase (10), maintaining intracellular redox by continuing to reduce endogenous antioxidants. Superoxide dismutase performs dismutation of superoxide anions, converting them to hydrogen peroxide, which is a less reactive oxygen species

(8). Catalase then assists with conversion of hydrogen peroxide to oxygen and water (20). Glucose oxidase and catalase reduce the levels of lipid hydroperoxide and hydrogen peroxide, preventing lipid peroxidation and contributing to antioxidant activity (23).

2.6 Measuring antioxidant capacity of honey

Evidence of the antioxidant effects of honey has been demonstrated through different methods widely used in the food industry. The antioxidant activity of honey can be measured through a variety of analytical tests which determine the antioxidant capacity of a sample. Antioxidant capacity refers to the number of converted molecules or donated electrons at full reaction under specific conditions, per mol of antioxidant. Popular analytical methods used to determine antioxidant capacity of honey samples include oxygen radical absorbance capacity (ORAC), Trolox equivalent antioxidant activity (TEAC), ferric reducing antioxidant power (FRAP) and 1,1-diphenyl-2-picrylhydrazyl (DPPH). Each method assesses a slightly different facet of oxidative process (50), and so several tests are usually required to obtain reliable results (32).

Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assays is a widely used method. Commonly used within the bee industry for measuring the antioxidant activity in honey samples, ORAC allows the protective capacity of compounds to be quantified by fluorescence intensity. The reaction involves the oxidative degeneration of fluorescein or beta-phycoerythrin when combined with the free radical generator, 2,2'azodiisobutyramidine dihydrochloride (AAPH), resulting in a non-fluorescent product. The addition of antioxidants reacts with free radicals, preventing attack on the fluorescent probe. The extent to which antioxidants compete with fluorescent probe to quench free radicals is reflected by reduced fluorescent decay (50). The degree of antioxidant protection can therefore be quantitated by measuring fluorescent light absorption over time. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble vitamin E analogue, is the antioxidant used as a reference standard in ORAC antioxidant testing. Results for samples are compared to Trolox equivalent (TE) values and used to represent the antioxidant capacity. ORAC is able to sensitively measure antioxidant capacity in honey, however the complex nature of this assay makes consistency of results, especially between laboratories, difficult (50).

Trolox Equivalent Antioxidant Capacity (TEAC)

TEAC uses a large and sterically-hindered radical with nitrogen centre. The assay can be used to compare antioxidant changes during processing or storage (50). TEAC is useful in determining whether antioxidants are hydrogen atom transfer (HAT) or single electron transfer (SET) dominant in reactions. The reaction is more heavily weighted on the steric accessibility of an antioxidant than chemical properties of highly-reactive radicals (50). Discontinuation of the use of TEAC for quantitative evaluation of antioxidant capacity has therefore been recommend (50).

Ferric Reducing Ability of Plasma (FRAP)

FRAP indirectly measures the transfer of electrons from antioxidants to Fe^{3+} reducing it to Fe^{2+}. Fe^{2+} forms a coloured complex with 2,4,6-tripyridyl-s-triazine (Fe II -TPTZ), in acetate buffer, at low pH (32, 51) and can be measured spectrophotometrically at 593 nm (9). Limitations of the method include false positives and inaccurate representation of slow reacting antioxidants (52).

1,1-diphenyl-2-picrylhydrazyl (DPPH)

DPPH works on a similar mechanism of electron transfer used in the FRAP assay. DPPH is a stable, nitrogen based free radical (22). Interaction with antioxidants reduce DPPH radicals to hydrazine (9), resulting in a loss of purple colour intensity (9). Spectrophotometric analysis of organic samples combined with DPPH allows measurement of free radical scavenging activity based on loss of DPPH, and hence colour intensity at 518nm. DPPH lacks information on antioxidant efficacy, unlike ORAC assay which measures the reaction over time, DPPH fails to measure reaction curves (50). The high correlation of results found between DPPH and FRAP assays (51) indicates a redundancy in the use of both assays. Issues with colour interference and slow colour development observed in FRAP assays (51) suggests DPPH would be a preferable assay to select for use in combination with another complimentary assay.

Enzymatic activity assays

Limitations experienced with each individual analytical antioxidant test highlight a need to perform several complimentary assays. The use of ORAC, FRAP, TEAC and DPPH do not assess the contribution of antioxidant enzymes to antioxidant activity in living cells, or in honey samples. Enzymatic activity assays are available to more thoroughly understand the true antioxidant potential of honey samples. Catalase, superoxide dismutase, glutathione reductase and glutathione peroxidase found in honey can be measured (53) to determine enzymatic antioxidant capacity of honey. Catalase assays measure the amount of catalase by the decomposition of hydrogen peroxide

per minute (52). Superoxide Dismutase assay uses tetrazolium salt to detect superoxide radicals generated by xanthine oxidase (52), effectively measuring the levels of superoxide dismutase in a sample required to perform the reaction. Glutathione Reductase assays use oxidized glutathione as a substrate for a reaction initiated by NADPH, where oxidized glutathione is recycled to its reduced state. Glutathione reductase and peroxidase activities are indirectly determined through oxidation or reduction of NADPH, measured by spectrophotometry (52).

Cell-based methods

Cell-based assays allow physiological responses in human cells to be measured, to determine the impact of an antioxidant stimulus on human cells. Due to the role of macrophages in the inflammatory response of many disease states related to oxidative stress, these cells are ideal for investigating the antioxidant potential of a sample. The presence of the prostaglandin derivative, 8-isoprostane is elevated in many pathological states associated with oxidative stress (2). The release of 8-isoprostane occurs in response to oxidative stress in human macrophages, after ROS generated peroxidation of arachidonic acid. This chemically stable lipid peroxidation product (54) is therefore commonly used as a reliable marker for oxidative stress in human cells (54).

Lipid peroxidation

Lipid peroxidation is elevated in many oxidative stress related disease states (31) or injuries (55). The process of lipid peroxidation can occur when an electron is taken from polyunsaturated fatty acids (3), present in lipid cell membranes. The self-perpetuating chain reaction (10) causes oxidative degradation of lipids, resulting in damage to cellular membranes (10) and compromised the structural integrity of the cell (8). Lipid peroxidation (LPO) assay or thiobarbituric acid reactive species (TBARS) assay are frequently used to measure lipid peroxidation. Malondialdehyde (MDA), an advanced product of unsaturated lipid oxidation reacts with 2-thiobarbituric acid (TBA) in acidic conditions and at high temperatures (9, 52). The pink adduct resulting from the reaction is detectable at 352/553nm excitation/emission (9). Inhibition of lipid peroxidation is used to measure antioxidant activity of honey samples (10). Although results from this method are unable to distinguish between the kinetics and the stoichiometry of the reaction, it allows peroxyl radicals involved in lipid peroxidation to be measured (52). LPO assays accurately depict antioxidant reactivity with fast-reacting peroxyl radicals, which differ substantially from long-lived DPPH nitrogen radicals (56).

2.7 Medical applications for antioxidant properties of honey

The correlation between *in vitro* and *in vivo* benefits of honey is based on evidence of the ability to relieve oxidative stress in cells, tissues, organs and body fluids. Consumption of honey improves the antioxidant activity of human plasma, increasing blood vitamin C, β-carotene and glutathione reductase (23). The benefits of honey consumption have been reported in many cells (26), organs and systems including the gastrointestinal tract, liver, reproductive organs, pancreas, cardiovascular system and immune system (20).

Gastroprotective effects of honey

Oxidative stress is thought to be responsible for impairment to membrane fluidity and the redox state of mineral ion transporters of the small intestinal brush border (1). Glutathione has beneficial effects on mineral ion transporters, possibly influencing the bioavailability of other nutrients (1). Manuka honey has been shown to decrease ethanol-induced gastric damage in rat models, by enzymatic and non-enzymatic antioxidants preservation mechanisms (57). Furthermore, several phenolic compounds present in honey (eg. chlorogenic acid and caffeic acid) are protective in the small intestine in a rat model of ischemic reperfusion injury (58).

Influence of honey on reproductive health

In the reproductive organs, antioxidants present in honey can reduce cadmium-induced oxidative stress caused by the depletion of cellular glutathione. Cadmium is known to cause vascular endothelial damage in testes and reduces prostate function and sperm volume in men (59). In women, cadmium affects female fertility by preventing of cell progression to the blastocyst stage (59). Honey may be valuable in inhibiting cadmium-induced alterations to cellular redox states.

Benefits of honey to the cardiovascular system

Benefits of honey to the cardiovascular system include improvement to plasma lipid profile and endothelial function and reduced oxidative damage to red blood cell (RBC) membranes (24). The high lipid content and oxygen supply, as well as iron and copper content, make RBCs susceptible to oxidation (8). Antioxidants prevent lipid peroxidation, resulting in antihemolytic properties (24), with honey-derived flavonoids shown to reduce oxidative damage to erythrocytes through cell membrane incorporation (8). A major risk factor for cardiovascular disease is hypertension. A high level of oxidative stress in vascular structures has been linked to the pathogenesis of hypertension (54), with 8-isoprostane found to be at elevated levels in patients with resistant hypertension (54). Previous studies (24) indicate a role of honey consumption in the reduction of systolic blood pressure and

malondialdehyde, a marker for lipid peroxidation (48, 53). The reduction in systolic blood pressure is associated with downregulation of Nuclear factor-erythroid 2 (Nrf2) (31) Leading to reduced susceptibility of kidneys to oxidative stress (31). Furthermore, endothelial dysfunction associated with resistant hypertension was improved in the aortas of animal models when ROS generation was inhibited (54).

Benefits of honey to chronic inflammation

A reduction in NADPH through actions of TNF-α and IL-1β are implicated in the excessive formation of ROS (60). An excess of ROS initiates activation of cell signalling pathways resulting in increased transcription of inflammatory genes (60). Macrophages detect tissue injury and infections when exposed to immune regulators such as cytokines (61). In response to ROS, classically activated (M1) macrophages release inflammatory cytokines (61). The continuous ROS activation of pro-inflammatory cytokines, stimulates further ROS production, preventing the activation of anti-inflammatory M2 macrophages (61). If resolution is not reached, resulting chronic inflammation can lead to pathological disease states such as Alzheimer's disease, rheumatoid arthritis, arthrosclerosis, non-alcoholic fatty liver disease (62) and inflammatory respiratory diseases (60). Honey samples reduce inflammation (63) and inhibit nitric oxide *in vitro,* (64) in a dose-dependent manner. Whilst small increases in nitric oxide provide beneficial vasorelaxant and anti-platelet activities, large increases in nitric oxide are toxic and considered pro-inflammatory in pathological situations. Intravenous injection of honey in rat models reduces lipopolysaccharide-induced production of tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and increases in levels of heme oxygenase-1 (65). Manuka honey was found to protect macrophages from the inflammation induced by LPS stimulation through stimulation of p-AMPK, SIRT1 and PGC1α expression (26). ROS is associated with inflammatory disease and mitochondrial dysfunction (66). The use of natural compounds such as honey to treat inflammation is an attractive alternative to many current anti-inflammatory drug therapies that are often associated with unwanted side effects (26).

Role of honey in blood sugar regulation

Increased oxidative stress is an early symptom preceding the development of insulin resistance and type 2 diabetes. (67). Administration of honey has shown to increase levels of C-peptide associated with insulin resistance, thought to be through stimulation of ß-cells of the pancreas (68). Co-administration of honey improves effectiveness of hypoglycaemic therapeutics. Metformin reduces glycemia, glycosylated haemoglobin concentration and insulin resistance in patients with diabetes (24). Studies have shown (53) honey works synergistically with metformin, further relieving

oxidative stress in the kidneys of diabetic rats, which metformin failed to achieve when administered alone.

Anti-tumour properties

Manuka honey inhibits tumour growth, and improves chemotherapy treated host survival rates (69). These effects attributed to the antioxidant properties of phenolic acids, including vanillic acid (44) and chrysin (70). Antioxidant therapy has been used as an adjunct to chemotherapy drugs to prevent treatment-induced cognitive decline caused by oxidative stress (71). The mechanism of reducing risk of cancer through reduction in ROS generation represents a novel therapeutic in anti-tumour therapy, particularly in combination with other cancer therapies. For instance, honey's synergistic activity when used in conjunction with metformin additionally inhibits ROS and reduces damage to DNA (53) and somatic cell mutations (72).

Gene expression

Oxidative stress and ROS are associated with chromatin remodelling, such as histone acetylation, through activation of transcription factors (60). Treatment of cells with the flavonoid, chrysin, increased gene expression of Bax protein and reduced gene expression of Bcl-2, leading to apoptosis of lung cancer cells (70). Nrf2 induction regulates expression of genes encoding for key components of antioxidant systems involving glutathione and thioredoxin (49). Polyphenols produce an indirect antioxidants effect by increasing Nrf2 activity (49). Keratinocytes treated with honey prevented the translocation of nuclear factor kappa- β (NFK-β) to the nucleus (73) and inhibited the expression of inflammatory cytokines, nitric oxide synthase and cyclooxygenase-2 (COX-2) and PGE2 (73) in response to UV-B. The presence of particular antioxidant compounds in honey (e.g. Catechin) contribute to protective effects against free-radical induced DNA strand scission and degradation *in vitro* (46). Oxidative damage causing degradation of DNA by hydroxyl radicals was reduced in plasmid DNA treated with honey (46).

Benefits to the nervous system

The abundance of polyunsaturated fatty acids, low antioxidant capacity and high oxygen consumption of the central nervous system (CNS) increases its vulnerability to oxidative stress (71). Free radicals damage susceptible neurons, glial cells and mitochondrial DNA which can lead to neurodegeneration (4). The accumulation of ROS observed in brain hypoxia (74) and Alzheimer's disease has been linked to the down-regulation of tight junction proteins in brain tissue (75). Changes to the tight junctions compromises the integrity of the BBB, increasing permeability (74)

and promotes neuroinflammation. Antioxidants play a role in reversing cerebral amyloid angiopathy-generated ROS (75). This highlights the importance of exogenous antioxidant supplementation as a neuroprotectant in oxidative stress-related neurological disorders.

Levels of honey required for therapeutic effect

Phenolics from mono-floral honeys were shown to protect human erythrocyte membranes against oxidative damage at concentrations between 10–80µg/mL (8). Antioxidants in Manuka honey inhibited cancer cell growth in cells cultured containing Manuka at final concentrations of 0.3-2.5% (69). Protocatechuic acid, implicated for its properties in reducing diabetes, was detectable in plasma following consumption 8-10mL of honey (44). A pilot study investigated the antioxidant effects of oral administration of honey on human plasma, following consumption of honey between 0.75g/kg to 1.5g/kg body weight (76), observing increased total phenolic content and antioxidant activity, coupled with reduced MDA and ROS levels in plasma of blood samples 1-2 hours later. The occurrence of increased total phenolic content in plasma in human subjects was noted (24) in other studies, following consumption of 1.5 g of honey/kg body weight. The levels of honey required to accomplish therapeutic benefit depends on the route of administration. Intravenous administration requires lower doses, while the amount of honey required to reduce oxidative stress by consumption and digestive absorption is largely dependent on the bioavailability of antioxidant compounds within each sample.

2.8 Bioavailability of antioxidants in honey

Bioavailability of phenolics and flavonoids

Flavonoids are among polyphenols absorbed into the bloodstream through the intestinal epithelium, where they undergo phase II and phase III metabolism (24). The complex metabolism of ingested polyphenolic compounds can occur through several mechanisms during passage through the GI tract. Glycosidases present in the bee salivary glands convert phenolics to an aglyconic form, where they can be readily absorbed through the intestinal wall (48). Cleavage and modification of molecules into metabolites and catabolites allow entry from the small intestine into the circulatory system, where some undergo phase II metabolism (77). Chlorogenic acid is hydrolysed to caffeic acid in the small intestine, where it protects against cerebral ischemic reperfusion injury in the rat (58). Many polyphenols are modified by microbiota in the large intestine into low-molecular-weight phenolic acids, which can be readily absorbed into the circulatory system (77). Low molecular weight

phenolic catabolites were shown to be more likely to pass the BBB to protect human neuronal cells from oxidative stress than parent compounds with a molecular weight of more than 400Da (77). The implications of delivery of antioxidant compounds into the CNS could be of substantial therapeutic benefit to neurological disorders and brain injuries. Recently, antioxidants have been packaged in solid lipid nanoparticles, protecting them from enzymatic degradation, reducing clearance, increasing permeability across intestinal membranes and allowing penetration across the BBB (55). Nanoparticles synthesized to target delivery of antioxidants to the brain are a current focus for the treatment of Alzheimer's disease (78). The method of liposomal encapsulation of compounds found in honey, including carotenoids (79) and quercetin (80), has already been applied, showing promising results in the effectiveness of increasing bioavailability of antioxidants (80).

2.9 Properties of Australian honey

Australian native plants are known for their therapeutic properties (81), their use in traditional medicine is legendary. Investigations of archaeological evidence (82) has suggested long-term sustainable honey production by indigenous Australians pre-dates European settlement. Economic, social, and symbolic significance among indigenous communities has been placed on honey produced by the Australian native stingless bee (82). Popular Australian mono-floral honeys include a variety of eucalyptus species, each with a distinctive chemical profile unique to each native species. Types of mono-floral eucalypt honey include Jarrah, Yellow Box, Grey box, Blue Gum, River Red Gum, Stringybark, Messmate and various ironbark species. Unique biochemical characteristics of a botanical species are often processed into corresponding mono-floral honey.

Antioxidant properties of Australian mono-floral honey

Many species of Australian plants have antioxidant properties. *Euphorbia drummondii* extracts have DPPH free radical scavenging activity and reducing power (83). Extracts from Australian native wattle species including *Acacia kempeana* and *Acacia ligulate* show significant free radical scavenging activity (83). Tasmanian pepper leaf (*Drimys lanceolate*) was found to perform well in ORAC assays. This plant species contains derivatives of chlorogenic acid and quercetin (81). Anise myrtle and lemon myrtle contain ellagic acid and flavonoids such as catechin, myricetin, hesperetin, and quercetin. Flavonoids including 2',3',4'-trimethoxychalcone, 2'-hydroxy-3', 4'-dimethoxychalcone, 2',4'-dihydroxy-3'- methoxychalcone, 5, pinobanksin 3-acetate and 5,7-dihydroxy-6-methoxy-2,3-dihydroflavonol 3-acetate have been identified in *Acacia paradoxa* propolis, extracted from bee hives (84). Distinct compounds from plant pollen or nectar can be transferred into the honey. Acacia

honey is generally composed of lower phenolic content and higher flavonoid content, by comparison to other types of honey (37), consistent with the limited phenolics and abundant flavonoids found in pollen derivatives (84). *Acacia mellifera* honey has shown to have a flavonoid, catechin (37) equivalent of 5–10mg/kg (19), variances were related to geographic origin (19). Australian leatherwood (*Eucryphia lucida*) honey is known to contain 0.65 mg trans-abscisic acid per 100g honey (85). Novel honey types such Cheeseberry (*Cyathodes juniperina*) exist among private honey producers. However, the antioxidant capacity of unique species of Australian mono-floral honey is currently lacking.

Geographical influence on biochemical characteristics of Australian honey

It has been established that environmental influences relating to geographical locations of hives including sunlight, moisture, soil composition and species of bee native to a region (86), influence the composition of honey. The plant species from which pollen is collected from depends on the preference of the species of local honey bee populations. Species of honey bee, such *as Apis mellifera var ligustica*, found on Australia's Kangaroo Island, preferentially collect propolis from *Acacia paradoxa*. The abundance of flavonoids 2,3,4-trimethoxychalcone, 2-hydroxy-3,4-dimethoxychalcone, 2,4-dihydroxy-3-methoxychalcone, 5,7-dihydroxy-2,3-dihydroflavonol 3-acetate (pinobanksin 3-acetate) and 5,7-dihydroxy-6-methoxy-2,3-dihydroflavonol 3-acetate in *Acacia paradoxa* (84), result in honey rich in flavonoids derived from this region. The wide range of climate and environmental characteristics found between regions of Australia creates a platform for production of a diverse range of honey with unique chemical profiles. The growth of specific plant species can vary between ecosystems within the same region of Australia. Proliferation of Cooktown ironwood (Erythrophleum chlorostachys); associated with wild honey (82); occurs in *Eucalyptus tetradonta* woodland ecosystems and not coastal or mangrove areas (82) of the Cape York Peninsular region of Australia. This region has a unique environment, composed of aluminous bauxite gravel plateaus (82) with high annual rainfall and humid conditions. The range of environmental conditions in Australia provides an excellent platform for comparing the effect of geographical location on the phenolic composition of honey from the same botanical origin.

Australian honey has high non-peroxide antibacterial activity compared to honey from other countries (34). Non-peroxide activity is attributed to the presence of phytochemical components (34). The highest non-peroxide activity was found in *Leptospermum* honeys from Northern Rivers, New South Wales and the southeast coast of Queensland (34). In addition, high peroxide activity is found in Jarrah (*Eucalyptus marginate*) and Marri (*Corymbia calophylla*) honeys, native to Western

Australia (34). The influence of factors such as high osmolarity, low pH and presence of hydrogen peroxide and methylglyoxal contribute to antibacterial properties of samples (34). Therefore, the results for antibacterial activity in Australian honey samples is difficult to use as an indication of phenolic content.

The mineral content and antioxidant properties of eucalyptus honey is influenced by the geographical origin (87). Volatile compounds used to distinguish eucalyptus honey vary, depending on geographical origins. Eucalyptus samples from Europe contain 1-Octene, 2,3-pentanedione, acetoin, diacetyl, 2,3-pentanedione, dimethyldisulfide or 2-Hydroxy-5-methyl-3-hexanone, 3-hydroxy-5-methyl-2-hexanone marker compounds, compared to the ethylphenyl acetate or phenethyl alcohol, used to distinguish eucalyptus honey from Turkey (86). Existing literature has indicated gallic acid, coumaric acid and ellagic acids as major phenolic compounds present in selected Australian eucalypt honeys (88). The presence of minor quantities of chlorogenic acid, caffeic acid and ferulic acids were also found among samples. Flavonoid profiles of Australian eucalyptus honeys contain quercetin, luteolin, kaempferol and tricetin (85). It has been reported that Australian Eucalyptus honeys contains smaller quantities of pinobanksin, pinocembrin and chrysin flavonoids (17), and larger quantities of ellagic acid and gallic acid (16) compared to Eucalyptus honey produced in Europe.

Leptospermum, commonly referred to as tea tree belongs to the Myrtaceae family and encompasses 83 unique species, found in New Zealand, Southeast Asia and Australia. *Leptospermum* species that are a source of Australian honeys include *L. polygalifolium*, *L. liversidgei* and *L. whitei,* found predominantly in NSW and Queensland. *L. scoparium* is native to NSW, Victoria, Tasmania and New Zealand. New Zealand Manuka honey, obtained from bees foraging on *L. scoparium,* is currently considered the gold standard of anti-microbial (44) and antioxidant honey (26, 44). Comparisons of Australian and New Zealand *Leptospermum* honeys showed similar flavonoid profiles of quercetin, luteolin and quercetin 3-methyl ether (85). Gallic acid and abscisic acid in *L. scoparium,* and chrysin levels in *L. polygalifolium* were dominant in honeys originating from New Zealand. It is yet to be investigated how the antioxidant properties of Australian *L. scoparium* honeys compare to its New Zealand counterpart.

2.10 Future directions

It has been well established that honey has demonstrated therapeutic health benefits in numerous cell types and organ systems, attributed to the presence of multiple phenolic constituents. Phenolic and flavonoid compounds are responsible for the antioxidant properties of honey, although the presence and quantity of these compounds varies extensively between honey samples. The relationship between antioxidant activity and the botanical origin of a range of Australian honey types is necessary to determine which mono-floral species are suitable for use as a potential antioxidant supplement. Due to the variations in phenolic compounds between honeys from the same floral source, further investigation is required to determine the influence of geographical origins on the antioxidant activity of honey from the same floral origin. Extensive research on the composition of different varieties of mono-floral honey has been performed thus far. However, the antioxidant activity of individual phenolic and flavonoid constituents, unique to each species, requires further investigation to better understand the mechanisms responsible for the antioxidant activity of honey samples.

Implications for honey industry

In Australia, approximately 10,000 beekeepers with around 500,000 hives produce 25,000 to 30,000 tonnes of honey every year (89). Of the honey produced in Australia, about a third is exported worldwide, predominantly to the USA, UK and Asia (89). The Australian honey industry generates around 60 million AUD annually (89), and identification of floral sources and geographical origins responsible for producing honey with high antioxidant properties could increase the value of Australian honey. The comparison of Australian Manuka with its New Zealand counterpart could have major implications if regions yielding honey with comparable or higher levels of major active compounds are identified, as this adds significant value to the price of a honey sample. The identification of novel antioxidant constituents in honey could lead to the discovery of potentially unique, potent or bioactive compounds, valuable in treatment or prevention of diseases and disorders related to oxidative stress.

The aims of this study were:

1) To compare the antioxidant activity of Australian mono-floral honey from a range of botanical origins and determine which mono-floral species have greatest therapeutic potential for treatment of pro-oxidant disease states.
2) To determine the influence of geographical origin on the antioxidant activity of samples from the same botanical origin.
3) To compare total antioxidant activity and total phenolic content of honey samples and determine if samples with high phenolic content will have greater antioxidant activity than those with lower phenolic content.
4) To isolate chemical constituents of Australian honey samples and test their individual antioxidant bioactivities.

The format of this thesis follows traditional thesis formatting. The introduction is combined with the literature review.

3. Materials and Methods

3.1 Honey samples

A diverse range of mono-floral honeys consisting of Jarrah, Cheeseberry, Leatherwood, Eucalyptus and Leptospermum species were selected for analysis. Where possible, triplicate mono-floral honey samples sourced from different regions of Australia were selected to obtain comparison of geographical influence on composition. Honey samples were supplied by Capilano Honey Pty Ltd, Blue Hills Honey, Chemcentre (Chemisty Centre WA) and small private honey producers. Samples were stored in a dark, airtight container at room temperature for the duration of the study. A total of 56 samples (Table 3.1) provided an overview for compositional comparison between Australian honey varieties.

Table 3.1: Honey samples selected for analysis, including botanical and geographical origin.

Honey	**Plant-derived species**	**Harvest location**	**Identifier**
Cheeseberry	*Cyathode glauca*	Triabunna, TAS	CB
Bluetop Ironbark	*Eucalyptus fibrosa*	Cecil Plains, QLD	177134
		Warwick, QLD	177010
		Miles, QLD	177832
Caley's Ironbark	*Eucalyptus caleyi*	Inverell, NSW	177575
		Glen Innes, NSW	180796
		Bundarra, NSW	175650
Coolibah	*Eucalyptus microtheca*	Moree, NSW	176944
Grey Ironbark	*Eucalyptus paniculata*	Imbil, QLD	183017
		Warwick, QLD	174622
		Grafton, NSW	175799
Hillgum	*Eucalyptus fasciculosa*	Warwick, QLD	182699
		Gundagai, NSW	181449
		Tumut, NSW	182506
Jarrah	*Eucalyptus marginata*	Western Australia	Jarrah
Messmate	*Eucalyptus obliqua*	Torrington, NSW	183389
		Torrington, NSW	177740
		Cooma, NSW	177890
Mugga Ironbark	*Eucalyptus sideroxylon*	Narrabri, NSW	180911
		Narrabri, NSW	179430
		Wee Waa, NSW	176943
Narrowleaf Ironbark	*Eucalyptus crebra*	Cecil Plains, QLD	177135
		Cecil Plains, QLD	177131
		Dinmore, QLD	177260
Peppermint	*Eucalyptus radiata*	Tumbraumba, NSW	183493

		Sydney South, NSW	182502
		Marulan, NSW	175055
River Red Gum	*Eucalyptus camaldulensis*	Warwick, QLD	181338
		Dalby, QLD	181841
		Cootamundra, NSW	182160
White box	*Eucalyptus quadrangulata*	Glen Innes, NSW	177835
		Wauchope, NSW	178082
		Manilla, NSW	177735
Yellow box	*Eucalyptus melliodora*	Wauchope, NSW	181711
		Tamworth, NSW	180668
		Inverell, NSW	180467
Leatherwood	*Eucryphia lucida*	Mawbanna, TAS	1827
		Mawbanna, TAS	1829
		Mawbanna, TAS	1839
		Mawbanna, TAS	Teepo3
		Mawbanna, TAS	Collingwood
Olive tea-tree	*Leptospermum liversidgeii*	Northern Rivers, NSW	333
		Northern Rivers, NSW	334
		Northern Rivers, NSW	338
		Northern Rivers, NSW	339
Jelly bush	*Leptospermum polygalifolium*	Northern Rivers, NSW	377A
		Northern Rivers, NSW	378
		Northern Rivers, NSW	380
		Northern Rivers, NSW	382
Whitei	*Leptospermum whitei*	Northern Rivers, NSW	311B
		Northern Rivers, NSW	313
		Northern Rivers, NSW	S2
Manuka	*Leptospermum scoparium*	Mawbanna, TAS	1806
		Mawbanna, TAS	1815
		Mawbanna, TAS	1818
		Mawbanna, TAS	1822
		Mawbanna, TAS	FI:2

3.2 Analysis of unfractionated honey samples

Reverse Phase-High Performance Liquid Chromatography of phenolics and flavonoids

Honey samples were diluted with 9 times weight of honey using deionized H_2O (dH_2O) to obtain 1.0:9.0 honey: water solution. The mixture was vortexed until completely homogenized. Analytical RP-HPLC with UV detection and reverse-phase column of the honeys was used to identify and

quantitate constituents present in the samples. Analytical RP-HPLC of honeys utilized Agilent 1290 Infinity II quaternary pump, autosampler column and diode array detector. The column was a Synergi Fusion RP (50mm x 4.6mm x 2.5µm) column. Mobile phase (MPA) consisted of 94.95% H_2O; 5% acetonitrile (ACN) and 0.05% formic acid. The mobile phase (MPB) was composed of 99.95% ACN and 0.05% formic acid. The flow rate was 0.5mL/min, and injection volume was 20µL. Detection wavelengths were set to 220, 260, 290 and 340nm. The mobile phase program began at 100% MPA isocratic for 2.0min, grading to 53:47 MPA: MPB from 20.0min, grading to 20:80 MPA: MPB at 20.5min, holding isocratic to 21.5min, grading back to 100% MPA at 22.0min, and isocratic to 23.0min.

The retention times and UV spectra from OpenLAB CDS ChemStation Edition-generated chromatograms were used to quantitate phenolics and flavonoids. The five highest peak areas above a minimum 20 milli-Absorbance Units (mAU) were recorded, excluding peak areas observed before 2.0min elution time. The phenolic composition was compared between samples using chromatograms. The peak areas were used to select honeys with the highest phenolic content of each mono-floral species with unique composition for further analysis.

Reverse Phase-High Performance Liquid Chromatography of Leptosperin and lepteridine

Analysis of 16 samples of *Leptospermum* honey was performed to determine the presence of leptosperin and lepteridine. Approximately 3-4g of each of the *Leptospermum* honey samples (Table 3.1) were weighed into test tubes. Each sample was diluted (1.0:9.0) with dH_2O and vortexed until the honey was completely dissolved. Samples were run on an HPLC (Agilent Technologies 1260 Infinity II quaternary pump auto-sampler) equipped with diode array detector (DAD), Inline Fluorescence Detector and Synergi Fusion-RP 4um (75 x 4.6mm) column. The two mobile phases consisted of 90% H_2O/10% ACN (MPA) and 100% ACN (MPB), at a flow rate of 1.5mL/min and injection volume of 20µL. The mobile phase program started at 100% MPA, isocratic for 3.0min, graded to 90:10 MPA: MPB at 5.0mins, graded to 50:50 MPA: MPB at 6.0min, isocratic until 7.0min, grading back to 100% MPA at 7.7min, isocratic until 8.5min. The chromatograms were analysed to identify lepteridine by fluorescence at 330/475nm excitation/emission, and detection of leptosperin by UV at 262nm DAD absorption.

2,2-diphenyl-1-picrylhydrazyl (DPPH) of unfractionated honey

Free radical scavenging activity was measured to determine antioxidant activity of all honey samples. The presence of antioxidants in honey samples neutralize deep violet DPPH radicals, resulting in a loss of colour absorbance at 518nm.

Approximately 0.1-0.3g of each honey sample was dissolved in dH_2O (1g/mL). A control was included to replicate the sugar content of honey. The control sample contained 40.2% fructose, 33% glucose, 7.5% maltose, 1.3% sucrose and 18% H_2O.

Gallic acid monohydrate (Sigma-Aldrich) (0.2M in methanol) was used to prepare gallic acid standards (methanol solvent) ranging from 1.5-150µM. L-Ascorbic acid (2.5mM) and butylated hydroxytoluene (BHT) (1.0mM), both prepared using methanol, were included as positive controls. A total volume of 100µL of ascorbic acid, BHT and gallic acid standard solutions were aliquoted into 1.5mL Eppendorf tubes. Honey solutions (1mg/µL) were aliquoted into Eppendorf tubes at volumes of 3µL, 10µL, 30µL, 50µL and 75µL. Methanol was added to obtain a volume of 100µL. A negative control consisting of 100µL methanol served as a reagent blank.

To each sample/standard/control, 400µL of DPPH solution (117.5µM, in methanol) was added to obtain 500µL of DPPH at 94µM. The final concentrations of gallic acid standards were 5.644×10^{-6} to 2.258×10^{-3}. Positive controls of ascorbic acid and BHT were 5×10^{-4}M and 2×10^{-4}M, respectively. Mixtures contained final concentrations of honey ranging from 3-75mg for each sample. Tubes were mixed using a vortex and immediately incubated in the dark for 30 minutes at 22°C. The presence of antioxidants resulted in a loss of deep violet colour.

Reaction mixtures (n=3) were added in duplicate 200µL aliquots into a 96 well plate and the plate was read at 518nm using an Enspire multimode plate reader (Perkin Elmer).

The antioxidant activity was expressed as percentage inhibition of DPPH radicals. Percentage inhibition of DPPH oxidation using equation 1.

Equation 1:

$$\text{Percentage inhibition} = [(\text{Abs. control} - \text{abs. sample})/\text{abs. control}] \times 100$$

The amount of honey that caused 50% inhibition of DPPH oxidation was determined and expressed as micromoles of gallic acid equivalents per gram of honey (GAE/g of honey).

Oxygen Radical Absorbance Capacity (ORAC) of unfractionated honey

Samples of mono-floral honey (Table 3.2) containing high phenolic content (RP-HPLC analysis) or high antioxidant activity (DPPH assays) were selected for further analysis using the ORAC assay.

Table 3.2: Honey samples selected for the ORAC assay.

Honey	***Identifier***	**Honey**	***Identifier***
Cheeseberry	CB1	River Red gum	182160
Bluetop Ironbark	177134	Whitebox	177835
	177010		178082
Caley's Ironbark	177575	Yellowbox	181711
	175650		180467
Coolibah	176944	*L. liversidgeii*	333
Grey Ironbark	183017		339
	174622	*L. polygalifolium*	377A
	175799		378
Hillgum	182699	*L. whitei*	311B
	182506		313
Messmate	177740	*L. scoparium (Manuka)*	1806
	177890		1815
Mugga Ironbark	180911		1818
	176943		1822
Narrowleaf Ironbark	177135		FI:2
Peppermint	183493	Leatherwood	1829
	182502	Leatherwood	Teepo3
River Red Gum	181338	Leatherwood	Collingwood
	181841	NZ Manuka	1804

Phosphate buffer solution (PBS) was prepared from 8g NaCl, 0.2g KCl, 1.44g Na_2HPO_3 and 0.24g KH_2PO_4 dissolved in 800mL of dH_2O. The pH of the solution was adjusted to 7.4 with HCl, dH_2O added to a total volume of 1.0L. All other materials required for ORAC assays including assay diluent and free radical initiator were provided in the OxiSelect™ ORAC Activity Assay Kit (Cell Biolabs, Inc).

A 1.0:9.0 ratio of honey to 1x assay diluent was made by homogenising approximately 0.1-0.2g of each honey sample with 1x assay diluent to a concentration of 100mg/mL. Samples were centrifuged for five minutes at 150 x g. The samples (10µL) were diluted 20-fold with 1x assay diluent to obtain a 5mg/mL final concentration of honey.

Trolox is a vitamin E analogue, and due to its water solubility, it is a commonly used antioxidant standard. A 0.2mM Trolox solution was prepared by combining 16µL of 5mM Trolox stock solution with 384µL 1x assay diluent. Trolox standards (200µL) in concentrations ranging from 2.5µM to 50µM were prepared by dilution of Trolox solution (0.2mM) with 1x assay diluent. Assay diluent served as the blank control. To examine the possible interference by the sugar component of the honeys, additional 30µM Trolox standard was prepared containing the sugar solution at a ratio of 1:100 and 1:200.

Duplicate 25µL aliquots of each sample and standard were dispensed into black 96 well plates. In a trial experiment, variable absorbance readings were obtained in the outer-most wells of the plate. To address this edge effect the outside 36 wells along the border of the plate were filled with 200µL H_2O for insulation. Fluorescein solution (1:100) was prepared by combining 95µL fluorescein probe with 9.405mL 1x assay diluent. Fluorescein solution (150µL) was added to each well containing sample or standard. The plate was covered with parafilm and incubated at 37°C for 30 minutes. Free radical initiator solution (25µL of 80mg/mL PBS) was added to samples and standards using a multi-channel pipette and mixed thoroughly. Antioxidants in honey reacted with the radicals incubated from 2,2'azodiisobutyramidine dihydrochloride (AAPH), preventing oxidative degeneration of fluorescein. The extent to which the antioxidants competed with the fluorescent probe to quench free radicals was measured by fluorescent light emission over time and compared to the antioxidant standards. Fluorescent readings were measured in triplicate using an Enspire multimode plate reader (Perkin Elmer), (480/520nm excitation/emission) at 37°C, every five minutes for 1-hour. The relative fluorescent value (RFU) for each sample over time was plotted using Microsoft Excel software and area under the curve (AUC) was determined after subtracting AUC of the reagent blank. The Trolox equivalent concentration of each sample was determined by comparing the AUC values to the standard curve. The values obtained for each sample were multiplied by the dilution factor (200) and divided by the conversion factor (1000) to calculate µmole Trolox equivalent (TE) per litre.

Free radical initiator 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH)

Thermolysis of a single molecule of AAPH produces two hydrophilic free radicals. The nature of this reaction allows identification of antioxidant compounds in honey involved in the reaction, through disappearance of compounds from RP-HPLC traces of honey that have interacted with free radicals.

The HPLC results were used to select 15 samples to represent a broad range of active Australian mono-floral honey, with unique phenolic profiles. The samples with the highest phenolic content from each mono-floral botanical origin were selected. Where samples from different botanical origin showed identical phenolic fingerprints, one mono-floral species was selected.

Table 3.3: Honey samples selected for AAPH analysis.

Honey	***Identifier***
Cheeseberry	CB1
Caleys Ironbark	175650
Coolibah	176944
Grey Ironbark	175799
Hillgum	182506
Messmate	177740
Messmate	183389
Narrowleaf Ironbark	177135
Peppermint	183493
River Red Gum	181338
Yellowbox	180467
L. liversidgeii	333
L. polygalifolium	377
L. whitei	311
L. scoparium (Manuka)	1818

For each sample (Table 3.3), equal parts of honey solution (diluted 1.0:4.0 with dH_2O) and AAPH solution (16mg/mL dH_2O) were combined. Controls were prepared by substituting AAPH solution with an equal volume of dH_2O. All samples were incubated for 2-hours at 65°C.

RP-HPLC was performed on incubated samples as previously described in RP-HPLC (Agilent 1290 Infinity II) analysis of whole honey phenolic and flavonoid samples. Peak areas observed at 260 and 340nm UV wavelength were examined for presence and quantities of phenolics and flavonoids. Peak areas of controls and AAPH-treated samples were compared to identify constituents which decreased >20% following AAPH treatment.

3.3 Isolation of active compounds from whole honey

Extraction of phenolic compound from honey

A range of five mono-floral honeys were selected for isolation of phenolic compounds (Table 3.4). The most active sample for each species was selected based on RP-HPLC, DPPH and ORAC results.

Table 3.4: Honey samples selected for phenolic extraction.

Honey sample
Cheeseberry
Coolibah
Grey Ironbark 175799
Messmate 177740
Tasmanian Manuka 1818
L. whitei 311

Approximately 60-80g of each honey sample was combined with 800µL absolute ethanol per gram of honey in a 250mL conical flask. The flask was swirled in a 55°C water bath until the ethanol and honey formed a homogenous mixture. The solution was gradually cooled to room temperature for 30 minutes prior to 1-hour refrigeration at 4°C and then -18°C for a minimum of 2-hours. The supernatant was collected, and the remaining sugar pellet was homogenized at 55°C with 250µL absolute ethanol/g honey. Samples were cooled as described above and the supernatant was collected. Supernatants collected from both extractions were transferred to a round bottom flask and evaporated to a thick consistency using a Rotavapour (Buchi R-205, 110 RPM) in a 45°C water bath. The yield was approximately 10% of the original weight of honey. The method was repeated with the phenolic extract used in place of honey. The volume of ethanol was adjusted according to the mass of extract obtained (800µL/g then 250µL/g). The second extractions yielded a product that was about 1% of original mass of the honey. The extraction process was repeated a third time yielding approximately 0.1% of the original honey mass in extracted phenolics.

Analysis of phenolic extracts

Samples of phenolic extracts were diluted 1.0:99.0 with dH_2O and RP-HPLC was performed as previously described in RP-HPLC (Agilent 1290 Infinity II) analysis of whole honey phenolic and flavonoid samples. Peak areas of phenolic extracts were compared to peak areas and retention times from chromatograms of whole honey analysis to compare phenolic fingerprints. New identical

phenolic fingerprints of whole honey and the phenolic extracts were observed, consistent with successful phenolic extraction.

DPPH assays were performed on the phenolic extracts (diluted 1.0:9.0 with methanol) as previously described for whole honey samples.
ORAC assays were performed on the samples of phenolic extracts as per methods described for whole honey samples. Each sample of phenolic extract was diluted 1:5000 with 1x assay diluent.

3.4 Cell-based assays

Whole blood was obtained from the antecubital vein of men and women from the Sunshine Coast region (HREC approval number: HREC/16/QPCH/114) Blood was collected in EFTA tubes. Human peripheral blood mononuclear cells (PBMC) were isolated and autologous serum prepared from the blood of abdominal aortic aneurysm patients, as described by Meital et al. (2018). PBMC were seeded into a 24-well plate at a density $3x10^5$ cells per well in Iscove's Modified Dulbecco's Medium (IMDM) containing 5% autologous serum and incubated in a 5% CO_2 incubator at 37°C. Following 2-hour and 24-hour and 4-day incubation periods, the supernatants were replaced with IMDM containing 5% autologous serum, 1000 U/ml penicillin, 1000µg/ml streptomycin, 2mM L-glutamine and 50 ng/ml macrophage colony-stimulating factor (M-CSF, Sigma, USA).

On 7-day, PBMCs were treated with Cheeseberry phenolic extracts at 10µg/mL and 1µg/mL. IMBM, excluding M-CFS was added to each well. Cells were incubated in a 5% CO_2 incubator at 37°C for 60 minutes. Following incubation, 4.2µL of water (control) or 1.2µL interferon gamma (IFN-γ) and 3µL *Escherichia coli*-derived lipopolysaccharide (LPS) (Serotype 0111: B4; Sigma, USA) were added to duplicate wells. Cells were incubated in a 5% CO_2 incubator at 37°C for 24-hours. Supernatants were collected, and cells were lifted from the plate using a cell scraper. Supernatants centrifuged (10,000 x g, 5min, 4°C), and the supernatant was stored at -80°C for later GPx and 8-isoprostane analysis.

Glutathione peroxidase (GPx) enzyme assay

PBMC cell extracts were thawed and homogenized with a handheld rotor-stator homogenizer (TissueRuptor). The homogenates were centrifuged (10 000 x g, 15min, 4°C) and the supernatants collected for protein and glutathione peroxidase enzyme activity determination.

A glutathione peroxidase assay kit (Cayman Chemical, Ann Arbor, USA) was used on cell lysates in accordance with manufacturer's specifications. The assay used $NADP^+$ formation to indirectly measure GPx activity through reduction of oxidised glutathione by glutathione reductase and the coupled oxidation of NADPH. Absorbance readings were immediately recorded following initiation of the reaction, then every minute for 6.0min after initiation, at 340nm wavelength. The rate of decrease of NADPH at 340nm allowed GPx activity of samples to be measured.

A Lowry protein assay was performed on extracted cell supernatant to determine GPx activity in proportion to total protein content. Cell supernatant (50µL), dH_2O (100µL) and 2M sodium hydroxide (50µL) were combined using a vortex and incubated for 30min. Reagent C was prepared from 1mL copper sulfate (0.1g/10mL dH_2O), 1mL potassium sodium titrate (0.2g/mL dH_2O) and 100mL sodium carbonate (2g/100mL dH_2O). Reagent C (1mL) was added to each of the mixtures, combined using a vortex and incubated for 10 minutes. Folins reagent (sodium 1,2-naphthoquinone-4-sulfonate) diluted 1:2 in dH_2O (100µL) was added, combined using a vortex and incubated for 30 minutes. Following incubation 200µL of each sample were dispensed into wells of a 96 well plate in duplicate and absorbance was read at 750nm. Microsoft excel software was used to express GPx activity per µg protein.

TNF-α and IL-6 assays

Cytokine levels in cell supernatants were measured to determine the inflammatory response of PBMC treated with phenolic extracts. Assays were performed using commercial immunoassay kits (Affymetrix eBioscience, San Diego, USA) in accordance with manufacturer's specifications.

8-isoprostane Assay

Levels of 8-isoprostane were measured in cell supernatants to determine if treatment of PBMCs with phenolic extracts changed the oxidative stress response of cells. Assay was performed using a commercial 8-isoprostane immunoassay kit (Cayman Chemical Company, Ann Arbor, USA) in accordance with manufacturers specifications.

The results from the GPx, 8-isoprostane and pro-inflammatory cytokine assays were analysed by Microsoft Excel student t.test, to determine statistical significance.

3.5 Isolation of antioxidant compounds from phenolic extracts

Preparative HPLC

Phenolic extracts from Tasmanian Manuka (1818), *L. whitei* (311) and Cheeseberry phenolic were selected for preparative HPLC. Preparative HPLC was performed on an Agilent 1200 series quaternary pump, autosampler and diode array detector. Each sample was dissolved in MPA (90% H_2O and 10% ACN). The RP-HPLC program used a flow rate of 25mL/min. The duration of solvent gradients for MPA and MPB (100% ACN) were adjusted for each sample. Each sample solution was injected onto a Synergi Fusion 50T 4µm column 100mm x 21.2mm with UV detection at 220, 260, 290 and 340nm. Significant peaks at 260 and 340nm were separately collected. Fractions were run through analytical RP-HPLC using a previously described protocol for phenolic extracts. Results of RP-HPLC for fractions were compared to chromatograms from phenolic extract and whole honey samples to confirm the identity of each compound. Fractions containing distinguishable significant peaks at 260nm were selected.

Selected fractions were distilled in a rotavapour (Buchi R-205, 110 RPM) in a 50°C water bath to remove ACN. Fractions were frozen at -80°C overnight in preparation for freeze drier process (ThermoSavant, ModulyoD) to remove remaining H_2O.

Table 3.5: Number of separated fractions recovered from each of 3 selected phenolic extracts.

Phenolic extract	**Number of isolated fractions**
Cheeseberry	5
Tasmanian Manuka 1818	5
L. whitei 311	7

Analysis of phenolic fractions of honey

RP-HPLC of extracted honey fractions were performed as previously described in RP-HPLC (Agilent 1290 Infinity II) analysis of whole honey phenolic and flavonoid samples.

The weight of each fraction was obtained and relevant H_2O was added to each fraction to achieve 2mg/mL concentration. DPPH assay was performed on each fraction (2mg/mL), as previously

described for samples of whole honey and phenolic extract. ORAC assays were performed on each fraction (diluted 0.1 to 0.01mg/mL with assay diluent) as previously described for samples of whole honey and phenolic extracts.

RP-HPLC was performed on known chemical standards (Table 4.6), to obtain retention time and UV spectrum for comparison with isolated phenolics. RP-HPLC followed methods previously described in RP-HPLC (Agilent 1290 Infinity II) analysis of whole honey phenolic and flavonoid samples. Known chemical standards of ascorbic acid, gallic acid, 4-hydroxyphenyllactic acid, lepteridine, chlorogenic acid, 4-Hydroxybenzoic acid, leptosperin, vanillic acid, caffeic acid, p-coumaric acid, 4-methoxyphenylLactic acid, trans-ferulic acid, methyl syringate, quercetin, naringenin, apigenin, kaempferol, chrysin, pinocembrin and 2-methoxyacetophenone were used.

4. Results

4.1 Whole honey

RP-HPLC of phenolics and flavonoids

RP-HPLC analysis of honey with detection at 260, 290 and 340nm produced chromatograms (Figure 4.1) for analysis of phenolic acids and flavonoids. From the chromatograms, the top five peak areas over 20mAU were selected to represent total phenolic content for each honey sample (Table 4.1). Peaks observed prior to 2.0min elution time were not included in results to eliminate readings of sugar interferences.

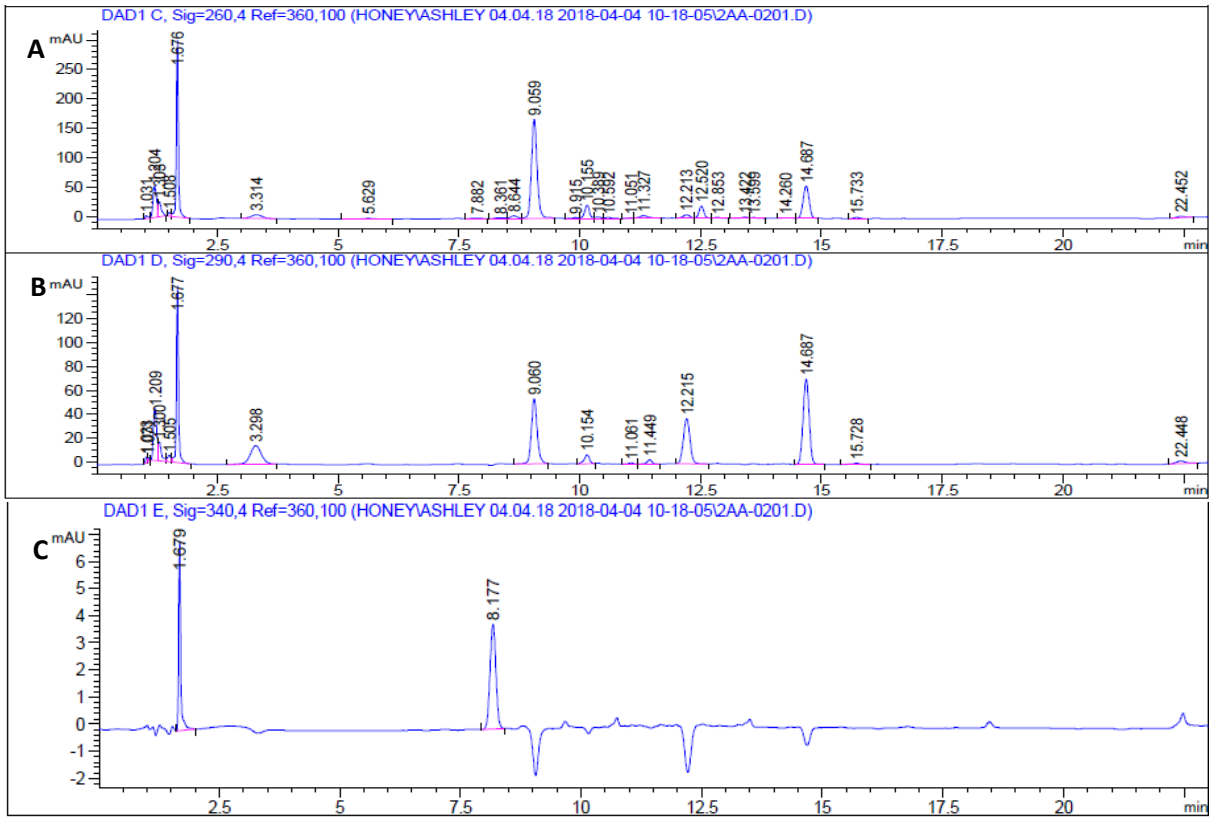

Figure 4.1: Example of chromatogram of RP-HPLC results for *L. polygalifolium* 378 sample. 260nm (A), 290nm (B) and 340nm (C) wavelengths for phenolic compounds over 23.0min elution time. Several significant peaks representing the presence of phenolic acids were observed at 260 and 290nm, and a single flavonoid at 340nm. All 3 wavelengths showed peaks prior to 2.0min elution time, which were regarded as sugar interference and excluded from results.

Table 4.1: The combined peak areas (mAU) of the 5 largest peaks for each sample. Peak areas below 20mAU were excluded.

Honey sample	Top 5 peak areas (mAU)
Bluetop Ironbark 177134	349
Bluetop Ironbark 177010	416
Bluetop Ironbark 177832	265
Caleys Ironbark 175650	116
Caleys Ironbark 177575	1322
Caleys Ironbark 180796	643
Cheeseberry	1063
Coolibah	831
Grey Ironbark 174622	677
Grey Ironbark 175799	2538
Grey Ironbark 183017	689
Hillgum 182699	510
Hillgum 182506	303
Hillgum 181449	704
L. liversidgei 333	1313
L. liversidgei 334	1051
L. liversidgei 338	1313
L. liversidgei 339	926
Messmate 177740	576
Messmate 177890	482
Messmate 183389	787
Mugga Ironbark 176943	340
Mugga Ironbark 180911	611
Mugga Ironbark 179430	237
Narrowleaf Ironbark 177135	332
Narrowleaf Ironbark 177131	428
Narrowleaf Ironbark 177260	476
Peppermint 182502	558

Honey sample	Top 5 peak areas (mAU)
Peppermint 183493	1741
Peppermint 175055	484
L. polygalifolium 377	4292
L. polygalifolium 378	2260
L. polygalifolium 380	2642
L. polygalifolium 382	3699
River red gum 181338	1560
River red gum 182160	418
River red gum 181841	514
Whitebox 177835	658
Whitebox 178082	526
Whitebox 177735	751
L. whitei 311	2630
L. whitei 313	1779
L. whitei S2	2023
Yellowbox 181711	462
Yellowbox 180467	713
Yellowbox 180668	602
Leatherwood 1827	805
Leatherwood 1829	858
Leatherwood 1839	817
Leatherwood Collingwood	709
Leatherwood Teepo 3	902
Tasmanian Manuka 1806	6076
Tasmanian Manuka 1815	4162
Tasmanian Manuka 1818	5434
Tasmanian Manuka 1822	3618
Tasmanian Manuka FI:2	1935

Honey samples with the highest phenolic content include those from *L. scoparium* (Tasmanian Manuka), *L. polygalifolium*, *L. liversidgei* and Cheeseberry samples (Table 4.1). The lowest phenolic content was observed in various eucalyptus samples (Table 4.1). Samples from coastal geographical origins showed higher phenolic content compared to samples from inland regions, even between mono-floral samples from the same botanical origin.

Leptosperin and lepteridine analysis

Chromatograms obtained from RP-HPLC of all *Leptospermum* honey samples found traces of lepteridine at 330/475nm ex/em and leptosperin at 262nm wavelengths (Table 4.2). All *L. liversidgei* samples and *L. scoparium* 1806, 1815, 1818, 1822 contained the highest quantities of leptosperin, with retention times of approximately 4.7min at 262nm (Figure 4.2). The highest quantities of lepteridine were found in *L. polygalifolium* 377, *L. scoparium* 1806, 1815 and 1818, with a consistent retention time of around 3.2min 330/475nm ex/em (Figure 4.3). The concentration of lepteridine and leptosperin in samples were determined using standard curve for lepteridine and leptosperin.

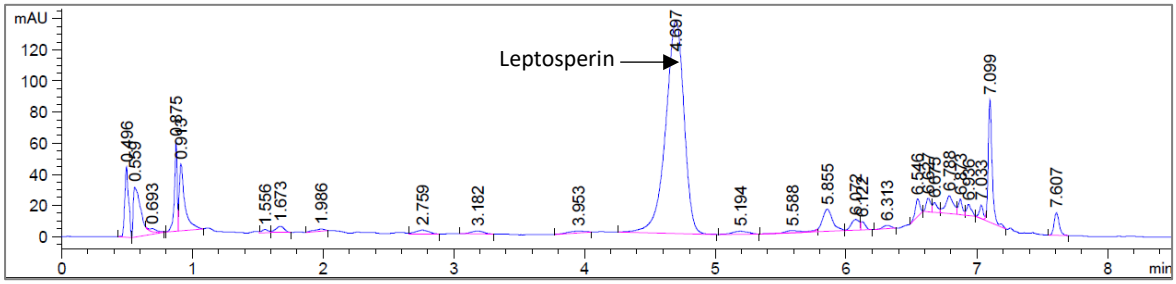

Figure 4.2: Detection of leptosperin by DAD absorption 262nm in Tasmanian Manuka 1818.

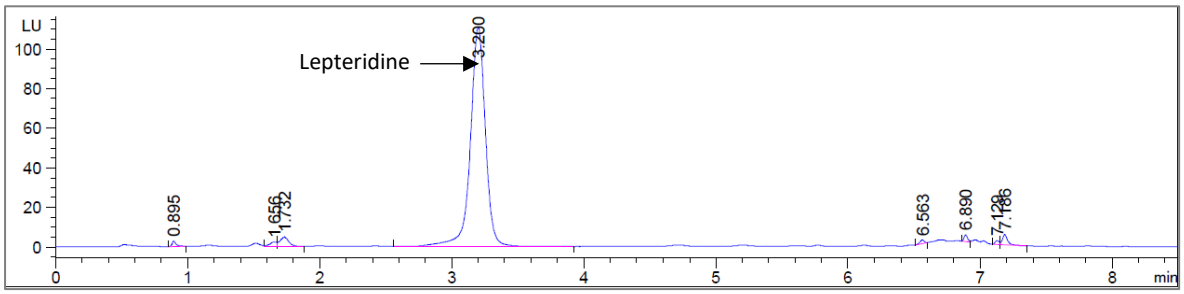

Figure 4.3: Detection of lepteridine by florescence at 330/475nm ex/em in Tasmanian Manuka 1818.

Table 4.2: Relative absorption units and retention times (RT) for 16 *Leptospermum* samples. Peak area determined using fluorescence detection (FLD) of lepteridine (330nm excitation/475nm emission) and UV absorption at 262nm (mAU) for leptosperin shown.

Leptospermum sample		**Lepteridine (330/475nm)**			**Leptosperin (262nm)**		
		Peak area (FLD)	RT (min)	ppm	Peak area (mAU)	RT (min)	ppm
L. liversidgeii	333	741	3.180	16.302	265	4.677	180.2
	334	782	3.183	17.204	167	4.676	113.56
	338	748	3.178	16.456	233	4.674	158.44
	339	605	3.182	13.31	112	4.678	76.16
L. polygalifolium	377	600	3.180	13.2	1045	4.670	710.6
	378	303	3.184	6.666	442	4.679	300.56
	380	302	3.179	6.644	486	4.670	330.48
	382	438	3.181	9.636	688	4.673	467.84
L. whitei	311	163	3.178	3.586	99	4.672	67.32
	313	239	3.179	5.258	104	4.675	70.72
	S2	254	3.189	5.588	289	4.685	196.52
L. scoparium	1806	881	3.209	19.382	1323	4.708	899.64
	1815	677	3.204	14.894	967	4.707	657.56
	1818	877	3.2	19.294	1335	4.697	907.8
	1822	850	3.196	18.7	618	4.693	420.24
	FI:2	160	3.193	3.52	224	4.687	152.32

Inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) by unfractionated honey samples

A dose-response curve of percentage DPPH inhibition vs concentration of honey (mg/ml) was constructed to determine the concentration of each honey sample required for maximal inhibition of DPPH (Figure 4.4).

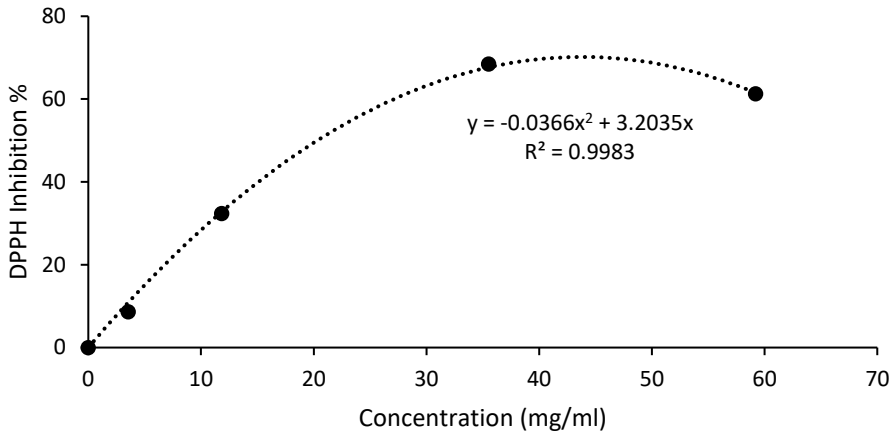

Figure 4.4: Dose-response curve of DPPH inhibition (%) vs increasing concentrations of Tasmanian Manuka 1818 honey sample.

A standard curve for percentage inhibition of DPPH by gallic acid in concentrations ranging from 5.644×10^{-6} - 2.258×10^{-3}mg/ml (Figure 4.5) was used to determine gallic acid equivalents EC_{50} values (concentration of honey required to produce 50% inhibition of the maximum inhibitory response), reported in Table 4.3.

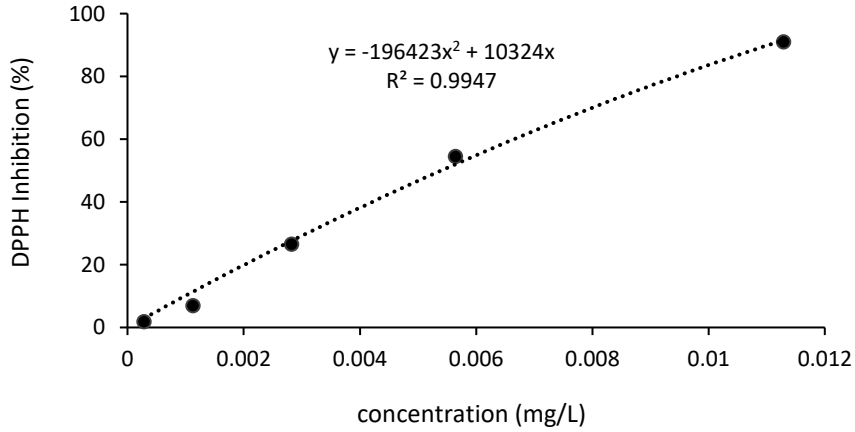

Figure 4.5: Standard curve for inhibition of DPPH with concentrations of gallic acid ranging from 5.644×10^{-6} - 2.258×10^{-3}mg/ml.

Table 4.3: Maximal DPPH percentage inhibition, EC_{50} (GAE mg/g) and IC_{50} (mg/ml honey) for honey samples.

	Max. Effect (%)	GAE (mg/g honey)	IC50 (mg/ml honey)
Bluetop Ironbark 177010	70.77 ± 0.59	0.035 ± 0.001	24.14 ± 2.61
Bluetop Ironbark 177134	68.14 ± 0.37	0.024 ± 0.002	29.63 ± 1.36
Bluetop Ironbark 177832	64.27 ± 1.02	0.015 ± 0.002	33.16 ± 2.98
Caleys Ironbark 175650	54.67 ± 0.51	0.014 ± 0.001	40.02 ± 2.53
Caleys Ironbark 177575	61.34 ± 1.90	0.038 ± 0.005	20.49 ± 2.29
Caleys Ironbark 180796	54.83 ± 2.01	0.015 ± 0.000	35.66 ± 0.82
Cheeseberry	70.37 ± 1.25	0.091 ± 0.007	12.62 ± 1.56
Coolibah 176944	70.40 ± 0.86	0.054 ± 0.005	18.51 ± 2.46
Grey Ironbark 174622	74.30 ± 1.21	0.052 ± 0.003	16.53 ± 0.79
Grey Ironbark 175799	71.05 ± 0.51	0.074 ± 0.005	14.97 ± 1.93
Grey Ironbark 183017	70.92 ± 0.15	0.056 ± 0.012	18.49 ± 0.25
Hillgum 181449	58.32 ± 2.44	0.020 ± 0.001	20.54 ± 1.39
Hillgum 182506	49.58 ± 1.13	0.033 ± 0.005	22.38 ± 0.62
Hillgum 182699	71.58 ± 0.22	0.045 ± 0.006	18.55 ± 0.88
Jarrah	70.89 ± 1.02	0.071 ± 0.004	12.76 ±0.85
L. liversidgei 333	28.33 ± 3.36	0.012 ± 0.001	32.43 ± 2.44
L. liversidgei 334	51.86 ± 0.58	0.015 ± 0.006	35.28 ± 7.01
L. liversidgei 338	56.15 ± 0.62	0.025 ± 0.002	21.75 ± 1.02
L. liversidgei 339	64.19 ± 0.76	0.039 ± 0.003	18.51 ± 0.44
L. polygalifolium 377	61.50 ± 0.17	0.044 ± 0.007	18.22 ± 0.84
L. polygalifolium 378	42.85 ± 6.10	0.033 ± 0.015	25.13 ± 3.45
L. polygaifolium 380	46.40 ± 2.57	0.025 ± 0.004	22.34 ± 2.00
L. polygalifolium 382	39.17 ± 1.14	0.013 ± 0.002	32.29 ± 4.99
L. whitei 311	67.30 ± 0.43	0.077 ± 0.006	14.48 ± 1.58
L. whitei 313	60.29 ± 0.46	0.039 ± 0.003	17.16 ± 0.68
L. whitei S2	64.29 ± 0.48	0.038 ± 0.005	17.10 ± 0.23
Leatherwood 1827	57.32 ± 2.23	0.019 ± 0.003	34.07 ± 4.44
Leatherwood 1829	59.66 ± 0.33	0.023 ± 0.002	24.03 ± 1.52
Leatherwood 1839	58.62 ± 0.68	0.025 ± 0.001	29.74 ± 1.72
Leatherwood Collingwood	62.27 ± 2.42	0.026 ± 0.003	27.24 ± 1.70
Leatherwood Teepo3	59.65 ± 1.52	0.022 ± 0.000	33.52 ± 2.58
Messmate 177740	47.87 ± 2.21	0.035 ± 0.003	25.39 ± 3.84
Messmate 177890	53.17 ± 1.53	0.027 ± 0.001	20.64 ± 0.85
Messmate 183389	26.95 ± 2.45	0.023 ± 0.005	21.44 ± 1.87
Mugga Ironbark 176943	64.72 ± 1.47	0.030 ± 0.006	29.17 ± 0.74
Mugga Ironbark 179430	57.27 ± 1.53	0.012 ± 0.005	44.84 ± 3.21
Mugga Ironbark 180911	67.91 ± 0.26	0.033 ± 0.004	22.63 ± 1.53
Narrowleaf Ironbark 177131	69.97 ± 1.49	0.032 ± 0.002	21.60 ± 1.27
Narrowleaf Ironbark 177135	73.94 ± 4.54	0.072 ± 0.040	23.21 ± 2.41
Narrowleaf Ironbark 177260	71.39 ± 0.74	0.049 ± 0.009	19.62 ± 0.53
NZ Manuka	76.57 ± 1.39	0.060 ± 0.001	17.41 ± 0.48
Peppermint 175055	54.33 ± 2.64	0.019 ± 0.003	30.49 ± 1.61
Peppermint 182502	66.05 ± 0.57	0.048 ± 0.002	24.12 ± 4.42

Peppermint 183493	33.09 ± 0.55	0.013 ± 0.003	24.64 ± 1.62
Red River Gum 182160	67.01 ± 0.75	0.052 ± 0.012	18.92 ± 0.21
River Red Gum 181338	70.62 ± 0.80	0.043 ± 0.001	18.60 ± 1.17
River Red Gum 181841	72.12 ± 3.21	0.060 ± 0.017	16.98 ± 0.82
Tasmanian Manuka 1806	64.12 ± 1.51	0.031 ± 0.002	24.64 ± 1.98
Tasmanian Manuka 1815	67.15 ± 0.63	0.030 ± 0.002	27.13 ± 1.58
Tasmanian Manuka 1818	67.27 ± 1.53	0.071 ± 0.011	18.33 ± 3.41
Tasmanian Manuka 1822	70.60 ± 2.94	0.063 ± 0.019	14.86 ± 1.45
Tasmanian mauka FI:2	73.27 ± 2.09	0.051 ± 0.007	23.96 ± 4.16
Whitebox 177735	63.19 ± 0.90	0.018 ± 0.002	34.13 ± 4.37
Whitebox 177835	60.69 ± 1.83	0.023 ± 0.003	36.86 ± 3.00
Whitebox 178082	61.55 ± 0.95	0.018 ± 0.006	33.49 ± 4.49
Yellowbox 180467	56.46 ± 1.13	0.018 ± 0.006	32.01 ± 3.03
Yellowbox 180668	56.15 ± 0.33	0.027 ± 0.003	23.24 ± 1.44
Yellowbox 181711	64.19 ± 0.73	0.034 ± 0.005	23.79 ± 1.86

Determination of Oxygen Radical Absorbance Capacity in unfractionated honey samples

Area under the curve (AUC) using normalized fluorescence intensity vs time was calculated in the absence or presence of the antioxidant (Figure 4.6; A). The results of ORAC assays (Table 4.4) using honey samples diluted 1:200, showed comparable activity in Tasmanian Manuka samples and a New Zealand Manuka sample. Activity in the Manuka samples was significantly higher than in all non-Manuka honeys. Lower ORAC values were recorded for eucalyptus honeys in general, with Yellowbox 180467 scoring lowest (1.84 µmole TE/g). Eucalyptus species such as Caleys Ironbark, Whitebox, Hillgum, Red river gum, Coolibah, Mugga Ironbark and Messmate produced lower scores compared to Bluetop Ironbark, Grey Ironbark and Peppermint. Overall *Leptospermum* honey samples produced higher ORAC values than Eucalypts. *L. polygalfolium* (4.05-4.06 µmole TE/g), *L. whitei* (3.53-4.28 µmole TE/g) and Leatherwood (3.95 µmole TE/g) species produced higher values than *L. liversidgei* (2.77-3.30 µmole TE/g). The highest antioxidant activity was shown in Cheeseberry (5.45 µmole TE/g) and Jarrah (5.35 µmole TE/g) samples.

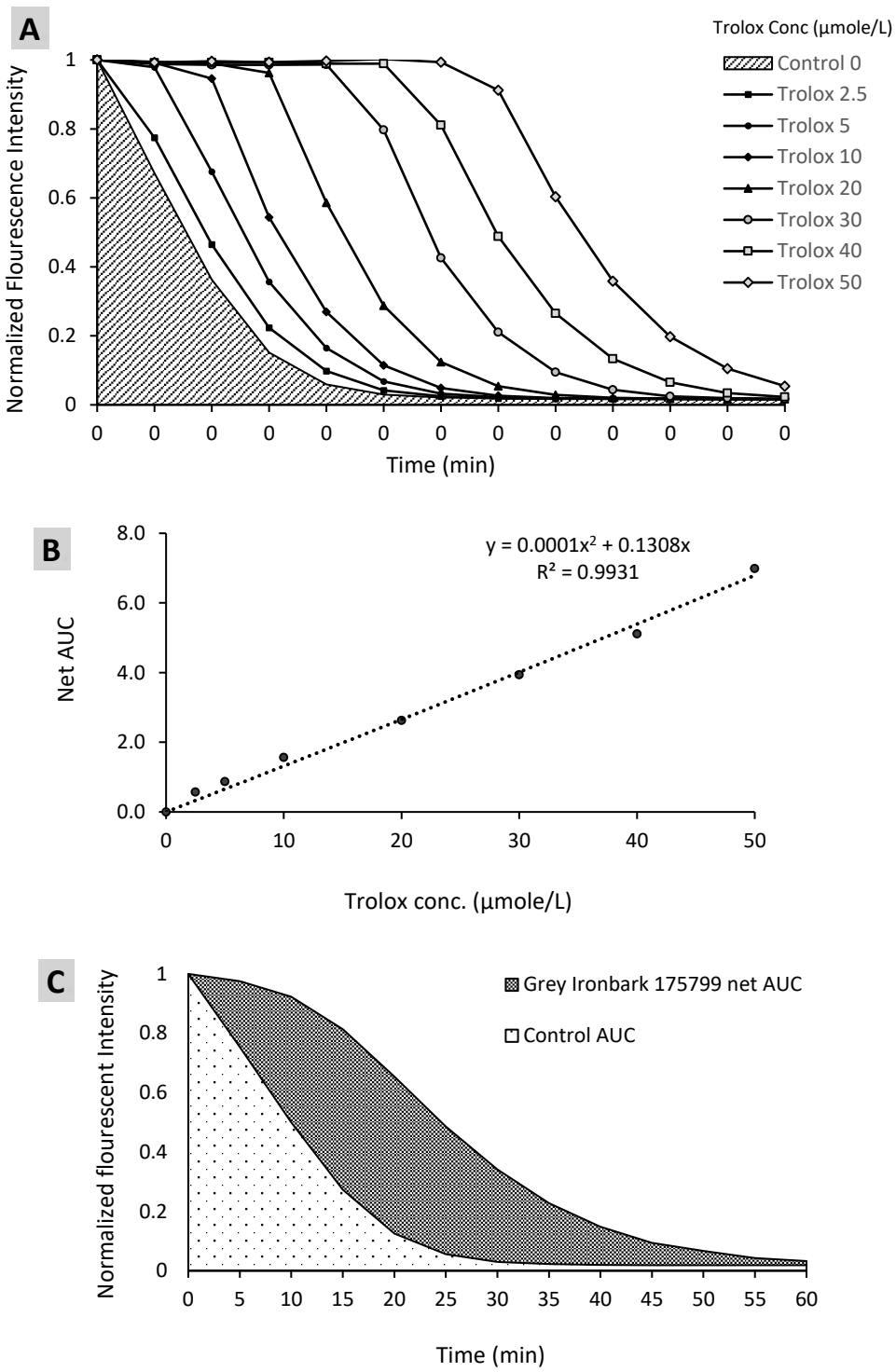

Figure 4.6: Determination of Oxygen Radical Absorbance Capacity (ORAC) of Trolox and honey sample. Normalized fluorescence intensity in the absence (control) and presence of increasing concentrations of Trolox (A). Area under the curve for control was subtracted from AUC for Trolox sample. Net AUC was plotted against Trolox concentration (B). ORAC was determined for honey sample 175799 (Grey Ironbark 1:200 dilution in dH_2O) (C) and converted to Trolox equivalent using the standard curve (B; Table 4.4).

Table 4.4: Samples selected for ORAC (Trolox equivalent).

Honey sample	**µmole TE/g**
Bluetop Ironbark 177010	2.56 ± 0.91
Bluetop Ironbark 177134	2.42 ± 0.91
Caleys Ironbark 175650	1.88 ± 0.68
Caleys Ironbark 177575	2.68 ± 0.92
Cheeseberry	5.45 ± 0.81
Collingwood (Leatherwood)	2.34 ± 0.53
Coolibah 176944	2.27 ± 0.42
Grey Ironbark 174622	2.50 ± 0.57
Grey Ironbark 175799	3.72 ± 0.62
Grey Ironbark 183017	2.73 ± 0.81
Hillgum 182506	2.19 ± 0.64
Hillgum 182699	2.10 ± 0.67
Jarrah	5.35 ± 0.38
Leatherwood 1829	3.95 ± 0.51
L. liversidgei 333	2.77 ± 0.64
L. liversidgei 339	3.30 ± 0.37
Messmate 177740	2.56 ± 0.76
Messmate 177890	3.12 ± 0.96
Mugga Ironbark 176943	2.31 ± 0.88
Mugga Ironbark 180911	2.48 ± 0.51
Narrowleaf Ironbark 177135	2.37 ± 0.70
NZ Manuka	4.46 ± 0.81
Peppermint 182502	3.25 ± 0.73
Peppermint 183493	2.65 ± 0.23
L. polygalifolium 377	4.05 ± 0.78
L. polygalifolium 378	4.06 ± 1.75
Redriver Gum 181338	2.28 ± 0.54
Redriver Gum 181841	2.18 ± 0.70
Redriver Gum 182160	2.41 ± 0.69
Tasmanian Manuka 1806	3.40 ± 1.11
Tasmanian Manuka 1815	3.57 ± 0.25
Tasmanian Manuka 1818	3.82 ± 0.88
Tasmanian Manuka FI:2	4.89 ± 0.89
Tasmanian Mauka 1822	3.52 ± 0.91
Teepo 3 (Leatherwood)	2.87 ± 0.80
Whitebox 177838	2.72 ± 0.99
Whitebox 178082	2.21 ± 0.73
L. whitei 311	4.28 ± 0.91

L. whitei 313	3.53 ± 1.16
Yellowbox 180467	1.84 ± 0.46
Yellowbox 181711	2.57 ± 0.81

Trolox standards had a steep rate of decline in AUC in comparison to the shallow curves (low rate of decline) for the honey samples (Figure 4.7).

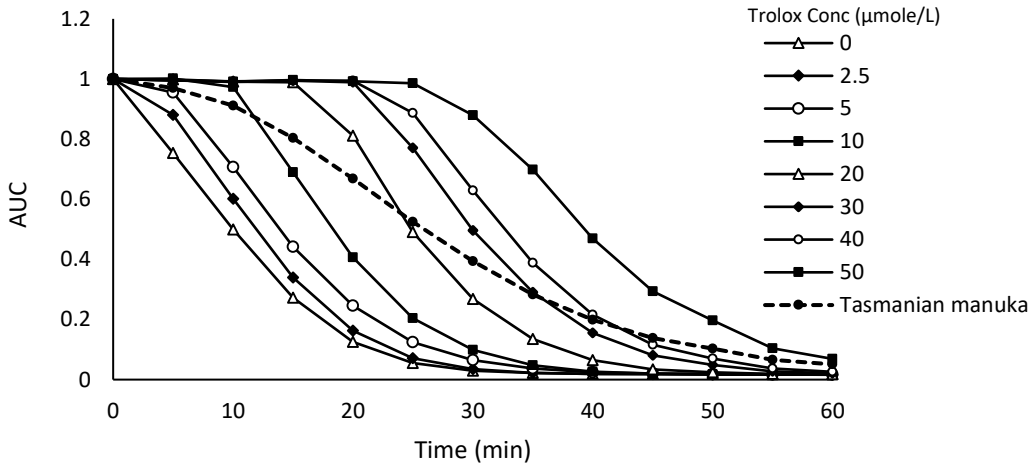

Figure 4.7: Antioxidant kinetics of Trolox standards and Tasmanian Manuka FI:2 honey sample.

Unlike honey, which comprises of approximately 82% sugars, the Trolox standards comprises of no sugars. To determine whether sugars affect the kinetics of the ORAC assay, a mixture of 82% sugars (40.2% fructose, 33% glucose, 7.5% maltose, 1.3% sucrose and 18% H_2O), diluted in dH_2O (1:100 and 1:200) was tested in the ORAC assay alone (Table 4.5) or in combination with Trolox (Table 4.6). The findings showed rightward shifts in curve compared to the control (Figure 4.8). The slope of the curves reduced with increasing ratios of sugar: H_2O, similar to that observed for honey alone. When added to Trolox, the sugars caused a rightward shift in the Trolox curve and reduced the slope of the Trolox curve (Figure 4.8).

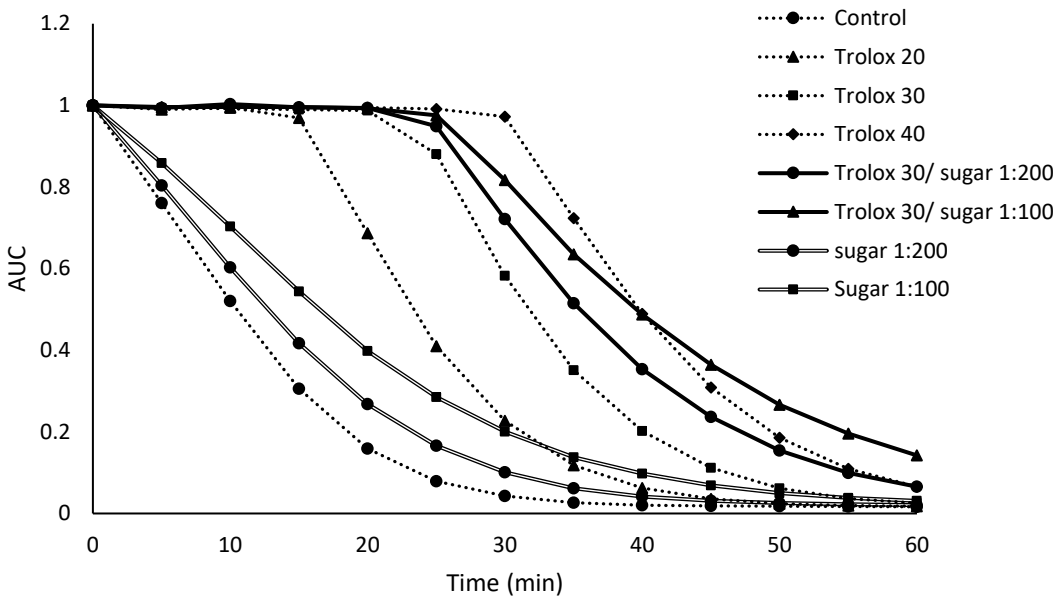

Figure 4.8: Antioxidant reaction kinetics for net AUC over 60 minutes. Dilutions (1:100 and 1:200) of sugar solution alone and combined with Trolox 30 µmole included. Trolox standards and control included for comparison.

Table 4.5: ORAC activity for three dilutions of 82% sugar solution.

Dilution Factor	µmole TE/g
1:200	0.255
1:150	1.021
1:100	1.650

Table 4.6: ORAC results for Trolox 30µM TE/L when combined with Increasing dilutions of sugar solution.

Sugar solution dilution factor	µmole TE/g
0	30
1:200	36
1:100	42

To determine if high concentrations of sugars in honey impact ORAC activity, selected honey samples were diluted 1:100 for the ORAC assay. ORAC activity was corrected for the dilution of honey sample to Trolox standard to give a final estimate of antioxidant activity (Table 4.7).

Table 4.7: Activity for honeys diluted 1:100 determined using the ORAC assay

Sample	**µmole TE/g**
Sugar solution	1.7
Jarrah	4.2
Red river Gum 182160	3.5
Tasmanian Manuka 1815	3.4
NZ Manuka	4.6

Antioxidant activity of honey constituents on 2,2'-azobis-(2-amidinopropane dihydrochloride)

Although DPPH and ORAC analysis report total antioxidant activity of whole honey samples, they do not provide information about antioxidant activity of individual constituents within the whole honey. HPLC allows separation of individual phenolics from whole honey. Addition of AAPH to honey samples resulted in the disappearance of antioxidant compounds which had interacted with AAPH radicals. By comparing HPLC traces from honey samples in the presence and absence of AAPH, it was possible to determine the retention times of individual antioxidant compounds in each sample.

Retention times (RT) were used to identify major peak area declines observed at 260nm and 340nm wavelengths from H_2O to AAPH treated samples (Table 4.8). The decrease for each significant peak is represented (Table 4.8).

Table 4.8: HPLC analysis of peak area (mAU) for phenolics and flavonoids, identified at 260 and 340nm, for honey samples incubated with dH_2O (control) or AAPH. The magnitude of the decline of peak area is indicative of antioxidant activity.

Honey sample	Wavelength (nm)	RT (min)	H_2O (mAU)	AAPH (mAU)	AAPH peak area (% of control)
Cheeseberry	260	5.4	32	17	53
	260	6.9-7.2	90	52	58
	260	8.4	163	128	79
	260	22.4	30	21	70
Coolibah 176944	260	2.2-2.4	385	192	50
	260	6.2	30	0	0
	340	14.7	15	0	0
	260	16.6	124	97	78
	340	16.5	27	12	44
Caleys Ironbark 175650	260	2.2-2.3	60	7	12
	260	6.2	20	0	0
	260	14.5	32	16	50
Grey Ironbark 175799	260	5.4	38	18	47
	260	9.8	24	15	63
	260	14.5	414	297	72
	260	16.6	32	24	75
Hillgum 182506	260	2.3-2.4	159	67	42
	260	5.4	16	0	0
	260	9.8	21	0	0
	260	14.5	28	0	0
***L. liversidgei* 333**	260	9.7	35	24	69
	260	10.8	28	18	64
	260	11.9	180	84	47
	260	12.7	24	14	58
	260	14.6	156	0	0
***L. polygalifolium* 377**	260	3.9	117	37	32
	260	5.4	32	23	72
***L. whitei* 311**	260	3	302	199	66
	260	3.9	141	81	57
	260	5.5	21	0	0
	260	12	37	0	0
	260	13.3	12	7	58
	260	13.4	22	0	0
	260	14.1	77	19	25
	260	14.5	1295	1133	70
	260	15.6	16	0	0
Messmate 177740	260	2.2-2.3	51	5	10
	260	5.4	34	25	74
	260	7.9-8	32	10	31
	260	12.2	30	20	67
	260	16	109	84	77
	260	17.3	24	0	0
Messmate 183389	260	2.3-2.5	229	89	39

	260	7.7	34	18	53
	260	8	30	0	0
	260	8.4	70	25	36
	260	9.6	50	18	36
	260	12.2	37	9	24
	260	13.1	94	66	70
	260	14.6	22	0	0
	260	15.4	90	49	54
	260	16	138	48	35
Narrowleaf Ironbark 177131	260	2.2-2.6	20	8	40
	260	3-3.1	61	49	80
	260	5.4	19	0	0
	260	6.2	18	0	0
	260	16.6	32	21	66
	340	16.6	16	0	0
Peppermint 183493	260	2.3	35	18	51
	260	4	37	26	70
	260	5.1-5.2	88	44	50
	260	7.6	54	37	69
	260	7.9	40	32	80
	260	8.4	130	46	35
	260	9.2	20	14	70
	260	15.4	29	20	69
	260	18.5	32	17	53
River red gum 181338	260	2.4	88	18	20
	260	5.4	27	0	0
	260	9.7	29	13	45
	260	10.1	37	16	43
	260	10.4	42	29	69
	260	10.9	42	34	81
Tasmanian Manuka 1818	260	2.4-2.6	33	16	48
	260	5.5	16	0	0
	340	8.6	57	0	0
	260	14.2	126	94	75
	260	14.6	468	153	31
	260	18	40	25	63
	260	22.4	20	0	0
Yellowbox 180467	260	2.8	102	63	62
	260	14.5	16	0	0
	260	14.9	14	0	0
	260	15.6	35	15	43
	260	16.6	25	18	72

*AAPH concentration and duration of incubation would affect the magnitude of response

Compounds that had antioxidant activity were identified by reduced peak area following incubation of honey with AAPH (Table 4.8). HPLC trace shows antioxidant activity of a compound in *L. liversidgei* 333, peak at 11.9 min RT decreased with exposure to AAPH (Figure 4.9). The UV spectra obtained by diode array detection of compounds in H_2O and AAPH treated samples visually compared to confirm compounds were identical. Identical UV spectra shown for antioxidant compound (11.9min RT) in H_2O and AAPH treated *L. liversidgei* 333 (Figure 4.9).

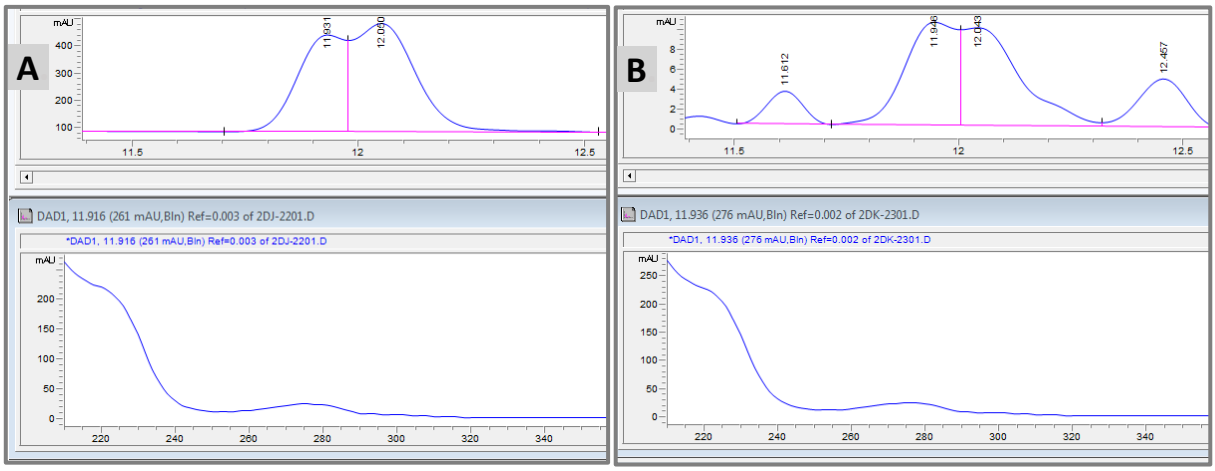

Figure 4.9: Chromatogram and UV spectra of compound at 11.9 min RT in *L. liversidgei* 333. AAPH treated sample has lower quantities of compound (B), compared to H_2O control (A). The UV spectrum are identical in both samples.

The total phenolic content of samples was compared to antioxidant activity determined by ORAC (Figure 4.10) and DPPH inhibition (Figure 4.11). Pearson's correlation coefficient determined there was a positive association between the total phenolic content and ORAC antioxidant activity of honey samples (r=0.5318). No correlation was found between total phenolic content and the DPPH antioxidant activity in honey samples.

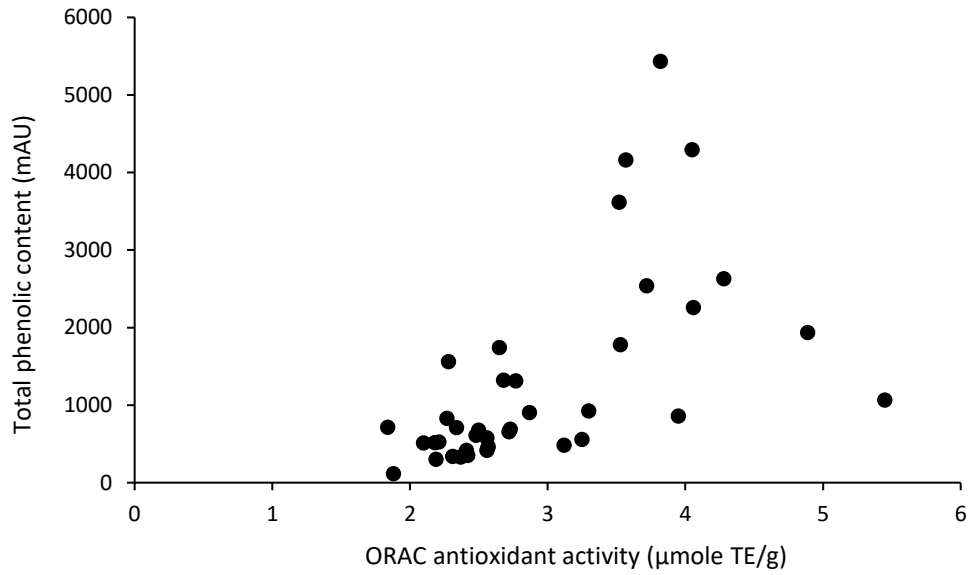

Figure 4.10: Comparison of total phenolic content (HPLC peak areas) vs antioxidant activity (ORAC µmole TE/g) for unfractionated honey samples.

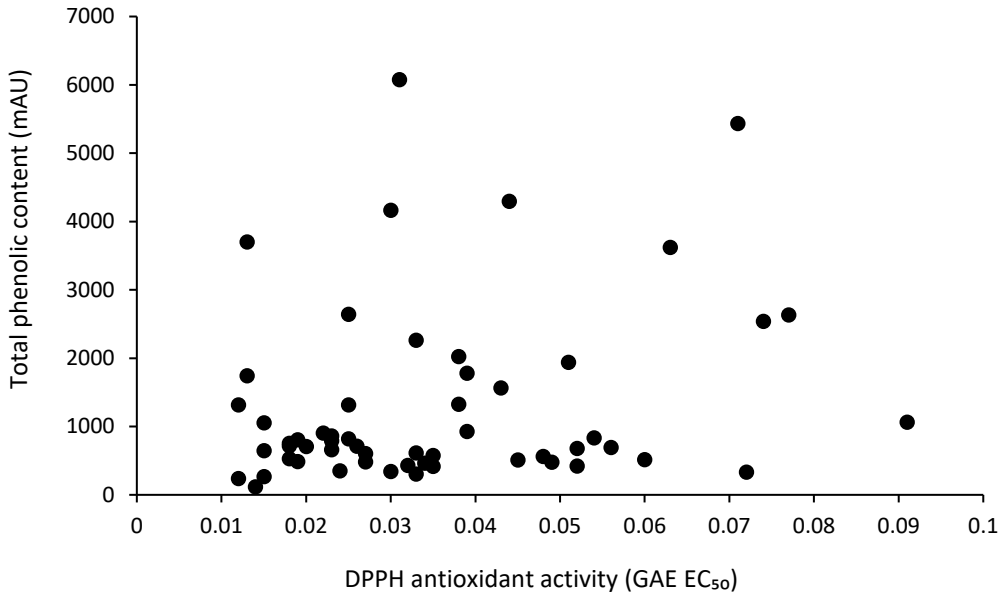

Figure 4.11: Comparison of total phenolic content (HPLC peak areas) vs antioxidant activity (DPPH inhibition EC_{50} GAE mg/L) for unfractionated honey samples.

4.2 Phenolic extracts

The first step in the process of isolating individual phenolics from honey samples requires the separation of phenolic compounds from the sugar and water components. Phenolic extracts require HPLC, DPPH and ORAC analysis to determine if the antioxidant constituents from honey were retained in the extract.

The results from HPLC, DPPH and ORAC analysis (Appendix 1) were used to select six honey samples for extraction of phenolic compounds. Cheeseberry, Coolibah, Grey Ironbark 175799, Messmate 177740, Tasmanian Manuka 1818 and *L. whitei* 311, were representative of a range of mono-floral honey species with different phenolic content and antioxidant activity.

Analytical RP-HPLC

Chromatograms from Tasmanian Manuka 1818 demonstrate that peaks observed in whole honey (Figure 4.12) were retained following extraction of the phenolics (Figure 4.13). Similar observations were made with the other five honey samples.

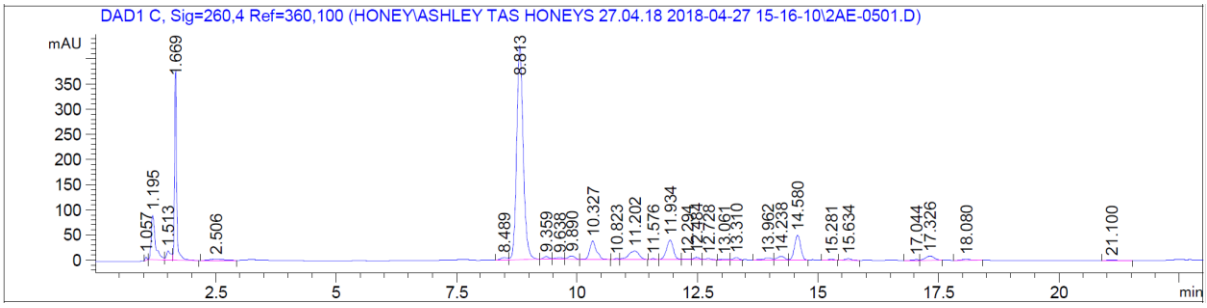

Figure 4.12: Chromatogram from RP-HPLC of unfractionated Tasmanian Manuka 1818 at 260nm

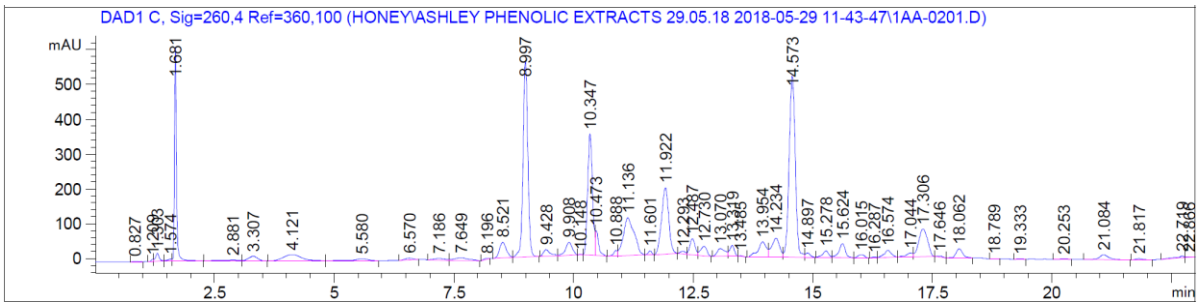

Figure 4.13: Chromatogram from RP-HPLC of Tasmanian Manuka 1818 phenolic extract at 260nm.

Determination of Oxygen Radical Absorbance Capacity (ORAC) in phenolic extracts of honey

The results using ORAC assays on 6 phenolic extracts (Table 4.9) showed *L. whitei* 311 as the sample with the highest antioxidant activity, followed by Cheeseberry, Tasmanian Manuka 1818, Grey Ironbark 799, Coolibah and Messmate 740.

Table 4.9: ORAC scores expressed as µmole TE/g for phenolic extracts of 6 honey samples (n=3).

Phenolic extract sample	**Mean µmole TE/g**
Cheeseberry	93.4 ± 12.8
Coolibah	44.9 ± 4.8
Grey Ironbark 175799	64.5 ± 8.6
Messmate 177740	32.9 ± 4.7
Tasmanian Manuka 1818	68.7 ± 4.3
L. whitei 311	129.0 ± 11.5

Inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) by phenolic extract of honey samples

DPPH activity was investigated for the 6 phenolic extracts. The maximal free radical scavenging activity was similar for all extracts (Table 4.10). The maximal inhibitory response (Figure 4.14) of 6 phenolic extracts were used to determine EC_{50} values (concentration of phenolic extract required to produce 50% inhibition of the maximum inhibitory response), expressed as gallic acid equivalent.

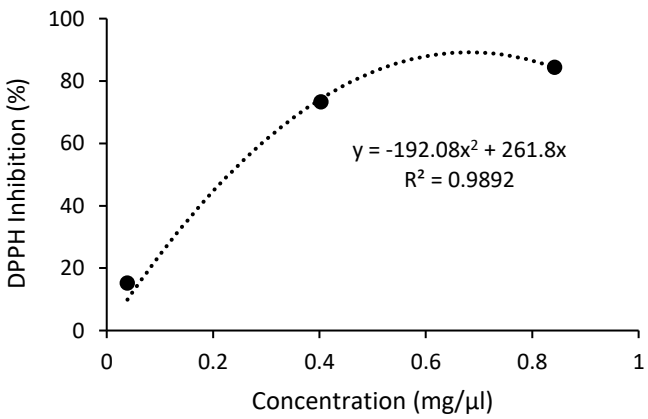

Figure 4.14: Percentage DPPH inhibition for increasing concentrations of the phenolic extract of Cheeseberry honey.

Table 4.10: Results for maximal DPPH inhibitory response for samples of phenolic extract (n=3).

Phenolic Sample	**Max effect (%)**
Cheeseberry	80.6 ± 0.8
Tasmanian Manuka 1818	84.4 ± 0.44
L. whitei 311	81.2 ± 2.2
Messmate 177740	84.3 ± 0.4
Coolibah	83.8 ± 0.2
Grey Ironbark 175799	82.8 ± 0.2

4.3 Cell based assays

HPLC analysis showed honey samples contained many phenolic constituents (Table 4.1), which were retained in phenolic extracts (Figure 4.2,4.3). The findings from ORAC and DPPH assays showed that antioxidant activity from the honey samples (Appendix 1) were concentrated in subsidiary phenolic extracts (Table 4.9, 4.10). The activity was mostly dependant on the mono-floral origin of a samples. Cell-based assays were used to test if the antioxidant properties of honey observed in cell-free assays could be effective *in vitro*.

Unstimulated blood-derived human macrophages (Figure 4.15, A) do not exhibit excessive oxidation, therefore determining the effects of antioxidant treatment such as phenolic extract on these cells (Figure 4.15, C) is difficult. As LPS has pro-oxidant effects on human macrophages (Figure 4.15, B), the increased oxidation in the cells allows the inhibitory effect of treatment (Figure 4.15, D) to be determined. Therefore, LPS stimulation was used to increase levels of oxidative stress in blood-derived human macrophages, to determine the protective effects of phenolic extracts of honey. Cheeseberry phenolic extract (10µg/ml) was selected to treat blood derived human macrophages for effect on Glutathione peroxidase (GPx) activity and 8-isoprostane levels prior to stimulation with LPS or media (control).

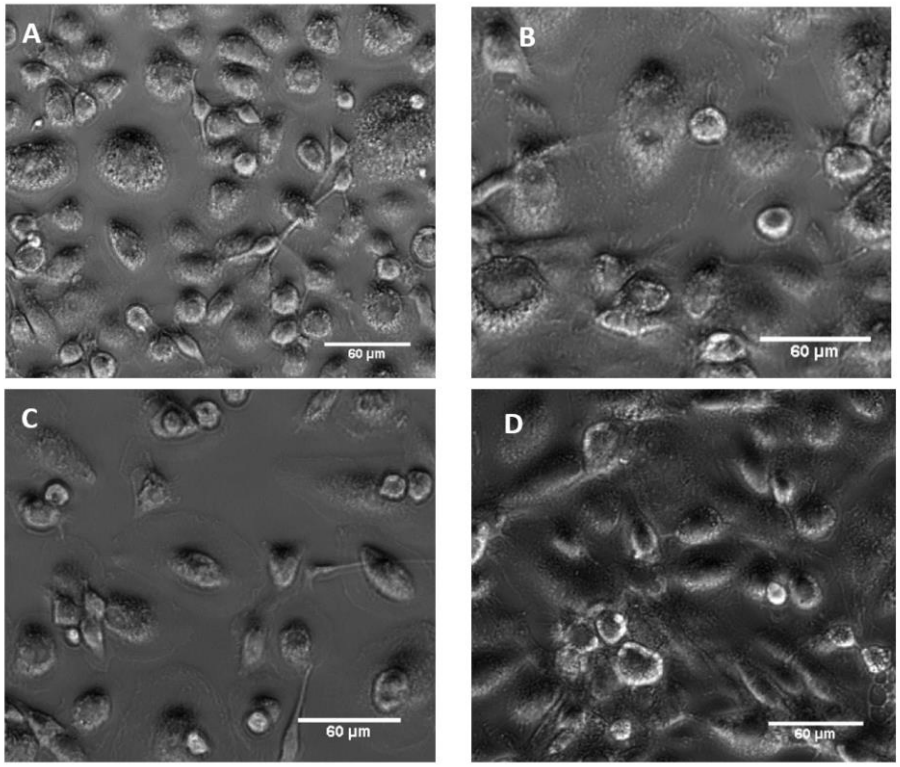

Figure 4.15: Phase contrast microscopy of blood derived monocytes. Control (A), LPS (0.1 µg/ml) stimulated (B, D) and treated with 10µg/ml Cheeseberry phenolic extract (C, D).

Glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) is an enzymatic antioxidant found in human cells. The role of this enzyme is to protect cells from oxidative stress by reducing free radicals. Elevated levels of intracellular ROS increase GPx activity in cells, to protect from oxidative damage. GPx activity was measured to determine if the phenolics in honey prevented an increase of ROS and corresponding GPx activity upon LPS stimulation. The results from GPx assay and Lowry protein assay (Figure 4.16) allowed relative GPx activity (pmol/min/ml/µg protein) to be calculated (Table 4.11).

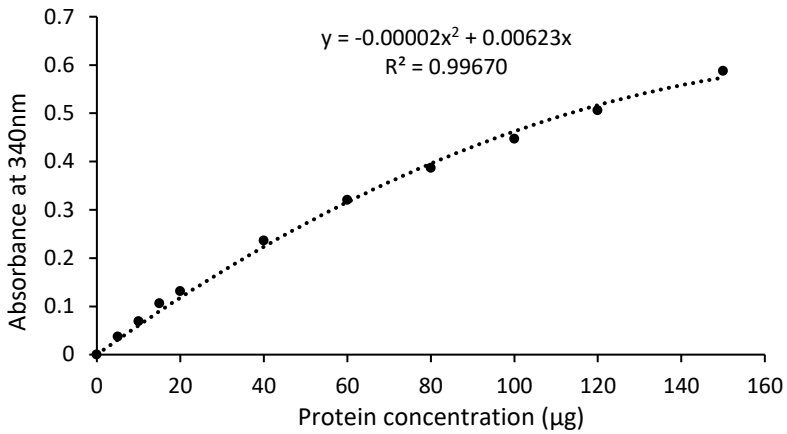

Figure 4.16: Standard curve for the Lowry protein assay

Although a slight reduction in GPx activity was observed in cells treated with phenolic extracts (10µg/ml) compared to control cells treated with media, differences in GPx activity was not significant. This occurred in the presence and absence of LPS stimulation (Table 4.11).

Table 4.11: Comparison of phenolic extract of Cheeseberry honey (10µg/ml) on GPx activity in cells, with and without LPS stimulation

	GPx activity (pmol/min/ml/µg protein)	
Treatment	**No stimulation**	**LPS**
Control (media) n=4	2.16 ± 0.39	2.28 ± 0.62
Phenolics (10µg/ml) n=4	1.97 ± 0.47	1.97 ± 0.34

Levels of 8-isoprostane detected in blood-derived macrophages

Macrophages release 8-isoprostane in response to peroxidation of fatty acids by free radicals. Elevated levels of 8-isoprostane are linked to the pathology of many disease states related to oxidative stress. The extent to which antioxidants in honey protect cells from oxidative damage was able to be determined from 8-isoprostane levels released by blood-derived human macrophages.

The results from 8-isoprostane assay (Table 4.12) showed higher levels of 8-isoprostane in LPS stimulated blood-derived macrophages, compared to controls. The results for the student t.test (paired 2-tailed) showed the treatment with Cheeseberry phenolic extract increased levels of 8-

isoprostane in non-stimulated cells (p=0.019), and significantly decreased 8-isoprostane in LPS stimulated cells (p= 0.008), compared to cells treated with media.

Table 4.12: Levels of 8-isoprostane in supernatant of blood-derived macrophages in the presence and absence of Cheeseberry phenolic extract 10µg/mL, with and without LPS stimulation.

	Cell supernatant 8-isoprostane levels (pg/ml)	
Treatment	**Non-stimulated**	**LPS**
Control (media)	18.31 ± 1.74	425.75 ± 39.28*
Phenolics (10µg/ml)	34.34 ± 2.15	182.96 ± 44.47*

*P=<0.05, compared to non-stimulated cells

Cytokine assays

Pro-inflammatory cytokines such as Interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) can stimulate excessive ROS production and lead to oxidative stress. The effects of antioxidants on the inflammatory response in human macrophages may assist in understanding of the links between oxidative stress and inflammatory diseases.

The concentration of IL-6 and TNF-α (Table 4.13) in supernatants obtained from human blood-derived macrophages were measured. LPS stimulation increased production of pro-inflammatory cytokines in all samples. Results for t.test (paired, 2-tailed) showed treatment with a phenolic extract of Cheeseberry significantly increased the concentration of TNF-α compared to media-treated cells (p= 0.0006) without LPS stimulation. The concentration of TNF-α in LPS-stimulated cells, with and without treatment with phenolic extract, were not significantly different. The concentration of IL-6 in cells with and without LPS stimulation were not significantly when treated with phenolic extract.

Table 4.13: Concentrations of IL-6 and TNF-α in blood-derived human macrophages.

	IL-6 concentration (pg/ml)		**TNF-α concentration (pg/ml)**	
Treatment	**Non-stimulated**	**LPS**	**Non-stimulated**	**LPS**
Control (media) n=4	1.4 ± 0.7	2543.4 ± 514.4	6.8 ± 4.2	67400.8 ± 5316.8
Phenolics (10µg/ml) n=4	17.5 ± 14.3	1936.4 ± 727.7	54.5 ± 3.7	69657.3 ± 7916.5

4.4 Isolated fractions from phenolic extracts of honey

RP-HPLC of isolated phenolic constituents

Isolation of phenolic compound from honey enables the antioxidant activity of each constituent to be measured. The identification of compounds with high antioxidant properties allows the presence of targeted compounds to be measured as an indicator of a sample's antioxidant capacity.

Antioxidant compounds in phenolic extracts of Tasmanian Manuka 1818, Cheeseberry and *L. whitei* 311 were separated by preparative RP-HPLC. The analytical RP-HPLC results showed that each of the isolated fractions contained predominantly one compound. Several fractions (Cheeseberry fraction 4, Tasmanian Manuka 1818 fraction 1 and 5) contained more than one compounds (Table 4.14).

Table 4.14: Analytical RP-HPLC retention times (RT) and quantity of extracted compounds for fractions from the 3 most active phenolic extracts of honey.

Sample	**UV Wavelength (nm)**	**RT (min)**	**Compound identity**	**Extract mass (mg)**
Tasmanian Manuka 1818				
Fraction 1	340	8.0		1.4
	260	8.5		
	260	9.9		
Fraction 2	260	10.3	Lepteridine	1.7
Fraction 3	260	11.9	4-methoxy phenylactic acid	1.3
Fraction 4	260	14.6	Methyl syringate	5.0
Fraction 5	260	17.3	Quercitin	1.3
	340	16.6		
Cheeseberry				
Fraction 1	260	2.8		1.4
Fraction 2	260	2.5		0.6
Fraction 3	340	1.6		0.3
Fraction 4	260	13.4		0.8
	340	13.4		
Fraction 5	260	15.6		0.5

L. whitei 311				
Fraction 1	260	3.8		0.8
Fraction 2	340	7.7	Lepteridine	<0.1
Fraction 3	340	7.7	Lepteridine	0.3
Fraction 4	260	8.8		0.4
Fraction 5	260	9.9	Leptosperim	0.6
Fraction 6	260	10.0		0.3
Fraction 7	260	14.6	Methyl syringate	1.3

Chemical standards of known antioxidant compounds were run on the RP-HPLC program used for honey samples and fractions. UV spectra and RT were used to compare phenolic compounds isolated from honey to known antioxidants (Figure 4.17, A). Lepteridine, 4-methoxyphenylactic acid, quercitin and methyl syringate were identified as compounds in Tasmanian Manuka 1818. *L. whitei* 311 contained Lepteridine, leptosperin and methyl syringate. The five fractions isolated from Cheeseberry honey were unable to be identified (Table 4.14).

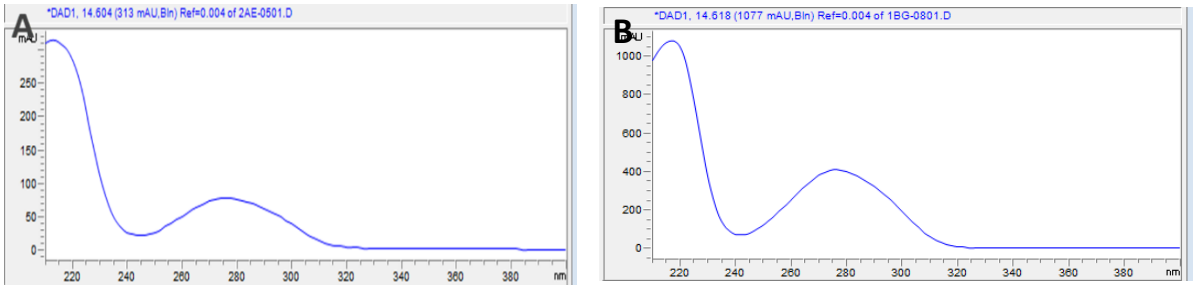

Figure 4.17: UV spectra at 14.6min RT for methyl syringate from HPLC of chemical standard (A) and fraction 4 of Tasmanian Manuka 1818 (B).

Inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) by fractionated compound of honey

Inhibition of DPPH free radicals using isolated fractions of concentrations up to 0.04mg/ml were unable to reach maximum effect due to insufficient quantity to establish a full dose-response curve (Figure 4.18). The EC_{50} values were therefore unable to be obtained through DPPH assay method of antioxidant analysis.

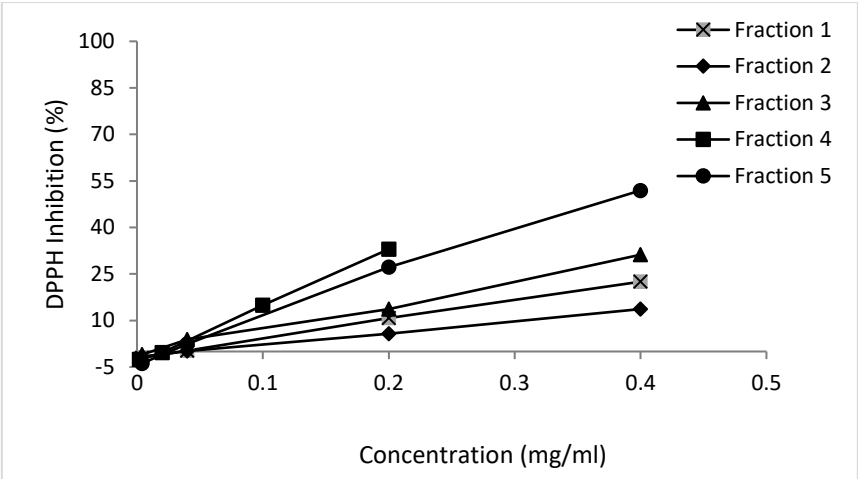

Figure 4.18: DPPH analysis of 5 fractions of Tasmanian Manuka 1818 at concentrations of 0.004mg/ml to 0.4mg/ml. A maximal response could not be attained with this concentration range

Oxygen Radical Absorbance Capacity (ORAC) of fractionated honey compounds

The antioxidant reaction kinetics from ORAC showed fast reacting honey constituents (Figure 4.19). From all compounds extracted from *L. whitei* 311, Tasmanian Manuka 1818 and Cheeseberry isolated fractions, each constituent showed antioxidant activity (n=3). Tasmanian Manuka (Fraction 4), Cheeseberry (Fraction 3) and *L. whitei* 311 (Fraction 5 and 7) showed significantly higher antioxidant activity than the remaining fractions (Table 4.15).

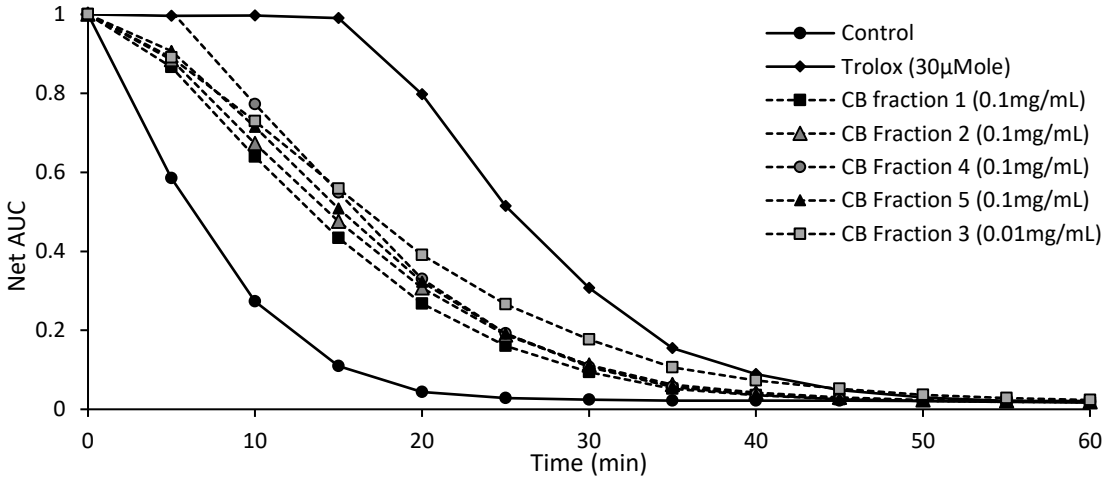

Figure 4.19: Antioxidant reaction kinetics for constituents extracted from Cheeseberry honey, compared to control and Trolox. Fraction 1, 2, 4, 5 (0.1mg/ml) and fraction 3 (0.01mg/ml).

Table 4.15: Trolox equivalent values for individual constituents isolated from honey phenolic extract.

Sample	**Fraction**	**Mean µmole TE/g**
Manuka 1818	Fraction 1 (0.1mg/ml)	530.74 ± 166.12
	Fraction 2 (0.1mg/ml)	91.71 ± 25.84
	Fraction 3 (0.1mg/ml)	505.10 ± 153.14
	Fraction 4 (0.01mg/ml)	4617.38 ± 711.27
	Fraction 5 (0.1mg/ml)	215.99 ± 38.55
Cheeseberry	Fraction 1 (0.1mg/ml)	310.26 ± 98.5
	Fraction 2 (0.1mg/ml)	382.76 ± 139.97
	Fraction 3 (0.01mg/ml)	5873.27 ± 2082.24
	Fraction 4 (0.1mg/ml)	470.07 ± 154.96
	Fraction 5 (0.1mg/ml)	385.44 ± 105.82
L. whitei 311	Fraction 1 (0.1mg/ml)	783.83 ± 94.87
	Fraction 2 (0.1mg/ml)	432.69 ± 117.73
	Fraction 3 (0.1mg/ml)	911.42 ± 332.04
	Fraction 4 (0.1mg/ml)	408.46 ± 151. 03
	Fraction 5 (0.01mg/ml)	3869.24 ± 1758.54
	Fraction 6 (0.1mg/ml)	929.38 ± 315.41
	Fraction 7 (0.01mg/ml)	5697.24 ± 3601.85

5. Discussion

5.1 RP-HPLC analysis of unfractionated honey

The antioxidant properties of honey are associated with the presence of phenolic acids and flavonoids (44). The phenolic content of honey samples is thought to be representative of their potential antioxidant activity (17). To determine the total phenolic content of various mono-floral honey samples from around Australia, RP-HPLC was used to detect the presence and quantities of active constituents in each sample. The chromatograms of honey samples showed several major peaks (Figure 4.1), the five highest peaks (>20mAU) at 260, 290 and 340nm were selected to represent total phenolic content (Table 4.1) and expressed as mAU. The sugars in the honey caused interference in the polar mobile phase, therefore peaks detected with an early retention time (<2min) were not included in the results.

The honey samples with the highest total phenolic content were from *Leptospermum* samples. *L. scoparium, L whitei* and *L. polygalifolium* showed significantly larger phenolic content than non-*Leptospermum* honeys (Table 4.1). *Leptospermum* honey has been found to contain a total phenolic acid content of up to 14.0mg/100 g honey, with gallic acid as the predominant phenolic component (85). Several samples of Eucalyptus honey had phenolic content above 1000mAU, these included Caleys Ironbark 177575, Grey Ironbark 174799, Peppermint 183493 and River Red Gum 181338, compared to the remaining eucalyptus honeys, which were all below 1000mAU (Table 4.1). The phytochemical composition of Eucalyptus honeys is known to be specific to each species (88). However, the significant difference between samples from the same mono-floral source, such as Grey Ironbark 174622 (677mAU), 175799 (2538mAU) and 183017 (689mAU) indicated that specific factors such as the geographical origin, may have also significantly influenced the composition between samples (47).

RP-HPLC was able to detect and quantitate phenolics and flavonoids in samples. HPLC equipped diode array detector and fluorescence detector enabled the presence of lepteridine and leptosperin to be quantified in *Leptospermum* honey samples. The highest levels of leptosperin (ppm) were found in *L. liversidgei* and *L. scoparium*, whereas *L. polygalifolium* 377, *L. scoparium* 1806, 1815 and 1818 contained the highest quantities of lepteridine (ppm) detected in *Leptospermum* samples (Table 4.2).

5.2 Antioxidant activity of unfractionated honey

To investigate if the total phenolic content of honey samples were reflective of the antioxidant activity, supporting evidence from previous studies (22), the samples were tested for their ability to reduce free radicals. DPPH is a reliable method to measure free radical scavenging activity in honey and provides similar results to antioxidant assays, such as Ferric Reducing Ability of Plasma (FRAP) (51). As DPPH is associated with fewer colour interference issues (51), it was selected to test the antioxidant activity of honey samples. The inhibitory effects of honey of DPPH at increasing concentrations (Figure 4.4) was assessed to determine the maximal inhibitory response for each sample. The EC_{50} values (concentration of honey required to produce 50% inhibition of the maximum inhibitory response), was reported as gallic acid equivalents. Honey samples with the highest DPPH free radical scavenging activity were Cheeseberry, *L. whitei* and Grey Ironbark 175799 (Table 4.3). *Leptospermum* honeys, which had high total phenolic content and performed well in the ORAC assay, were comparable to the antioxidant activity in Eucalyptus honey species in the DPPH assay (Appendix 1).

DPPH is a commonly used method for detection of free radical scavenging activity of food samples (51), however the reactivity of DPPH with oxidants such hydrogen peroxide also contribute to antioxidant activity of samples (9). Further limitations of this assay include the preference of steric accessibility over chemical characteristics in reactions, overestimation of polyphenols and underestimation of small phenols (50). Due to limitations of the DPPH assay, a complimentary method used to measure free radical scavenging was required to ensure the antioxidant activity of honey samples was accurately represented (32). The ORAC assay was selected to compare antioxidant activity using a different mechanism to DPPH (50). Samples from each mono-floral species were selected for ORAC analysis based on highest total phenolic content or DPPH inhibiting activity (Appendix 1). The free radical scavenging activity of antioxidants in honey were measured over time, allowing the kinetics of the reaction to be observed (Figure 4.7).

Trolox equivalent antioxidant activity of honey samples, provided by the ORAC assay (Table 4.4), found Eucalyptus honey species had lower antioxidant activity, compared to honey species such as Cheeseberry and *Leptospermum scoparium*, *L. whitei* and *L. polygalifolium*. The increased free radical scavenging activity of these honey samples can be attributed to high quantities of individual phenolics and flavonoids within samples (33), as determined by the total phenolic content (Table 4.1). *L. scoparium* (Manuka) honey contains higher levels of catechin, p-coumaric acid and caffeic acid than other varieties (22). Kojic acid, 4-methoxyphenyllactic, 2-methoxybenzoic acid and methyl

syringate (38) are antioxidant compounds found uniquely in Manuka honeys that may contribute to high antioxidant capacity. New Zealand Manuka honey has been used as the gold standard for investigating medicinal properties (26, 90), due to the abundance of therapeutic compounds it contains. The ORAC score for New Zealand Manuka was slightly higher than most Tasmanian Manuka samples (Table 4.4), interestingly Tasmanian Manuka FI:2 produced the highest ORAC score of all Manuka varieties. The finding that samples of Tasmanian Manuka could outrank the antioxidant potency of New Zealand Manuka could implicate it as a valuable source of therapeutic honey. Jarrah honey was highly active (5.35 µmole TE/g), the antioxidant activity of which may be due to its composition of phenolic acids and flavonoids, which correlate to the potent antimicrobial properties of this honey (34). Cheeseberry honey is a unique Tasmanian honey, derived from *Leptecophylla juniperina* (formerly *Cyathodes juniperina)* species. Although Cheeseberry produced the highest ORAC value (5.45 µmole TE/g, very little is known of the compositional properties of the *L. juniperina*, and no literature currently exists on this honey variety. The potent antioxidant properties of Jarrah and Cheeseberry are derived from the unique species of plants and environments from which they are sourced, which may account for the presence of novel antioxidant constituents in the sample.

Antioxidant activity varied between honey of the same botanical species, this is likely due to the geographical origin of the honey (46, 47). Honeys sourced from coastal areas generally produced higher ORAC values than their inland counterparts. Grey Ironbark and Peppermint species produced higher ORAC scores for coastally-derived samples than inland samples. Increased phenol and flavonoid content observed in areas with precipitation (45) may account for the increased antioxidant capacity observed in honeys derived from coastal areas. Tasmania produced the most active honeys, although these were collected from coastal locations, no mainland-based Manuka or Cheeseberry honey were available for comparison in this study. Therefore, the species of Tasmanian honey may have contributed more substantially to the content of antioxidant compounds than the geographical locations (22).

The results from ORAC assays showed a fast rate of reaction for Trolox standards compared to the slow-reacting honey samples (Figure 4.7). As honey contains a high concentration of sugars, which were found to influence ORAC results (91), a sugar mix was used to determine if any interference was caused (Table 4.5, Figure 4.8). The addition of sugar solution to 30µmole Trolox standard resulted in a slower rate of reaction (Figure 4.8). When data kinetics were observed, the rate of reaction for 40µmole Trolox was clearly more efficacious than all Trolox 30µmole/g combined with

sugar solution (Figure 4.8). The resulting data for Trolox 30µmole/g combined with sugar solution displayed comparable ORAC values to the 40µmole Trolox than the 30µmole Trolox equivalent (Table 4.6). Observation of the kinetics indicated the increased values were attributed to the improved duration of fluorescence protection in Trolox samples containing sugar solution. The results suggest the presence of carbohydrates in samples changes the stoichiometric factor of antioxidant reaction kinetics. Higher initial concentrations of honey were used to further investigate the interference of sugar in the ORAC assay. The increased dilution (1:100) was accounted for in the dilution factor used to determine TE ORAC values (Table 4.7). The honeys tested had ORAC values within the standard deviation of mean ORAC values for 1:200 dilution. The finding may implicate a limitation of sugar interference in the assay. Therefore, ORAC assay may not be ideal for measuring the antioxidant activity of unfractionated honey samples.

ORAC scores (µmole TE/g honey or sugar solution), obtained from samples (Table 4.2) were consistent with literature (8, 36). Although some studies reported higher ORAC scores (23, 35, 56), comparison of ORAC data obtained from different labs has proven inconsistent due to a lack of standard procedures (9). Several procedures were applied to minimise method-based error in data (92). The ORAC method was originally developed to measure the consumption of AAPH-derived peroxyl radicals (93). Recent evidence indicates that ORAC values are influenced by the interaction of antioxidants with both peroxyl radicals and azo-derived alkoxyl radicals (93, 94). The fluorescein probe employed in the use of ORAC assays has a greater affinity for alkoxyl radicals than peroxyl radicals (93), whereas Trolox has been suggested to efficiently trap both alkoxyl radicals and peroxyl radicals equally (94). Antioxidants with more influence in alkoxyl radicals trapping could produce a higher ORAC score compared to Trolox, regardless of peroxyl quenching capabilities.

ORAC assays have been shown to produce higher values for complex combinations of antioxidants, suitable for testing honey (91). When accurate ORAC methods are followed, other mechanisms contributing to antioxidant activity is not assessed in assay results. The focus on free radical scavenging activity as the basis for antioxidant capacity testing does not factor the contribution of other mechanisms to the activity (92), nor the synergistic effect of these mechanisms. Many antioxidant mechanisms such as hydrogen atom transfer, electron transfer and metal chelation play an important role in the overall antioxidant activity of a substance (92). Currently no individual analytical method reflects the diverse range of antioxidant mechanisms produced by honey (52), highlighting the importance of using several antioxidant testing methods, as was employed in this study.

5.3 Correlation of phenolic content to antioxidant activity

The *Leptospermum* samples displaying the highest total phenolic content also showed high antioxidant activity when analysed in ORAC assays. Statistical analysis showed ORAC activity was correlated to phenolic content (r=0.5318), supporting findings from previous studies (22). Although no correlation was found between total phenolic content and DPPH antioxidant activity, the correlation between ORAC activity and total phenolic content proved ORAC may be a more accurate method to determine antioxidant activity.

5.4 Justification for selection of samples for further analysis

Cheeseberry, Tasmanian Manuka 1818, *L. whitei* 311 and Grey Ironbark 175799 were among the top performing honeys in DPPH and ORAC assays (Appendix 1). Additionally, these samples possessed high phenolics content (Appendix 1), and thus were selected for further analysis. Coolibah and Messmate 177740 were also selected for phenolic extraction as they would allow Australian honeys with a range of antioxidant activity to be compared to provide useful information for the honey industry.

5.5 Analysis of phenolic extracts of honey

The compounds in traces from RP-HPLC of honeys were retained and concentrated in each of the six phenolic extracts. The similarity in traces from whole honey and phenolic extract of the same sample (Figure 4.11, 4.12) were observed. The maximum inhibitory concentration for the six phenolic extracts (Table 4.10) were more potent than the corresponding unfractionated honey (Table 4.3). This indicated that the presence of sugar in unfractionated honey samples limited the antioxidant activity. When samples were comprised of concentrated phenolics, the maximal effect of honeys such as Messmate 177740 (47.7%, Table 4.3) was markedly increased (84.3%, Table 4.10). The ORAC assay was performed on phenolic extracts as per methods used for honeys. The sensitive nature of ORAC assays required a 25-fold (1:5000) dilution of extracts compared to the 1:200 used for honey. The concentrations of the extracts were accounted for with dilutions factors. A 1:9 dilution of extract was required to obtain values for DPPH inhibition to compare to 1:1 honey dilution used for DPPH assays. The results for ORAC and DPPH provided an overview of the antioxidant activity of phenolic extracts of honey. To determine if antioxidant activity found in cell-free assays could be related to *in vitro* results, blood-derived human macrophages were treated with phenolic extract to assess the

effects. Cheeseberry honey is a unique, uncharacterised sample with high phenolic content and antioxidant activity. The powerful concentration of phenolics and flavonoids in the phenolic extract of Cheeseberry was therefore selected to use in cell-based assays.

5.6 Cell-based assays

Under homeostatic conditions, macrophages do not produce excessive radical species. In the pathological states of oxidative stress, macrophages are activated, changing their morphology and behavioural characteristics, including the formation of ROS for chemical signalling (61). For the purposes of investigating the effects of antioxidants in honey on preventing excessive ROS in macrophages, the cells required pro-oxidant stimulation to mimic pathological *in vivo* conditions. Bacterial LPS is known to have pro-oxidant effects on human macrophages, therefore LPS were selected to stimulate macrophages, allowing the effects of antioxidant treatment to be evaluated.

Glutathione peroxidase in cells treated with extracts of honey

GPx recycles glutathione in the cell, increased GPx activity is indicative of an increased demand of intracellular antioxidants and hence an elevated presence of ROS (49). To determine the levels of intracellular antioxidants, the GPx enzyme activity was measured in cells. GPx activity was measured to show if the phenolics in honey prevented the need for endogenous antioxidants to be produced. The (pmol/min/ml/µg protein) results (Table 4.11) were determined by GPx activity, relative to the protein content of samples. Reduced GPx activity was recorded in cells treated with phenolic extracts (10µg/ml) compared to control cells treated with media, with and without LPS-stimulation (Table 4.11). Statistical analysis determined that the reduction in GPx was not significant.

Oxidative stress in human macrophages treated with phenolic extract

The levels of 8-isoprostane are a reliable marker for oxidative stress in human macrophages (6) and allowed the oxidative stress to be measured in cells treated with phenolic extract of honey compared to controls. The results (Table 4.12) showed 8-isoprostane was present in low levels in unstimulated cells, and treatment with phenolic extracts increased these levels (0.019). The phenolic extracts of honey may have disturbed the cellular redox state in favour of reduction, possibly causing cells to compensate with 8-isoprostane to restore homeostatic conditions. In LPS-stimulated cells, the 8-isoprostane was increased, and phenolic extracts of honey significantly reduced the elevated 8-isoprostane levels ($p=0.008$). The protective effects of honey phenolics on oxidative stress in

human macrophages is reflective of the bioavailability of these compounds. As increased levels of 8-isoprostane is linked to numerous diseases and disorders (6, 54), these results indicate the use of honey may contribute to prevention, decreased severity or slowed progression of oxidative stress-related disease states.

Pro-inflammatory cytokines

Pro-inflammatory cytokines such as Interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) can stimulate the excessive ROS production (26), required to incite an immune response. To investigate underlying mechanisms surrounding the increased chemical production of mediators such as ROS in pathological conditions, the inflammatory response in human macrophages was studied. Significant increases in the concentration of TNF-α ($p= 0.0006$) were found in cells treated with honey extract, compared to media-treated cells. The increase was consistent with a previous study which found TNF-α were significantly elevated in macrophages treated with honey, promoting inflammation through independent mechanisms to the LPS-induced toll-like receptor-4 inflammatory pathway (95). As expected, LPS-stimulation increased the production of pro-inflammatory cytokines, however, the concentration of TNF-α were not significantly different in LPS-stimulated cells, with or without treatment with phenolic extract (Table 4.13). No significant increase in IL-6 occurred in cells treated with phenolic extract in the presence or absence of LPS (Table 4.13).

5.7 Evaluation of fractionated phenolic extracts of honey

To identify if the antioxidant activity of individual constituents and their contribution to the total antioxidant activity of samples varied, compounds were isolated and tested. Five fractions were isolated from Cheeseberry and Tasmanian Manuka 1818 honey samples, seven were extracted from *L. whitei*. HPLC analysis showed most fractions contained a single, pure compound (Table 4.14).

DPPH was selected to determine the antioxidant activity of fractions, however the mass of individual compounds produced quantities too low to exert a maximal response inhibition with DPPH (Figure 4.18). In contrast, the ORAC assay was able to determine antioxidant activity as it requires low quantities of antioxidants.

The ORAC assay results (Table 4.15) showed two compounds in *L. whitei,* (fraction 5 and 7) and a single compound in both Cheeseberry (fraction 3) and Tasmanian Manuka (fraction 4) produced considerably higher antioxidant activity than the remaining fractions (Table 4.15). These findings suggest the presence a few constituents in honey are responsible for most of the antioxidant activity. To identify such key compounds of interest, the UV spectra and RT for each compound were compared to known chemical standards. The chemical standards included ascorbic acid, gallic acid, 4-hydroxyphenyllactic acid, lepteridine, chlorogenic acid, 4-Hydroxybenzoic acid, leptosperin, vanillic acid, caffeic acid, p-coumaric acid, 4-methoxyphenylLactic acid, trans-ferulic acid, methyl syringate, quercetin, naringenin, apigenin, kaempferol, chrysin, pinocembrin and 2-methoxyacetophenone.

From extracts of Tasmanian Manuka 1818, lepteridine, 4-methoxyphenylactic acid, quercetin and methyl syringate were identified, and only one fraction was unable to be identified (Table 4.14). Lepteridine, leptosperin and methyl syringate were found in *L. whitei* 311, the remaining fractions 1, 4 and 6 were unidentifiable (Table 4.14). The identification of specific compounds in honey with high antioxidant activity assists with developing chemical markers for the antioxidant capacity of samples in a similar way in which methoglygoxal is used to measure antibacterial activity of Manuka honey (38). The high antioxidant activity of methyl syringate (4617 and 5697 µmole TE/g) and leptosperin (3869 µmole TE/g) in the isolated fraction of honey indicate these compounds could be used to measure the antioxidant activity of *Leptospermum* honey samples.

None of the phenolic compounds in Cheeseberry were able to be identified as they did not match any of the known chemical standards. A single key compound in Cheeseberry (Fraction 3) showed potent antioxidant activity (5873.27 µmole TE/g), most likely responsible for the reduced oxidative stress observed in blood-derived human macrophages (Table 4.12). The use of Nuclear Magnetic Resonance (NMR) in future studies is a recommended means of determining the chemical identity of these compounds for therapeutic use.

6. Conclusion

The antioxidant activity of honey derived from different botanical sources showed *Leptospermum* and Cheeseberry honeys to possess high a free radical-scavenging capacity. Although several Eucalyptus species showed elevated antioxidant activity, this activity was not consistent across samples from the same botanical origin and is therefore attributed to factors which are not associated with the mono-floral species.

Antioxidant activity and geographical origin of honey samples were compared to determine if a correlation existed. Samples of the same botanical origin expressed differing activity, appearing to be correlated to the proximity of hives to coastal regions. Samples from inland locations expressed lower antioxidant activity to coastal counterparts, even when originating from the same botanical source. All Tasmanian honeys showed high antioxidant activity, highlighting the ideal environmental characteristics of this region for honey production. There are several factors including coastal proximity, climate, soil composition, bee species and altitude, which may contribute to the preferred conditions for production of high-quality honey in Tasmania.

The isolation and identification of several significant antioxidant compounds were successful. Leptosperin and methyl syringate were extracted and positively identified as the major constituents responsible for the antioxidant activity observed in *Leptospermum* samples. The combination of RP-HPLC, ORAC, DPPH and cell-based methods have proven efficacious and robust for determination of antioxidant potential of a sample. Novel compounds in Cheeseberry, responsible for its antioxidant activity were unable to be identified by known chemical standards. One compound showed significant antioxidant activity and its bioavailability was evident in cell-based assays, implicating this novel compound as showing potent activity for potential therapeutic interest.

7. References

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8. Appendix

Appendix 1: Combined RP-HPLC, DPPH and ORAC results of Australian mono-floral honey

HPLC peak areas (Top 5)	mAU
Tasmanian Manuka 1806	6076
Tasmanian Manuka 1818	5434
L. polygalifolium 377	4292
Tasmanian Manuka 1815	4162
L. polygalifolium 382	3699
Tasmanian Manuka 1822	3618
L. polygalifolium 380	2642
L. whitei 311	2630
Grey Ironbark 175799	2538
L. polygalifolium 378	2260
L. whitei S2	2023
Tasmanian Manuka FI:2	1935
L. whitei 313	1779
Peppermint 183493	1741
River Red Gum 181338	1560
Caleys Ironbark 177575	1322
L. liversidgei 333	1313
L. liversidgei 338	1313
Cheeseberry	1063
L. liversidgei 334	1051
L. liversidgei 339	926
Leatherwood Teepo 3	902
Leatherwood 1829	858
Coolibah 176944	831
Leatherwood 1839	817
Leatherwood 1827	805
Messmate 183389	787
Whitebox 177735	751
Yellowbox 180467	713
Leatherwood Collingwood	709
Hillgum 181449	704
Grey Ironbark 183017	689
Grey Ironbark 174622	677
Whitebox 177835	658
Caleys Ironbark 180796	643
Mugga Ironbark 180911	611
Yellowbox 180668	602
Messmate 177740	576
Peppermint 182502	558
Whitebox 178082	526
River Red Gum 181841	514
Hillgum 182699	510
Peppermint 175055	484
Messmate 177890	482
Narrowleaf Ironbark 177260	476
Yellowbox 181711	462
Narrowleaf Ironbark 177131	428
River Red Gum 182160	418
Bluetop Ironbark 177010	416
Bluetop Ironbark 177134	349
Mugga Ironbark 176943	340
Narrowleaf Ironbark 177135	332
Hillgum 182506	303
Bluetop Ironbark 177832	265
Mugga Ironbark 179430	237
Caleys Ironbark 175650	116

DPPH	EC_{50} GAE (mg/g honey)
Cheeseberry	0.091
L. whitei 311	0.077
Grey Ironbark 175799	0.074
Narrowleaf Ironbark 177135	0.072
Tasmanian Manuka 1818	0.071
Jarrah	0.071
Tasmanian Manuka 1822	0.063
River Red Gum 181841	0.060
NZ Manuka	0.060
Grey Ironbark 183017	0.056
Coolibah 176944	0.054
Red River Gum 182160	0.052
Grey Ironbark 174622	0.052
Tasmanian mauka FI:2	0.051
Narrowleaf Ironbark 177260	0.049
Peppermint 182502	0.048
Hillgum 182699	0.045
L. polygalifolium 377	0.044
River Red Gum 181338	0.043
L. liversidgei 339	0.039
L. whitei 313	0.039
Caleys Ironbark 177575	0.038
L. whitei S2	0.038
Messmate 177740	0.035
Bluetop Ironbark 177010	0.035
Yellowbox 181711	0.034
L. polygalifolium 378	0.033
Hillgum 182506	0.033
Mugga Ironbark 180911	0.033
Narrowleaf Ironbark 177131	0.032
Tasmanian Manuka 1806	0.031
Mugga Ironbark 176943	0.030
Tasmanian Manuka 1815	0.030
Yellowbox 180668	0.027
Messmate 177890	0.027
Leatherwood Collingwood	0.026
L. polygaifolium 380	0.025
L. liversidgei 338	0.025
Leatherwood 1839	0.025
Bluetop Ironbark 177134	0.024
Messmate 183389	0.023
Whitebox 177835	0.023
Leatherwood 1829	0.023
Leatherwood Teepo3	0.022
Hillgum 181449	0.020
Leatherwood 1827	0.019
Peppermint 175055	0.019
Whitebox 178082	0.018
Yellowbox 180467	0.018
Whitebox 177735	0.018
L. liversidgei 334	0.015
Bluetop Ironbark 177832	0.015
Caleys Ironbark 180796	0.015
Caleys Ironbark 175650	0.014
Peppermint 183493	0.013
L. polygalifolium 382	0.013
Mugga Ironbark 179430	0.012
L. liversidgei 333	0.012

ORAC	µmole TE/g
Cheeseberry	5.45
Tasmanian Manuka FI:2	4.89
NZ Manuka	4.46
L. whitei 311	4.28
L. polygalifolium 378	4.06
L. polygalifolium 377	4.05
Leatherwood 1829	3.95
Tasmanian Manuka 1818	3.82
Grey Ironbark 175799	3.72
Tasmanian Manuka 1815	3.57
L. whitei 313	3.53
Tasmanian Manuka 1822	3.52
Tasmanian Manuka 1806	3.4
L. liversidgei 339	3.3
Peppermint 182502	3.25
Messmate 177890	3.12
Leatherwood Teepo 3	2.87
L. liversidgei 333	2.77
Grey Ironbark 183017	2.73
Whitebox 177838	2.72
Caleys Ironbark 177575	2.68
Peppermint 183493	2.65
Yellowbox 181711	2.57
Messmate 177740	2.56
Bluetop Ironbark 177010	2.56
Grey Ironbark 174622	2.5
Mugga Ironbark 180911	2.48
Bluetop Ironbark 177134	2.42
Redriver Gum 182160	2.41
Narrowleaf Ironbark 135	2.37
Leatherwood Collingwood	2.34
Mugga Ironbark 176943	2.31
River Red Gum 181338	2.28
Coolibah 176944	2.27
Whitebox 178082	2.21
Hillgum 182506	2.19
River Red Gum 181841	2.18
Hillgum 182699	2.1
Caleys Ironbark 175650	1.88
Yellowbox 180467	1.84