Investigation of the immunostimulatory effects of some New Zealand honeys and characterization of an active component

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Abstract

Medicinal use of honey has re-emerged recently indicating that honey accelerates wound healing activity. Honey has been shown to stimulate TNF-α production from monocytes and macrophages which is apparently correlated with a high molecular weight fraction, and not lipopolysaccharide (LPS, an immunostimulatory endotoxin) levels. Cytokine production by honey has been attributed to the endotoxin content. The aim of this study was to investigate the ability of Comvita sourced honeys to elicit a TNF-α cytokine response from acute monocytic leukemia (THP-1) cells as well as identify the responsible component.

Five honey samples were used together with sugar and methylglyoxal controls. The samples were incubated with THP-1 cells, with and without LPS. After incubation, the cell culture supernatants were collected and TNF-α was measured by the enzyme-linked immune sorbent assay (ELISA). The most active honey samples were further heat-treated to remove enzyme/protein/peptide-like stimulation; the samples were treated with polymixin B (PmB) to remove LPS-like stimulation and not protein fraction. The samples were then filtered by molecular weight centrifugal filters to separate constituents according to their size and the fractions were re-analysed.

All five honey samples in the absence of LPS stimulated TNF-α release from THP-1 cells, whereas untreated, sugar- and methylglyoxal-treated cells did not. The cytokine production was partially inhibited by heating, but mostly by PmB. In the filtered honey samples, the activity was observed in the >30 kDa fraction. These results suggest that the activity may be associated with one or more components which are partially heat-labile, LPS-like stimulated with a high molecular weight.

Further, honey samples were analyzed for the concentration of LPS present. The tests revealed that the cytokine stimulation was higher than would be expected from the concentration of LPS present in the honey. The possibility that this component was a plant-derived β-glucan, which is known to have LPS-like activity and can interfere with detection of LPS in the LAL assay, was investigated. Subsequent analyses confirmed the presence of arabinogalactan, a large complex carbohydrate.

The data presented in this study suggests that arabinogalactans in honey may stimulate inflammatory responses and the release of cytokines that are crucial in regulating wound-healing. This heralds a significant advancement in the usage and understanding of medicinal honey.

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May you live a long and happy life,
With all my love

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List of Abbreviations

NH_4OH	Ammonium hydroxide
ATCC	American Type Culture Collection
ANOVA	Analysis of variance
AG	Arabinogalactan
AGP	Arabinogalactan-protein
AH	Artificial honey
BSA	Bovine serum albumin
$(CH_3)_2SO$	Camptothecin
CO_2	Carbon dioxide
^oC	Degrees Celsius
DMSO	Dimethylsulfoxide
KH_2HPO_4	Dipotassium hydrogen orthophosphate
Na_2HPO_4	Disodium hydrogen orthophosphate anhydrous
ECM	Extra cellular matrix
ESR	Environmental Science Research
ELISA	Enzyme-linked immune sorbent assay
FBS	Fetal bovine serum
GC-MS	Gas chromatography-mass spectrometry
g	Gram or Acceleration due to gravity
>	Greater than
HPAEC	High-performance anion-exchange chromatography
h	Hour
THP-1	Human monocytic leukemia cells
HMF	Hydroxymethylfurfural
HRP	Horse radish peroxidase
H_2O_2	Hydrogen peroxide
IRL	Industrial Research Limited
IU	International Unit
IL-1	Interleukin-1

IL-6	Interleukin-6
Kg	Kilogram
KGy	kilo Grays
KDa	Kilo Daltons
<	Less than
L	Litre
LPS	Lipopolysaccharide
Ltd	Limited
$LiNO_3$	Lithium nitrate
pH	-Log $[H^+]$
MS	Mass spectroscopy
MHz	Megahertz
MGO	Methylglyoxide
CH_3I	Methyl iodide
MeOH	Methanol
ML	Millilitre
µL	Microliter
mg	Milligram
µm	Micrometer
µM	Micromolar
mM	Millimolar
mm	Millimetre
min	Minutes
M	Molar
ng	Nanogram
nm	Nanomolar
N_2	Nitrogen
NMR	Nuclear magnetic resonance
ppm	Part per million
%	Percent
% (w/v)	Percent by weight per volume
PMA	Phorbol 12-myristate 13-acetate
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline -Tween 20

PmB	Polymixin B
rpm	Revolution per minute
RPMI	Roswell park memorial institute medium
Complete RPMI	RPMI medium supplemented with 10 % FBS, penicillin at 50 IU units/mL and streptomycin at 5 µg/mL
SEC	Size-exclusion chromatography
NaN_3	Sodium azide
$NaBD_4$	Sodium borodeuteride
NaH_2PO_4	Sodium dihydrogen phosphate monohydrate
TMB	Tetra methyl benzidine
TFA	Tri fluoro acetic acid
TNF-α	Tumor necrosis factor
USP	United States Pharmacopeia
v/v	Volume per volume
WST-1 reagent	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzenedisulfonate

Chapter 1

Introduction and literature review

1.0 Introduction

The work outlined in this thesis investigated the immunostimulatory effects of New Zealand honeys. The ability of honey to elicit a cytokine response from human monocytic cells isolated from peripheral blood may explain the stimulation of inflammatory cytokines observed with honey during wound healing.

1.1 Honey

Honey is produced by bees using floral nectars. Nectar is gathered, modified and stored as honey in the honey comb by honey bees (Olaitan *et al*., 2007). Since ancient times people have been using honey as a food product and also as a medicine (Zumla and Lulat, 1989). The production, composition and use of honey have a long and varied history.

Honey is a known antimicrobial agent, and has been used historically as a medicine for the treatment of wounds and burns. During the past two decades, a number of laboratory studies and clinical trials have shown that honey is an effective broad-spectrum antibacterial agent that has no known adverse effects on wound tissues (Molan, 2002; Cooper, 2009). The reasons for good therapeutic effects on wounds, particularly on infected non-healing chronic wounds, include its high viscosity which helps to provide a protective barrier to prevent the infection by providing lack of free water, which inhibits the growth of microorganisms (Ndip *et al.*, 2007); high sugar content and osmolarity (Molan, 2001); acidity, hydrogen peroxide content, flavonoids and the phenolic acids (Miyata and Sato, 2000), methylglyoxal (Mavric *et al*., 2008), and by maintaining a moist wound environment (Timm *et al*., 2008). Recently some studies reported that antimicrobial peptides are responsible for antimicrobial effects in honey (Lee *et al*., 2008; Kamran Azim and Sajid, 2009). Although evidence exists for the antibacterial effects of honey there is a limited evidence for its effect on direct mechanisms of healing. To date, honey efficacy in wound healing remains largely anecdotal with claims that it reduces inflammation, reduces oedema, debrides necrotic tissues, promotes angiogenesis,

epithelialisation and granulation. However it has been suggested it contains an unidentified component (Tonks *et al*, 2003) which induces the release of inflammatory cytokines from monocytic cells (Timm *et al*., 2008).

The wound healing activity of honey is widely accepted. This activity is probably dependent upon a variety of mechanisms and also the composition of the honey (Molan, 1992). Consequently, it is important to validate these effects and determine how honey composition influences them.

1.2 Past review on honey

1.2.1 Composition of honey

Honey is predominantly carbohydrate, with 95% to 99.9% of the solids being sugars; other constituents include water, organic acids, amino acids, enzymes, vitamins, proteins, and phytochemicals (Molan, 1996).

The major sugars that are found in honey are fructose (48%), glucose (45%) and sucrose (1%), (Echigo *et al*., 1976), where the exact ratio of these sugars differs due to floral sources and level of honey synthesis by the bees. Other sugars (Doner *et al*., 1977) presented in smaller quantities include disaccharides such as maltose, sucrose, maltulose, turanose, isomaltose, trehalose, nigerose, laminaribiose, gentiobiose and kojibiose (White and Hoban, 1959). Trisaccharides also known to be found in honey include l-kestose, centose, erlose, melezitose, maltotriose, isopanose, theanderose, 3-a-isomaltosylglucose, panose, isomaltotriose, isomaltotetraose and isomaltopentaose (Siddiqui, 1970).

Water in honey contributes to the quality, granulation and body, but after extraction the water content may change due to storage conditions. The water content may also change because of various environmental factors that the honey is exposed to during production due to weather and humidity inside the hives (Olaitan *et al*., 2007).

Several acids are found which include acetic, a-ketoglutaric, butyric, citric, glycolic, gluconic, glucose 6-phosphate, lactic, maleic, oxalic, pyruvic, pyroglutamic, and 2- or 3-phosphoglyceric acid (Nozal *et al*., 2003). Gluconic acid is the major acid, and it comes from

glucose through the action of bee-derived glucose oxidase (Anon, 2006). Honey contains hydroxyl- and methoxy-derivatives of benzoic and cinnamic acids. These ingredients contribute the sensory quality of the product and may also indicate the floral origin (Anklam, 1998). Eleven to twenty one essential and non-essential amino acids are present in honey which varies according to floral source, including glutamic acid, alanine, aspartic acid, leucine, isoleucine, tyrosine and proline (Del Nozel, 1998). Proline is the most prevalent amino acid with lysine being the second most prevalent. By heating honey the amino acids can react with sugars slowly or more rapidly, which causes the colour change from yellow to brown in honey. This is one of the reasons for honey darkening with age or heating.

In recent years, researchers have identified a number of phytochemicals in honey, many of which may possess beneficial health properties (Fahey and Stephenson, 2002; Gheldof *et al*., 2002; Baltrusaityte *et al*., 2007). The major phytochemicals are anti-oxidants, which reduce the risk of tissue oxidative damage. These include ascorbic acid, alkaloids and flavonoids (Baltrusaityte *et al*., 2007). Honey that is made by bees fed with herbal extracts has greater anti-oxidant activity than normal honey. The high anti-oxidant components are found in dark honey that contains more water content (Gheldof *et al*., 2002).

Honey contains some enzymes such as invertase, catalase, diastase (amylase), acid phosphatase and glucose oxidase (Molan, 1992; Dustmann, 1971; White, 1975b). Invertase converts sucrose to fructose and glucose. Diastase is a mixture of enzymes which splits starch to simple compounds to produce dextrins and maltose that is added by bees during honey production. The reaction catalyzed by glucose oxidase converts glucose to a gluconolactone intermediate that in turn forms gluconic acid. It also produces glutamic acid and hydrogen peroxide which contributes to the basis of heat sensitive antimicrobial properties of honey. Honey also contains catalase in a small amount, which produces oxygen and water from hydrogen peroxide.

Honey contains low levels of minerals, trace elements (Rashed and Soltan, 2004) and proteins (White, 1975a). Their nutritional significance is likely to be negligible because of their low concentrations (Haydak *et al*., 1975). Fresh honey contains small amounts of hydroxymethylfurfural (HMF). It is formed in honey by the breakdown of monosaccharides in the presence of honey acids (Jiminez *et al*., 1994). HMF content in honey is directly related to its quality, aging and heating (Marcavia *et al*., 2008). Generally honey is a stable

product (White *et al*., 1960) but changes can occur over time. Certain honeys can also vary in their composition due to the continuous action of enzymes. For example the sucrose content decreases with time (White *et al*., 1960; Browne, 1908).

1.2.2 Production of honey

Production of honey starts with the collection of nectar and pollen by bees from flowers. To make honey only nectar is used. Nectar is mainly composed of water with dissolved sugars, and the amount of sugar varies greatly (Olaitan *et al*., 2007). The majority of the sugar in nectar is sucrose (Pais, 1986) and bees produce invertase to breakdown sucrose to fructose and glucose (White, 1975). Nectar is harvested by honey bees by inserting their proboscis into the flowers nectar, and the collected material is passed through the oesophagus to the thorax and finally to the abdomen. Nectar is transported into the stomach whereas pollen is transported back to the hive in the posture pollen baskets on the hind leg. On return to the hive the nectar is regurgitated into the wax honey comb cells and the excess water evaporates until the honey is approximately 83% sugar. This process may take up to few days. The cells containing ripened honey are covered with a layer of wax and the layer is removed when the bees need the honey store. When bees collect large amount of nectar, the hive is ventilated to speed up evaporation by using their wings. By evaporating the excess water, the bees make the sugar concentration in honey too high for yeast and other microorganisms to grow.

To extract honey, a sharp knife is used by the beekeeper to remove the wax cap, the combs are spun in a centrifuge and honey is removed. Knowledge of the process of honey production is important to understand the various ways in which bacteria and other microorganisms may contaminate the honey, and how this potentially influences the honey immunostimulatory profile.

1.2.3 Microorganisms in honey

The medical significance of honey contains a contradiction, in that on one hand honey contains microorganisms; yet and on the other hand it is active against many microorganisms. Microorganisms in honey will influence the quality and safety of the product (Snowdon and Cliver, 1996). However cleanly processed honey is a natural product that contains minimal species and levels of microorganisms due to its innate antimicrobial natural properties.

Microorganisms that survive in honey are those that withstand the high sugar concentration, acidic nature and antimicrobial properties of honey (Olaitan *et al*., 2007). Microorganisms do not replicate in honey solution but certain vegetative microorganisms can survive at low temperatures for many years. Accordingly it is typically expected that honey contains low numbers and a limited variety of microorganisms.

The microorganisms found in honey include bacteria, yeasts and moulds (Snowdon and Cliver, 1996). Sackelt (1919) reported that *Bacillus, Micrococcus* and *Saccharomyces* were readily isolated from combs and bees and number of microorganisms could be isolated from the faeces of bee larvae (White, 1996). *Actinomycetes, Enterobacteriaceae,* moulds, *Penicillin* species and frequently *Torulpsis* species have been recovered in the intestinal microflora of honeybees (Gilliam and Prest, 1987). Anaerobic bacteria have not been recovered whereas aerobic spore-forming bacilli are the most frequently encountered microorganisms in the bee honey-crop, intestine and external surface of the bees (El-leithy and El-sibael, 1992). *Saccharomyces* and *Torula* yeasts can be found in high moisture sugars, *Leuconostoc mesenteroides* can be found in sugar refineries whereas *Enterobacter, Citrobacter, Erwinia, Lactobacillus, Lactococcus, Listeria* and *Pediococcus* species are found in plants and plant products (Snowdon and Cliver, 1996).

1.2.3.1 Sources of microbial contamination

Sources of microbial contamination can be mainly attributed to pollen, the digestive tracts of honey bees, dust, earth, air, flowers and nectar, all of which are very difficult to control. The other sources of contamination can be due to handling, cross contamination of equipment, containers and honey houses all of which can be controlled easily by good manufacturing practices.

Air and dust are the primary and important sources of *Clostridium, Bacillus and Micrococcus* species. The microorganisms found in honeycomb are mostly bacteria and yeasts. Pollen could be the original source of microorganisms in the intestine of honey bees and studies have been reported that flowers and hives are more important sources of microorganisms rather than the soil (Root, 1993). The primary sources of the sugar tolerant yeasts are flowers and soils; however nectar, the bee, soil, air, honey houses and equipments are all considered possible sources of yeast (Crane, 1979). Much research has been completed on primary

sources of microbial contamination in honey to understand the microbial ecology of the honey bee (Snowdon and Cliver, 1996). However it is likely more research is required to determine the relative importance of these various sources of contamination.

The secondary source of microbial contamination was reported by Tysset and Rousseau (1981). They included food handlers, cross contamination of equipments and containers, insects, water and animals. Most of the yeasts and vegetative microorganisms are recovered from this secondary source of contamination. Therefore, good manufacturing practices and standard sanitation is required to control the microorganisms in all food. These control measures have been developed by the food production industries.

1.2.4 Varieties of honey

There are more than 300 unique types of honeys which differ depending on the nectar source which give rise to colour, aroma or flavour and the composition of particular honeys (Molan, 2001). The variation in the honey composition constituents is also due to various physiological factors including climate, soil, flora and honey bees. Ihtisham-ul-haq (1997) reported that the climatic conditions and soil types makes honey vary from one region to another and influences its physio-chemical characteristics (Kamal *et al*., 2002). The honey samples that are commercially available can be different in quality depending on various factors such as the season, packaging, processing conditions, floral sources, geographical origin and period of storage.

1.2.4.1 Floral origin

Honeys can be collected and labelled according to floral origin or plant source if it comes mainly from that particular plant source. The floral honeys are differentiated depending on the number of flowers from which the nectar is collected. There are monofloral honeys and polyfloral honeys described. Identifying the extent of purity, particularly for monofloral honeys, primarily relies upon pollen analysis (melissopalynology) drawn from samples found in the honey itself (Louveaux *et al*., 1978), although alternative methods using honey phenolic composition have been suggested (Molan, 1998 b; Stephens *et al*. 2010).

Honeys from hives where the bees have collected nectar from only one variety of flowers are known as monofloral honeys (Gutierrez *et al*., 2009). As this type of honey contains the properties from only one plant it is believed to be the best type of honey. Only professional beekeepers readily attempt these collections allowing the honey bee's access to only particular flower from which they produce honey. The honey from hives where bees have collected nectar from different types of plants is known as poly-floral honeys (Louveaux *et al*., 1978; Gutierrez *et al*., 2009). Sometimes, another type of honey is commercially available and that is blended honey. Blended honey is also a mixture of different honeys.

1.2.4.2 Geographical origin

The pollen grains in honey reveal the types of plants that nectar has been collected from and determines the geographical origin of honeys. In the past melissopalynology was used to determine the geographical origin of honey but it is less valid for determining the botanical origin of honey (Molan, 1998 b). Sanz *et al*. (2006) reported the possibility of characterizing the geographic origin of honeys using multivariate statistical analysis. Currently there is a rapid progress to determine the geographical origins of honey based on protein finger printing and barcoding using Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Wang *et al*., 2009).

1.3 Properties of honey

Honey has several important properties in addition to composition and taste. The properties in honey contribute to its various roles in human health. Over the past two decades, scientific studies have revealed therapeutic uses for natural honey (Molan, 1998a). Honey possesses antibacterial, antifungal (French *et al*, 2005 and Weston, 2000) and anti-inflammatory properties (Postmes, 1993). Honey also possesses anti-oxidant activity, phenolic content (Nasuti *et al*., 2006; Zegerac et al., 2009) and alimentary applications (Nagai *et al*., 2001), which may involve the anti-inflammatory properties (White, 2005). Honey is also useful in wound healing and burn healing (Subrahmanyam, 1998).

1.3.1 The antibacterial activity of honey

The medicinal use of honey has been known for centuries, but only recently has the specific roles for honey been investigated and identification of the responsible bioactives responsible been attempted. Of these medicinal roles, the antimicrobial activity of honey has been the most widely accepted and understood. It was recognized for the first time by van Ketel in 1892 (Dustmann 1979).

The importance of the antimicrobial activity of honey mostly applies to its external uses as a wound dressing or skin-care agent, even though it may also play a role in maintaining the balance of internal microflora after consumption. It has been found that the mechanism of antimicrobial action relates to its high osmolarity, acidity and content of inhibines such as hydrogen peroxide (Cooper *et al*., 1999), flavonoids and phenolic acids (Wahdan, 1998). In the New Zealand honeys, potency ranges from the equivalent of <2% (w/v) phenol to 58% (Cooper *et al*., 1999), with significant differences between floral sources. This activity is dependent upon a number of factors, several of which are endemic to all or most honeys, geographical and floral origin and honey composition (Molan, 1992).

The mode of bacterial inhibition by honey is bactericidal (killing) and bacteriostatic (growth-inhibiting) (Wahdan, 1998; Cooper *et al.*, 2002). Studies have identified susceptible microbial species such as *Staphylococcus aureus, Enterococci, Klebsiella pneumoniae* and *Pseudomonas* (Cooper *et al.*, 1999*;* Allen *et al.*, 2000*;* Basualdo *et al.*, 2007). Yeasts and fungi can also be susceptible to honey (Brady *et al*, 1996). At present, some studies also showed the mode of action of honey on bacterial cells (Gencay *et al*., 2008; Henriques *et al*., 2010). Further studies need to be carried out on cellular and molecular events.

1.3.1.1 Osmotic effects

Honey is a supersaturated solution with high sugar content which possesses the osmolarity that restricts the growth of microorganisms (Lusby *et al*., 2002; White, 2005). Osmolarity is largely responsible for the antimicrobial activity of honey even at low concentrations (Molan, 2002). Due to its high sugar content, the osmotic pressure is high and the water activity (a_w) is low. For example, the growth of many microorganisms is completely inhibited when a_w is in the range of 0.94-0.99 (Leistner and Rodel, 1975). Therefore, the low water activity of

undiluted honey (approximately $a_w \approx 0.6$) is not suitable for the growth of microorganisms. The low water activity may primarily be due to fructose and glucose and disaccharides including maltose and sucrose in minor amounts with insignificant contribution of other sugars (Zamora and Chirife, 2006). This a_w effect is more important for antimicrobial activity during application as a wound dressing rather than as a food in the gut.

1.3.1.2 Hydrogen peroxide

Sackett (1919) has shown that the antimicrobial effects of honey are not solely due to the sugar content because the antimicrobial activity increased on dilution of the neat honey in water. The word inhibine was used by Dold (1937) to explain the involvement of an antimicrobial substance in the honey. There are two types of inhibines that are responsible for antibacterial effects. One of the major inhibines is hydrogen peroxide (H_2O_2) (White *et al*., 1963). It is a well known antimicrobial agent for clinical purposes. The amount of hydrogen peroxide in honey is typically around 1 mM (0.034%), which is much less than the concentration of hydrogen peroxide used as a clinical aseptic solution (3%). Therefore, any harmful effects of the hydrogen peroxide in honey are minimal (Molan, 2001). Hydrogen peroxide is heat and light sensitive and produced by glucose oxidase. This enzyme is normally secreted from the hypopharyngeal glands of bees, and is introduced into honey during production (White, 1996). Undiluted, the enzyme is largely inactive, but upon dilution, conversion of glucose into gluconic acid and H_2O_2 in the presence of oxygen commences (Equation 1). The rate of H_2O_2 production is dependent on the honey variety and concentration. It has been reported that H_2O_2 can have a more potent antibacterial activity when present in honey than on its own (Molan, 1992).

(1)

The hydrogen peroxide activity in honey revealed higher antibacterial effects than hydrogen peroxide alone (Chanchao, 2009) which suggests that some indigenous substances in honey may raise its activity. It has been shown that the bactericidal potency of H_2O_2 could be increased by addition of 0.1 mM ascorbic acid and metal ions. In addition, McCulloch,

(1945) reported that the antibacterial potency of H_2O_2 activity in honey can be increased by the addition of 0.83 mM of iron, copper, chromium, cobalt or manganese.

Some researchers have claimed that the non-peroxide activity of honey is more important (Gonnet and Lavie, 1960; Bogdanov, 1984) and have also suggested that it is heat and light-stable. The non-peroxide activity of honey could be extracted by organic solvents but the chemical nature of the component was not characterized (Radwan *et al*., 1984). There are many studies showing the antimicrobial potency of honey (Malika *et al*., 2004; Mboto *et al*., 2009; Al-Jabri *et al*., 2003) yet on the other hand research on the limitations of the antimicrobial spectrum and the nature of the honey are few.

1.3.1.3 Methylglyoxal

Investigations have been carried out to determine the non-peroxide activity of Manuka honey. Recently methylglyoxal (MGO) has been identified as the substance that is responsible for the non-peroxide antibacterial effects of Manuka honey (Mavric *et al*., 2008). MGO is present in New Zealand Manuka honeys at high concentrations and has been shown to be directly responsible for the non-peroxide antimicrobial properties (Atrott and Henle, 2009). The non-peroxide substance was formerly termed as the Unique Manuka Factor (UMF®). This UMF® was suggested to be insufficient to account for all the antibacterial activity (Weston, 2000) but Snow and Manley-Harris (2004) contradicted these findings. The presence of catalase in honey, H_2O_2removal and anti-oxidant activity peroxide scavengers in Manuka honeys (Inoue *et al*., 2005; Henriques *et al*., 2006) may account for some of these differences in opinion. If the antibacterial activity is not only due to the presence of MGO and hydrogen peroxide then an unidentified component may also be involved.

1.3.1.4 Acidity

The third factor that is responsible for antimicrobial activity of honeys is acidity. Honey contains approximately 30 organic acids (Mato *et al*., 2003). The complex mixture of these acids in honey contributes to its low pH and prevents bacterial growth. Interestingly, Bogdanov (1977) reported that the non-peroxide antimicrobial activity in honey correlates significantly with its acid content, but not necessarily with pH.

1.3.1.5 Phytochemicals

Phytochemicals are described as non-peroxide antibacterial compounds (Molan, 2002). The relative abundance of plant compounds in honey relates to the age, floral and geographical origin, as well as extraction and storage conditions. Antibacterial phenolics acids include ferulic, caffeic, syringic and methyl syringic acids and flavonoids such as quercetin, luteolin and isorhamnetin have been identified (Russell *et al.*, 1990; Weston *et al*., 2000). However the concentrations of these substances were considered too low to cause antibacterial effects alone in honey, and as both studies (Russell *et al*., 1990; Weston *et al*., 1998) were carried out on Manuka honey only, it is still unknown whether these substances occur in honeys originating from other floral sources at high enough concentrations to exhibit an antimicrobial effect.

The antimicrobial effects of honey may vary according to floral sources (Molan, 1992; Weston, 2000; Snow and Manley-Harris, 2003); however Revathy and Banerji (1980) demonstrated that the raw honey processing before marketing reduced its antibacterial activity. Yet Allen *et al.* (1991) in a survey of 345 New Zealand honey samples from different floral sources demonstrated that there was no correlation between age, extraction, storage and processing with the levels of antimicrobial property. Additionally it was also demonstrated that many honeys of dark colour, a feature often associated with high levels of hydrogen peroxide possesses more antibacterial activity over the light coloured honeys. Weston *et al*. (1999) suggested that phenolic compounds in Manuka honey from nectar, pollen or propolis were partially responsible for non-peroxide antibacterial activity and in addition suggested that all of this activity in Manuka honey was associated with the carbohydrate fraction of the honey.

1.3.1.6 Antimicrobial peptides

Recently some studies have reported that antimicrobial peptides can be isolated from different honey sources and are responsible for some antimicrobial effects in honey (Lee *et al*., 2008; Kamran Azim and Sajid, 2009). Lee *et al*. (2008) found antimicrobial production by bacteria from different honey sources and suggested that they are bacteriocin-like compounds and peptide antibiotics. Kamran Azim and Sajid (2009) reported that honey is also useful in curing helminth diseases such as ascariasis hookworm infections etc. and they

speculated that bacterial peptides in honey may be responsible. Chongsiriwatana *et al*. (2008) reported that antimicrobial peptides and their mimics are antibiotics with a helical structures and biomimetic sequences and several members have antibacterial effects at low (µM) concentrations.

1.3.2 Antifungal activity of honey

Honey also demonstrates antifungal effects against the *Candida* species (Irish *et al*., 2006). A limited number of observations have shown that honey has an inhibitory effect against *Candida* species *in vitro* (Theunissen *et al*., 2001; Koc *et al*., 2009). Sissons *et al.* (2004) reported that especially Manuka honey may be of benefit in oral infections where *Candida albicans* are prominent. There are only a few investigations into the antifungal activity *in vitro* and clinical application and further studies are required to demonstrate antifungal activity.

1.3.3 Anti-oxidant activity of honey

It has been shown that oxidative stress plays an important role by producing oxidant free radical molecules and contributes in the development of numerous pathological conditions including coronary heart disease, stroke and cancer (Peto *et al*., 1981; Schramm *et al*., 2003; Zegarac *et al*., 2009). Over the past 10 years many studies have been carried out on the anti-oxidant capacity of honey *in vitro* (Frankel *et al*., 1998; Chen *et al*., 2000; Gheldof and Engeseth, 2002). Furthermore, Mathew *et al.* (1998) and McKibbon and Engeseth (2002) reported that honey has the capacity to prevent lipid oxidation in cooked ground turkey meat. In general, the anti-oxidant capacity of honey appears to be associated with the combination of wide range of compounds including enzymes, organic acids, phenolics, peptides, products of the Malliard reaction and possibly some other minor components (Gheldof *et al*., 2002). It has also been reported that the anti-oxidant capability of honey directly correlates to the phenolic content and honey colour intensity (Fahey and Stephenson, 2002; Beretta *et al*., 2005). Many researchers also reported that there is a significant difference between anti-oxidant capacity and honey colour and suggested that darker coloured honeys have higher anti-oxidant capacities (Frankel *et al*., 1998; Gheldof and Engeseth, 2002; Bertoncelj *et al*., 2007). In particular, past research suggested that the anti-oxidant capacity is largely due to its phenolic content (Gheldof *et al*., 2002) and many studies have shown that several honeys

have a rich phenolic profile consisting of benzoic acids, cinnamic acids and flavonoids (Ferreres *et al*., 1992; Martos *et al*., 2000; Gheldof *et al*., 2002).

Within the last few years, studies have investigated the anti-oxidant capacity of honey *in vivo* (Gheldof *et al*., 2003; Schramm *et al*., 2003; Perez *et al*., 2006). Gheldof *et al*. (2003) claimed the serum anti-oxidant capacity was increased by 7% in individuals who had consumed 160 g/L of Buckwheat honey compared to those who had consumed the same amount of mixed sugars (fructose and glucose) in black tea. Similarly, Schramm *et al*. (2003) showed that plasma anti-oxidant capacity was increased by 12-25% six h after ingestion of a single dose of Buckwheat honey and total phenolic content was also increased by 4-8%. Honey also has the capacity in preventing deteriorative oxidation reactions in food (Antony *et al*., 2000) and enzymatic browning of fruit and vegetable homogenates (Chen *et al*., 2000), and it is likely honey has potential as a natural food anti-oxidant. Today there is a reasonable interest in the usage of honey in food due to its anti-oxidant effects even though there is contrasting data as to whether the anti-oxidant activity of honey is comparable to other food sources (Gheldof and Engeseth, 2002).

1.3.4 Wound-healing activity of honey

Honey has been observed to be beneficial as a wound dressing in the following ways (Simon *et al*., 2008).

- The antibacterial activity of honey rapidly clears existing infection and protects wounds from additional infection;
- Honey debrides wounds and removes malodour;
- The anti-inflammatory activity of honey reduces edema and minimizes scarring;
- Honey stimulates growth of granulation and epithelial tissues to speed healing.

Figure 1.1 summarizes the activities performed in a non- healing wound when honey is applied.

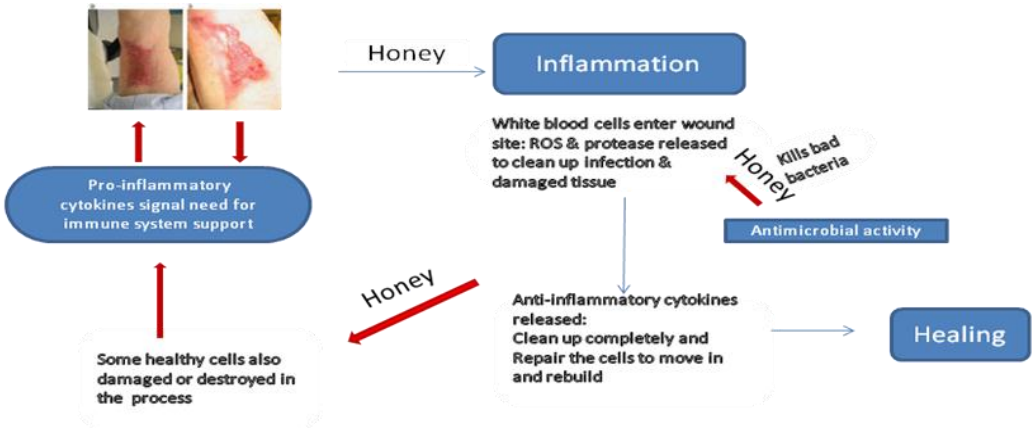

Figure 1.1 Flow diagram summarizing the activities of wound-healing when honey is applied (indicate source).

1.3.4.1 Historical usage of honey

Honey has had a long historical usage as a wound dressing and an antiseptic due to its medicinal properties. Egyptians used up to 30 % honey as shown prescribed papyri and Sumerian clay tablets (Stomfay-Stitz, 1960). About 2500 B.C Egyptians had a standard salve containing honey for treating diseases of eyes and skin (Al Walli, 2003). Honey was also mentioned having healing properties in the Holy Quran 1400 years ago (Al-Waili, 2003a). Greeks also reported that honey treats fatigue (Wilson and Crane, 1975). Hippocrates (46-357 BC) found that honey cleaned sores and ulcers of the lips and healed buncles and running sores. Beck and Smedley (1997) reported that honey could treat gastric and intestinal problems, especially as a laxative. Additionally, honey has been reported as a remedy for all sorts of inflammation of the kidneys for both gravel and stones (Beck and Smedley, 1997). In ancient times honey was also used as an ayurvedic medicine for various conditions (Subrahmanyam, 1996). The Hindu people had great faith in natural honey as a medical treatment; using it mainly for coughs gastric disorders and pulmonary issues. In China, ancient people used honey as a component of diets and medicine. In western countries, honey has played only a minor medicinal role because antibiotics and other pharmaceuticals have been remedies of choice. Slowly honey is gaining popularity in the west as researchers have started to reveal the efficacy of honey. In particular, the antimicrobial effects of honey and wound healing properties (Molan, 2006) have gained substantial recognition in the last 15 years, although scientists are only now starting to fully research the components involved and the processes by which these health benefits occur. Scientific studies are beginning to

demonstrate that honey has a number of health benefits including anti-bacterial, anti-inflammatory, immunostimulatory and anti-oxidant properties, beneficial effects on glucose levels and cancer prevention and the components responsible and the mechanisms involved are starting to be defined.

1.3.4.2 Wound healing compounds in honey

The physical and chemical properties of honey play an important role in wound healing. Honey provides a protective barrier due to the viscosity preventing cross infection. The osmolarity of honey draws away the harmful substances that are produced by bacterial contaminants from the tissues in the wound (Molan, 2001). Due to the moist wound environment that it generates it helps to deal with bacterial infection and nourishes new growing tissues (Mathew, 2010). Burdon (1995) suggested that hydrogen peroxide stimulates proliferation of fibroblasts and epithelial cells to repair the wound. The acidity of the honey releases oxygen from haemoglobin, increasing the oxygen available to new cells (Molan, 2006a). Murphy *et al*., (1982) and Tonnesen *et al*., (1988) reported that there are digestive enzymes present in wound tissues including metalloproteases of the connective tissue, and serine proteases produced by the neutrophils, and they are active due to hydrogen peroxide (Molan 2001). Rushton (2007) reported that excessive protease activity in a wound can prevent wound healing by growth factors. This protease activity results from increased inflammation. The anti-inflammatory effects of honey can remove this impediment to healing, also resulting in reduced pain and swelling (Molan, 2002). The high sugar content of honey also prevents pain during dressing changes, by keeping the wound surface moist and by mobilizing the oedema from the surrounding tissues.

In addition to these actions low concentrations of hydrogen peroxide activate insulin receptor complexes that trigger the molecular events in the cells which stimulate the uptake of glucose and amino acids, promoting anabolic metabolism supporting cell growth (Czech *et al.* 1974, Helm and Gunn 1986, Koshio *et al.* 1988). It has also been reported that honey may stimulate an appropriate inflammatory response in leukocytes (Abhuharfeil *et al*., 1999; Tonks *et al*., 2001; Tonks *et al*., 2003). Tonks *et al*. (2001) have reported that honey stimulates cytokine release from monocytes, giving rise to activation of tissue repair mechanisms. Tonks *et al.* (2007) have also demonstrated that there is a 5.8 kDa component in honey responsible for the

stimulation of TNF-α production through Toll-like receptor 4 (TLR4), but this component has not yet been identified.

1.3.4.3 Clearing infections

Glucose from honey allows maximal activity of phagocytes which clear infecting bacteria. In macrophages the glucose is the source of energy for the respiratory burst that generates the hydrogen peroxide which destroys microorganisms. Honey also provides substrates for glycolysis by this process; allowing macrophages to perform their function in damaged tissues (Ryan and Majno, 1977). As stated earlier, honey is antimicrobial, particularly Manuka honey. The UMF antimicrobial compound (MGO) diffuses deeply into skin tissues and acts against deep infections. Both MGO and hydrogen peroxide together are believed to have a synergistic effect (Sherlock *et al*., 2010).

1.3.4.4 Deodorizing and debriding

For chronic wounds malodour is common, as the wound is colonized with both anaerobic and aerobic microorganisms such as *Bacteroides* spp., *Peptostreptococci* spp., and *Prevotella* spp. which originate from the oral cavity and the gut (Bowler *et al*., 2001). Due to the antimicrobial effects, rapid deodorizing of wounds can be observed when honey dressings are used (Simon, 2008). In addition, honey contains high glucose levels which are used by bacteria instead of amino acids from the serum and dead cells, resulting in increased lactic acid instead of the malodour-causing ammonia, amines and sulphur compounds (Molan, 2002). Van der Weyden (2003) reported that patients with pressure ulcers showed quick and complete healing by the use of honey alginates and they observed both de-odorizing and anti-inflammatory effects. Haynes (2004) stated that there no odour was observed in 5 out of 20 patients and debridement in variety of wounds in three patients.

Autolytic debridement is facilitated by the development of a clean and granulating wound bed (Subrahmanyam, 1998; Stephen-Haynes, 2004). The debridement initiates the repair process of dormant wounds (Efem, 1998; Stephen-Haynes, 2004). Honey initiates this process by stimulating the growth of epithelium and minimizing scarring (Molan, 2001). Honey can also reduce inflammation through the stimulation and release of anti-inflammatory cytokines by cells of the immune system (Subrahmanyam, 1998) as well as

reducing oedema and smoothing the skin when applied to burns (Hejase *et al*, 1996; Subrahmanyam, 1993). However, honey has a stinging sensation upon application which has been reported by some patients (Vandeputte and Van Waeyenberge, 2003). For my knowledge, this may be due to the things present in honey such as acidity of honey, sugar concentration, LPS or it may be due to phytochemical such as simple phenolic acid.

1.3.4.5 Anti-inflammatory activity of honey

Inflammation is a part of the normal reaction to infection or injury, and involves a series of events involving cells of the immune system, cytokines and other inflammatory mediators (Wan *et al*., 1989). However, if inflammation is in excessive amounts or for prolonged periods of time, it causes more damage to the wound (Molan, 2001). The most important consequence of inflammation is the production of free radicals in the tissues (Flohe *et al*., 1985) leading to the breakdown of lipids, proteins and DNA (Cochrane, 1991). In some situations the reactive oxygen species produced in excessive amounts directly prevents wound healing.

Honey applied to wounds reduces the swelling, pain and heat associated with inflammation (Burlando, 1978; Efem, 1993; Subrahmanyam, 1998). The anti-inflammatory effects of honey in animals have also been studied (Burlando 1978, Gupta *et al.* 1992, Postmes *et al.* 1997, Oryan and Zaker 1998). Anti-inflammatory activity is responsible for minimizing scars (Molan, 2002). Though the process by which honey acts as an anti-inflammatory agent has yet to be fully elucidated, the effect has been widely observed clinically (Molan, 2006). The anti-oxidant content of honey accounts for its anti-inflammatory activity by preventing activation of nuclear factor kappa enhancer binding protein (NF-κB) (Grimble, 1994). Honey anti-oxidants showed reduction in inflammation of burns (Subrahmanyam, 1991). The anti-oxidant activity of honey has also been demonstrated by inhibition of chemiluminescence in a xanthinexanthine oxidase-luminol system that works via generation of superoxide radicals (Mobarok Ali and Al- Swayeh, 1997). These studies suggest that honey has a direct anti-inflammatory effect, not a secondary effect resulting from the antibacterial action removing the bacteria which cause inflammation.

1.3.4.6 Wound healing with stimulation/immunomodulation

Honey has been demonstrated to stimulate immune function *in vitro*. Abuharfeil *et al.* (1999) have found that concentrations of honey as low as 0.1% stimulates the proliferation of B- and T-lymphocytes in cell culture, and activate phagocytes from blood and demonstrated that this activity is probably due to low concentration of the active ingredient and commented that honey may contain mitogen isolated from higher plants that activates both the cells. The anti-oxidant components have been shown to stimulate immune function *in vitro* (Sabngi *et al*., 1997) and *in vivo* (De La Fuente *et al*., 2002). This is an important bioactivity of honey, as a feedback loop which allows the reactive oxygen species produced as a consequence of the inflammatory response to destroy bacteria to initiate a greater inflammatory response which can be deleterious to the healing process. It is important to identify the component that is stimulating this activity in honey.

Apalbumin1 (Apa1) glycoprotein in honey stimulates macrophages to release TNF-α (Majtan *et al*., 2006). Honey stimulates antibody production during primary and secondary immune responses (Al Wali and Haq, 2004). Tonks *et al.* (2001 and 2003) found that honey at a concentration of 1% stimulated monocytes in cell culture to release TNF-α production, which in turn activated the many facets of the immune response to infection.

Tonks *et al*. (2007) demonstrated that a 5.8 kDa component may be responsible *in vitro* for immunostimulatory activity in honey, as discussed earlier. However, Tonks *et al*., (2007) reported that the 5.8 kDa component from the honey was not a lipopolysaccharide, amino acid, vitamin or mineral. Therefore, the identity of the compound of interest remained unknown. In contrast, Timm *et al*. (2008) claimed that the immunostimulatory effects in honey were due to LPS because natural honeys contains substantial amounts of LPS and they observed the responses induced by honey were similar to the responses induced by LPS alone. If honey is to be used in wound healing the observation that it has immunostimulatory activity is important to facilitate more rapid healing, particularly in wounds that are proving difficult to heal.

1.3.4.7 Honey wound healing clinical trials

De-odorising and debridising effects were described earlier (Section 1.4.4.4). Clinical studies have also shown that honey contributes to clearing wound infections quickly (Cavanagh *et al*., 1970; Efem, 1993; Subrahmanyam, 1998; Cooper, 2007; Simon, 2008) and facilitates the healing of deeply infected surgical wounds (Vardi *et al*., 1998; Ahmed *et al*., 2003). Molan (1998a) briefly reviewed the importance of honey as clinical dressings. Some studies reported that honey promotes healing of wounds that are not responding to treatment by antiseptics or antibiotics (Dunford *et al*., 2000; Ahmed *et al*., 2003). Dunford *et al*. (2000) have observed that honey healed the wounds that were infected with methicillin-resistant *Staphylococcus aureus* which is an antibiotic resistant bacterium.

Further, honey has been successfully used on skin grafts, infected skin graft donor sites, pressure, leg, diabetic, tropical, sickle cell and malignant ulcers (Efem, 1988). It is also claimed to be a reliable alternative to conventional dressing for managing skin excoriation around stomas (reviewed by Molan, 2001). A recent study suggested that the topical application of honey was essential for the treatment of wound healing (Medhi *et al*., 2008).

1.4 Infection and immunity

The immune system is a system of cells and signalling cytokines that protect against invading pathogenic microorganisms. These microorganisms include bacteria, viruses, fungi and parasites, which are prevalent in the environment. The microorganisms enter into the host and their establishment can result in disease resulting from infection. Infection occurs when microorganisms invade epithelial surfaces such as the skin, the respiratory tract and the gastrointestinal tract. One of the highest risks of infection occurs when there are wounds (Anon, 2003) as it is relatively simple for bacteria to enter through the broken skin and penetrate the rest of the body.

Higher organisms have mechanisms to resist infection and are able to protect themselves against reinfection via immunity (Klein and Horejsi, 1977). The immune system acts as an integrated defense system to eliminate or control the infectious agent. The immune system resists infection with two mechanisms, innate and adaptive immune responses, both of which function to protect the individual from invading microorganisms. The innate immune system

provides immediate defence against infection whereas the adaptive immune system provides long lasting protective immunity.

Inflammation is one of the first responses of the immune system to infection. Inflammation can be stimulated by chemical factors that are released by injured cells and establish a barrier to prevent the spread of an infection; and it promotes the healing of any damaged tissues following the clearance of pathogens. The chemical factors that are produced during inflammation include histamine, bradykinin, serotonin, leukotriens and prostaglandins (Hertel, 1997) and attract the phagocytes, especially neutrophils.

Normally, the immune system resists infection with white blood cells, the complement system, and cytokines (Derkins, 2001). Macrophages and dendritic cells recognise foreign antigens; bind these and activate the rest of the system. Complement factors bind to and mark invading organisms so that the immune cells can more readily recognise the foreign antigens. Furthermore it plays another role, in the formation of an attack complex which kills bacteria (Delves, 2008). A group of white cells involved in protecting from infectious microorganisms are known as phagocytes. These include granulocytes, macrophages (which are converted monocytes), neutrophils and dendritic cells. These cells are the first line defense against infections (Poeta, 2004) engulfing bacteria in a process known as phagocytosis.

Another group of white cells fundamentally important in the active response part of the immune system are the lymphocytes. These cells determine the specificity of the immune response against foreign substances and microorganisms. Lymphocytes feature two main types of cells: B cells that grow to maturity independent of the thymus, and T cells that are processed in the thymus. The T cells are the main regulators of the immune system while B cells produce immunoglobulins (Ollila and Vihinen, 2005).

Finally, there are substances called cytokines. These molecules have a variety of roles; as signalling molecules and growth factors; activation, recruitment and inactivation of immune cells; and may also act as hormones. Cytokines can be pro-inflammatory or anti-inflammatory compounds (Moore, 2002). Cytokines are critical to a myriad of pathophysiological processes including fever, inflammation, wound repair, tissue repair and fibrosis. They play an important role in regulating cell function including proliferation, migration and matrix synthesis. The balance between these mediators appears to play the

main role in regulating the initiation, progression and resolution of wounds (Gharaee-Kermani and Phan, 2001).

1.4.1 Wound healing

Wound healing is a complex physiological and biological process involving a series of overlapping chemical, cellular and biological events (Singer and Richard, 1999) involving both in degenerative and reparative phases (Clarke, 1996). The sequence of wound healing can be described as inflammation, angiogenesis, matrix deposition, wound contraction, epithelialisation, and scar remodelling. Repair of injured tissue begins with the aggregation of platelets and formation of clot which maintains homoeostasis. The activation of inflammatory cells debriding the wound at the wound site marks the beginning of inflammation. Inflammatory cells include neutrophils, macrophages and lymphocytes and they release a variety of growth factors and cytokines that assist in wound repair (Werner and Grose, 2003). These signalling substances stimulate both pro-inflammatory and anti-inflammatory responses.

1.4.2 Anti-inflammatory cytokines

The pro-inflammatory response that is part of the normal response to infection is controlled by anti-inflammatory cytokines to ensure that inflammation is resolved (Opal and Depalo, 2000). The major pro-inflammatory cytokines are interleukin-1 (IL-1) and tumour necrosis factor (TNF-α) produced by monocytes and macrophages (Seymour and Henderson, 2001). Anti-inflammatory cytokines functioning antagonistically include interleukin (IL)-10, IL-11, and IL-13. Furthermore specific cytokine receptors for IL-1, TNF-α and IL-18 also function as pro-inflammatory cytokine inhibitors (Opal and Depalo, 2000).

1.4.3 Pro-inflammatory cytokines

Pro-inflammatory cytokines are produced normally by the activation of immune cells and involved in the amplification of inflammatory reactions. These cytokines play an important role in wound healing (Hubner *et al*., 1996). Macrophages, which differentiate from monocytes, migrate into various tissues during the inflammation process, and have three major functions: phagocytosis, antigen presentation, and immunomodulation through

production of growth factors and cytokines. Macrophages remove foreign debris, and will remain in the wound for few months depending on the extent of injury; clearing infection by foreign and necrotic debris (Anderson, 2001). Macrophages play a critical role in the initiation, maintenance, and resolution of inflammation and are activated and deactivated in the inflammatory process (Fujiwara and Kobayashi, 2005). The capacity of macrophages in producing both inflammatory cytokines and growth factors is considered to play a major role in wound repair (Gillitzer *et al*., 2001).

After injury the macrophage induces pro-inflammatory cytokines immediately such as IL-1, IL-6, and TNF-α (Stroncek and Reichert, 2008). TNF-α is a regulatory pro-inflammatory cytokine which plays an important role in the defense against intracellular microorganisms (Ciacci-woolwine *et al*., 1999) and induces fibroblast collagen synthesis in initiating healing (Van den Berg *et al*., 2008). TNF-α possesses a wide range of functions and shares many of the physiological and pathological effects of IL-1 (Beutler and Cerami, 1988). Together with IL-1, TNF-α is the first cytokine to upregulate the inflammatory phase of wound healing and contributes to the oxidative stress within the wound by generating reactive oxygen species and intracellular proteins that are released by necrotic cells which are crucial in wound healing.

1.4.4 Tissue re-growth

In wound healing tissue re-growth is essential to establish the scaffolding that is necessary to rebuild the damaged tissues. During inflammation, the tissue strength of a wound is minimal, so tissues do not regain their normal functional strength until inflammation transitions into repair. These transitions are mediated by macrophages and their anti-inflammatory cytokines which occur during the week after injury (Stroncek and Reichert, 2008). Granulation tissue develops during the proliferative stage, characterized by growth factor production, fibroblast growth and the synthesis of extracellular matrix (ECM) to fill in the damaged tissue that has been cleared during inflammation (Writte and Barbul, 1997). The ECM is composed of collagen, elastin, glycosaminoglycans and chondroitin-6-sulfate. During the granulation and remodelling stage the ECM reforms and restores. During ECM remodelling the fibroblasts break down and re-synthesize collagen fibrils. Collagen fibre is a prominent component of the ECM and plays an important role in wound healing (Caria *et al*., 2004). The remodelling phase proceeds until fibronectin and hyaluronic acid are replaced by collagen bundles which

lend strength to the tissue (Stroncek and Reichert, 2008). The impaired wound healing of acute and chronic wounds are characterized by more complicated process with more inflammation and more granulation than normal wound healing (Menke *et al*., 2007).

To heal wounds, with an impaired wound-healing process, the use of some natural products such as plants and herbs has been suggested. Amongst natural products reputed to aid wound healing, honey has a particularly good anecdotal reputation.

The aim of this thesis was firstly to investigate the highest non-toxic concentration of honey that stimulates THP-1 cells to produce pro-inflammatory cytokine (TNF-α) which plays an important role in the wound healing process. It was hypothesized that this study may lead to the identification the component(s) that may be responsible for the immunomodulatory ability of Comvita sourced New Zealand honeys (Section 3.4.1). Activation was assessed by measurement of TNF-α production. The majority of this work was carried out using an *in vitro* cell-based culture system to measure the immunostimulatory effects of honey samples.

This thesis, presents the data from *in vitro* and subsequent analyses that were designed to investigate the component(s) in honey are responsible for immunomodulatory effects.

1.5 Aim of this project

Investigate the ability of Comvita New Zealand honeys to elicit a TNF-α cytokine response from acute monocytic leukemia (THP-1) cells, and to identify the component which is responsible.

The study is focused on the following specific objectives to achieve the aim:

Objective 1: Investigate the immunostimulatory properties of different types of honeys with or without LPS;

Objective 2: Assess the role of LPS and heat-labile honey components with or without LPS;

Objective 3: Identify the active compound(s) in honey associated with immuno-modulation.

Chapter 2
Materials and methods

2.1 Materials

2.1.1 Chemicals and reagents

Chemicals used in the study were obtained from various suppliers.

Some chemicals and reagents were obtained from the following manufacturers/suppliers:
Dimethylsulfoxide (DMSO) (Ajax Chemicals, Auburn, NSW, Australia).
Disodium hydrogen phosphate, sodium dihydrogen phosphate, potassium chloride, potassium dihydrogen orthophosphate (BDH Laboratory Supplies, Poole, England).
4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzenedisulfonate (WST-1 reagent) (Roche Diagnostic NZ Ltd, Mt.Wellington, New Zealand).
Tween 20 (0.05 %), (BDH chemicals, Auckland, New Zealand) was added to 1 x PBS solution while washing.
Bovine serum albumin (BSA) low endotoxin (ICP bio Ltd, Auckland, New Zealand).

The following chemicals and reagents were obtained from Sigma Aldrich, Auckland, New Zealand:
Methylglyoxal (MGO), Camptothecin, Lipopolysaccharide (LPS) from *Escherichia coli*0127:B8, Fructose, glucose and sucrose, Phorbol 12-myristate 13-acetate (PMA), Polymixin B (PmB)

The following reagents were obtained from BD Biosciences, Auckland, New Zealand:
Purified mouse anti-human TNF monoclonal antibody (Capture antibody), Biotin mouse anti-human TNF (Detection antibody), Streptavidin – Horseradish peroxidase (HRP enzyme); TMB Substrate Reagent Set (Substrate), Tumor Necrosis Factor (TNF-α)

The following reagents were obtained from Invitrogen, Auckland, New Zealand:
Roswell Park Memorial Institute Medium (RPMI) 1640, Fetal bovine serum (FBS), Penicillin and Streptomycin.

2.1.2 Other materials

Amicon ultra centrifugal filter devices of 30,000 molecular weight cut-off and 3000 molecular weight cut-off were obtained from Biolab, Auckland, New Zealand. High binding 96 well plates for enzyme linked immune sorbent assay (ELISA) were obtained from Greiner, Bio-one microlon, New Zealand.

Other routine equipment, consumables and disposables such as pipette tips, serological pipettes, multichannel pipettors, culture vessels, 24-well plates, pipettors, 0.2, µm filters and barrier tips were obtained from manufacturers or suppliers including the following: Axygen Inc., Beckton Dickonson and Co., Sparks, MD; and Biolab, Auckland, New Zealand; Eppendorf; Nunc; Roskilde, Denmark; Sartorius AG, Goettingen, Germany; Sorenson BioScience Inc.

2.1.3 Cell line

THP-1 cells (American Tissue Culture Collection (ATCC): TIB-202, *Homo sapiens* male 1 year infant, peripheral blood acute monocytic leukemia) were obtained from the cell culture laboratory at the Mt Albert site of the New Zealand Institute for Plant and Food Research Ltd (Plant and Food Research), Auckland, New Zealand.

2.2 Solutions and media

Water

Distilled and de-ionized water (Millipore) (Biolab, Auckland, New Zealand) was used throughout.

Complete RPMI medium

Complete RPMI 1640 medium was prepared by supplementing RPMI 1640 with 10 % fetal bovine serum and penicillin at 50 IU units/mL and streptomycin at 5µg/mL and stored at 4°C

Cell freezing media

Freezing medium was prepared by adding 1% DMSO to complete RPMI medium.

Phosphate buffered saline

10x PBS was prepared for the washings by adding 80 g NaCl + 2 g KCl + 26.8 g Na_2HPO_4 + 2.4 g KH_2HPO_4 to 1 L with distilled water.

Coating buffer

Coating buffer was prepared for TNF ELISA by adding 11.8 g Na_2HPO_4 + 16.1 g NaH_2PO_4 to 1 L with distilled water, and then frozen in 10 and 50 mL aliquots in falcon tubes until required.

Camptothecin

Camptothecin ($(CH_3)_2SO$) was dissolved in DMSO to obtain a stock solution of 10 mM for the cytotoxicity analysis. The stock solution was kept at -20°C.

Lipopolysaccharide (LPS)

LPS from *Escherichia coli* 0127:B8 cell culture was tested and purified by gel-filtration chromatography (1 mg) and it has < 1 % protein. It was diluted in distilled water to give 1 mg/mL. The solution was aliquoted into 50 µL volumes and kept at –20°C.

Phorbol 12-myristate 13-acetate (PMA)

A stock solution at 1 mg/mL (162 µM) PMA was made up in DMSO. It was stored as 20 µL and 50 µL aliquots in small eppendorf tubes and kept at – 20°C.

Polymixin B (PmB)

A stock (1 mg/mL) of PmB was prepared freshly with distilled water every 2 weeks and stored at 4°C.

Methylglyoxal (MGO)

A representative concentration to simulate a high UMF® Manuka honey is 500 mg/kg reported by Stephens *et al*. (2010). The MGO was diluted using RPMI 1640 medium (1.28 µL of MGO in 4 mL of media, which is equal to a 25% (w/v) honey sample). Further dilutions were made using RPMI medium according to the honey dilution being simulated.

2.3 Honey samples

Eighteen test New Zealand honey samples (Table 2.1) were supplied by Comvita New Zealand Ltd in yellow screw cap polystyrene containers (Labserv, Biolab, Auckland, NZ). The honey samples were delivered at ambient temperature, and upon receipt were immediately transferred into a refrigerator (4ºC) until required for further use. The age of the honey samples ranged from 0.3 to 5.5 years.

Table 2.1 *New Zealand honey samples analyzed in the study*

Code	Honey type	Principal floral source (and floral contaminates[e])	Sample age (years)	Geographic origin
INTPH-01	Manuka[a]	*L. scoparium* var. *incanum* (*Trifolium spp.*)	0.5	Northland
INTPH-03	Manuka[b]	*L. scoparium* var. *incanum* (hive site not assessed)	5	Northland
INTPH-05	Manuka[a]	*L. scoparium* var. *incanum* (*Trifolium spp., K. excelsa)*	0.4	Northland
INTPH-06	Manuka[a]	*L. scoparium* var. *incanum* (*Trifolium spp. K. excelsa, K. ericoides)*	0.75	Northland
INTPH-09	Manuka[b]	*L. scoparium* var. *linifolium (*hive site not assessed)	4	Waikato
INTPH-10	Manuka[a]	*L. scoparium* var. *myrtifolium (Trifolium spp., K. excelsa)*	0.5	Whanganui
INTPH-12	Manuka[a]	*L. scoparium* var. triketone[d] (*Trifolium spp.)*	0.3	East Coast
INTPH-14	Manuka[b]	*L. scoparium* var. triketone[d] (hive site not assessed)	5.5	East Coast
INTPH-15	Kanuka[b]	*Kunzea ericoides* (hive site not assessed)	1.5	Northland
INTPH-16	Kanuka[b]	*Kunzea ericoides* (hive site not assessed)	2.5	Waikato
INTPH-17	Kanuka[b]	*Kunzea ericoides* (hive site not assessed)	3.5	East Coast
INTPH-18	Kanuka[b]	*Kunzea ericoides* (hive site not assessed)	5.5	East Coast
INTPH-19	Manuka[b]	*L. scoparium* (variety unknown, hive site not assessed)	1.5	Unknown
INTPH-20	Clover[c]	*Trifolium spp.* (hive site not assessed)	1	South Island
INTPH-21	Rewarewa[b]	*Knightia excelsa* (hive site not assessed)	5	Bay of Plenty

Notes: [a] Samples collected from hive sites; [b] Matured samples from drums supplied by apiarists and purchased as designated type; [c] Commercial labelled product; [d] Unclassified *L. scoparium* variety that carries an enhanced triketone essential oil profile; [e] *Knightia excelsa*; *Kunzea ericoides* (Stephens *et al*., 2010).

Honey samples listed in Table 2.1 were analyzed by Hill Laboratories Ltd and Comvita NZ Ltd, and the phenolic compounds and methylglyoxal content formed the basis of a report by Stephens *et al*. (2010). The floral sources, ages and geographic origins served as further distinguishing characteristics.

Table 2.2 *Phenolic compounds and methylglyoxal present in New Zealand Manuka, Kanuka, Rewarewa and Clover honeys*

	Concentration of phenolic compounds and methylglyoxal (mg/kg)[a]										
Sample code	Gal	Abs	Phe	Ph03	4MB	2MB	Ph01	MSyr	Ph02	Syr	MGO
INTPH-01	0.1	0.6	1790	5.0	Tr	28.9	12.5	5.4	91	0.4	651
INTPH-03	0.5	0.2	1880	2.1	Tr	14.8	143	103	371	1.4	1541
INTPH-05	0.3	0.1	790	8.1	Tr	5.7	27	15	97	0.2	218
INTPH-06	1.3	0.4	1100	25.2	Tr	4.3	75	99	320	0.9	297
INTPH-09	1.4	0.5	1120	25.6	Tr	4.5	97	111	334	0.8	1004
INTPH-10	0.7	0.3	330	7.4	Tr	1.2	26	9.4	56	0.2	102
INTPH-12	0.4	0.3	590	6.8	Tr	6.4	223	18	14	1.3	372
INTPH-14	0.5	0.2	1390	182	3.0	1.4	24	207	502	1.0	270
INTPH-15	0.5	1.5	720	87	2.0	0.7	3.0	60	108	1.0	37
INTPH-16	Tr	0.4	910	161	2.0	0.3	1.0	112	213	1.3	6
INTPH-17	0.3	2.9	910	157	5.0	1.1	22	130	351	1.9	174
INTPH-18	0.5	0.1	1610	18.8	5	1.7	22	305	602	1.6	230
INTPH-19	1.0	0.4	1120	8.8	Tr	9.9	106	103	440	1.2	1490
INTPH-20	Tr	2.8	20	5	Tr	Tr	2	1.7	8.0	0.1	nd
INTPH-21	0.5	2.2	80	Tr	Nd	0.4	27.2	12.7	4.6	0.4	nd

Notes: tr – trace, nd – not detectable;.[a] Gal – gallic acid, Abs – abscisic acid, Phe – phenyl lactic acid, Ph03 – 4-methoxyphenyllactic acid (tentative identification), 4MB – 4-methoxybenzoic acid, 2MB – 2-methoxybenzoic acid, Ph01 –trimethoxybenzoic acid (tentative identification), MSyr – methyl syringate, Ph02 – structural isomer of syringic acid (tentative identification), Syr – syringic acid, MGO – methylglyoxal (Stephens *et al*., 2010).

Interpretation by Stephens *et al*. (2010) suggests that the honey samples (Table 2.2) contain different degrees of monoflorality. Two of the four Northland Manuka honeys, INTPH-01 and INTPH-03, are principally monofloral, whereas INTPH-05 and INTPH-06 contain significant amounts of nectar collected from other plant sources. The Waikato Manuka is fairly monofloral; however, the Wanganui Manuka sample contains other forest nectars. The most monofloral East Coast Manuka is INTPH-12. The matured East Coast Manuka honey, INTPH-14, and the two older Kanuka honeys from the East Coast, INTPH-17 and INTPH-18, are Manuka/Kanuka honey blends typical of honeys from this region. The Kanuka honeys INTPH-15 and INTPH-16 are principally monofloral. The honey sample INTPH-19 displayed an elevated level of hydroxymethylfurfural (HMF), and had most probably been exposed to heating to accelerate aging and promote the development of methylglyoxal; the age of the sample and methylglyoxal concentration indicated significant heat manipulation.

The Clover and Rewarewa samples did not carry the phenolic markers typical of Manuka or Kanuka honeys, and would appear to be monofloral.

The most monofloral Manuka honeys were Northland INTPH-01 and INTPH-03, and East Coast INTPH-12; the monofloral Kanuka honeys were INTPH-15 and INTPH-16; the Manuka INTPH-19 had most probably been heated during processing or storage; and the Clover INTPH-20 and Rewarewa INTPH-21 honey samples were predominantly monofloral.

2.4 General Methods

2.4.1 Preparation of honey samples

Honey samples were weighed using a top-pan balance (Mettler, Toledo, USA). Honey samples were diluted (40 or 50 µg) in distilled water (1 mL) to give a final concentration of 4 % or 5 % (w/v) honey, respectively, and then incubated at 37 °C for 15 min to dissolve. Once the honey had dissolved, further dilutions were carried out in complete RPMI medium according to requirements.

2.4.2 Preparation of artificial honey (AH)

The AH was prepared by dissolving 192 mg fructose, 180 mg glucose and 4 mg sucrose (Sigma Aldrich, New Zealand) in 10 mL of RPMI medium, filtered through a 0.20 µm (DISMIC-13CP) disposable syringe filter (Millipore, Auckland, New Zealand) and stored at 4°C. The filtered AH contained fructose (48%), glucose (45%) and sucrose (1%), mimicking the composition of undiluted honey (Echigo *et al*., 1986).

2.4.3 Treatment of honey samples

Honey samples were treated in three different ways according to Figure 2.1.

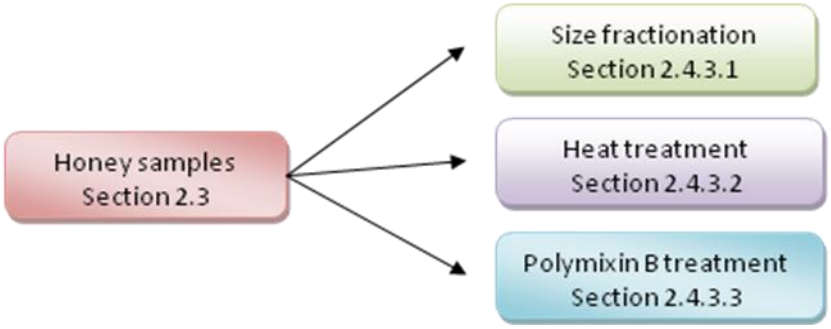

Figure 2.1 Summary of honey sample treatments to determine the component responsible for immunomodulatory effects. The methods are described briefly in the following sections.

2.4.3.1 Size fractionation of honey samples

Two filters were selected to fractionate the honey samples according to molecular weight. Honey samples (4 % (w/v)) were separated by filtration using an Amicon Ultra 30000 molecular weight membrane cut-off centrifugal concentrator (Biolab). The resultant filtrate was further separated using a 3000 filter, producing 3 fractions: >30,000, 30,000-3000 and <3000 Da. Four mL of honey samples (4 % (w/v)) were added into the upper chamber of the Amicon Ultra 30,000 molecular weight membrane cut-off centrifugal concentrator, and centrifuged at 3220 x *g*, at 25°C for 15 min. The retentate was collected and the volume adjusted to 4 mL using distilled water. The left over filtrate was further filtered using an Amicon Ultra 3000 molecular weight membrane cut-off centrifugal concentrator by repeating the centrifugation step as described above. The volume of the retentate was adjusted to the volume that was left after filtration from 30,000 molecular weight cut-off. Fractions of the filtrate (>3000) and retentate (<30,000 and 30,000-3000) were immediately used in the cell-based assay.

2.4.3.2 Heat treatment of honey samples and lipopolysaccharide (LPS)

The stock solution of honey samples (5 % (w/v)) and LPS 1000 ng/mL that were prepared separately in distilled water were heated in a shaking at 100 rpm water bath (80ºC for 30 min). After the heat-treatment, further dilutions were performed in complete RPMI medium.

2.4.3.3 Treatment of honey samples with polymixin B (PmB)

The stock solution of honey sample (5 % (w/v)) was further diluted to 4 % (w/v) and 2 % (w/v) using complete RPMI medium. Honey samples (500 µL) of 4 % (w/v) and 2 % (w/v) were treated with 500 µL of 80 µg/mL and 40 µg/mL of PmB at room temperature to give final honey concentrations of 2 % (w/v) and 1 % (w/v) and final PmB concentrations of 40 µg/mL and 20 µg/mL, respectively. The samples were added immediately to the cells

2.5 In vitro analysis

2.5.1 Culturing of THP-1 cells

The glassware and distilled water used to culture cells were sterilized by autoclaving at 121°C for 15 min. THP-1 cells were grown from 5 x 10^4 cells/ mL to 2 x 10^6 cells/mL in a Sanyo Model MCO-20AIC carbon dioxide (CO_2) incubator at 37°C with humidity control and CO_2 maintained at 5 % in air. Cultures were monitored microscopically, refreshed with new growth media every 2-3 days (Parthiban *et al*, 2007), and passaged prior to reaching the end of log phase growth by centrifuging at 130 x *g* for 5 min and resuspending to 2 x 10^6 cells/mL. Cells were counted using a haemocytometer chamber under microscope (Olympus CKX41).

2.5.2 Preparation of cells for frozen storage

THP-1 cells were centrifuged at 130 x *g* and resuspended at 1 x 10^5 cells/mL and stored in appropriate growth medium (complete RPMI) containing cryoprotectant 1% DMSO (v/v) that was added slowly in the medium for 1 to 2 min. Cryotubes containing cells were gradually frozen in a freezing box by successively placing at -20°C for 4-6 h, -80°C for overnight and finally transferred to liquid nitrogen (N_2).

2.5.3 Thawing cells

The cryotubes were placed in a 37°C water bath for 1-2 min with continual visual monitoring to ensure complete thawing occurred, prior to the contents being added dropwise to complete RPMI media and centrifuged at 130 x *g* to dilute out the DMSO. The cells were resuspended in fresh complete RPMI medium.

2.5.4 Cytotoxicity of honey samples

The method used to determine the cytotoxicity of honey samples, and artificial honey was based on the WST-1 cytotoxicity assay (Ngamwongsatit *et al.,* 2008). THP-1 cells were harvested during the log phase of growth, centrifuged at 130 x g for 5 min and resuspended in complete RPMI medium at 1 x 10^6 cells/mL. The cells (50 µL) were pipetted into each well of a 96-well plate giving a concentration 5 x 10^4 cells/well. Honey samples were prepared as described (Section 2.2.2) and diluted using complete RPMI medium into serial dilutions of 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03 and 0.015% (w/v). Serial dilutions of honey solutions (50 µL) were added, resulting in 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03, 0.15 and 0.007 % (w/v) final concentrations of honey in 100 µL final volume.

AH was prepared as previously described (Section 2.4.2) using complete RPMI medium (10 mL) to simulate a 4% (w/v) honey concentration. The same concentrations of artificial honey solutions were added to the cells as the honey solutions. The controls were the wells containing RPMI medium alone, cells in complete medium (negative control) and cells with camptothecin 0.1 µg/mL – 10 µg/mL final concentrations (positive control). The plates were incubated for 24 h at 37^{o}C in an incubator with humidity control and CO_2 maintained at 5% in air. Cell proliferation reagent (WST-1) was added to each well (10 µL) and mixed on a shaker for 1 min and the plate was incubated at 37^{o}C for 1 h as previously indicated. The absorbance of the samples were measured against the background control as blank using a multiscan plate reader (Medica Pacifica Ltd, Auckland, New Zealand) at 450 nm set at a reference wavelength of 620 nm. The amount of cytotoxicity was calculated according below to equation 2.

$$\text{Cytotoxicity (\% of cell survival)} = \frac{\text{ATS - Blank}}{\text{ANC - Blank}} \times 100 \quad (2)$$

ANC = Absorbance of negative control, ATS = Absorbance of test samples

2.6 In vitro assay for immunostimulation

A summary of *in vitro* immunostimulation protocol is shown in Figure 2.2.

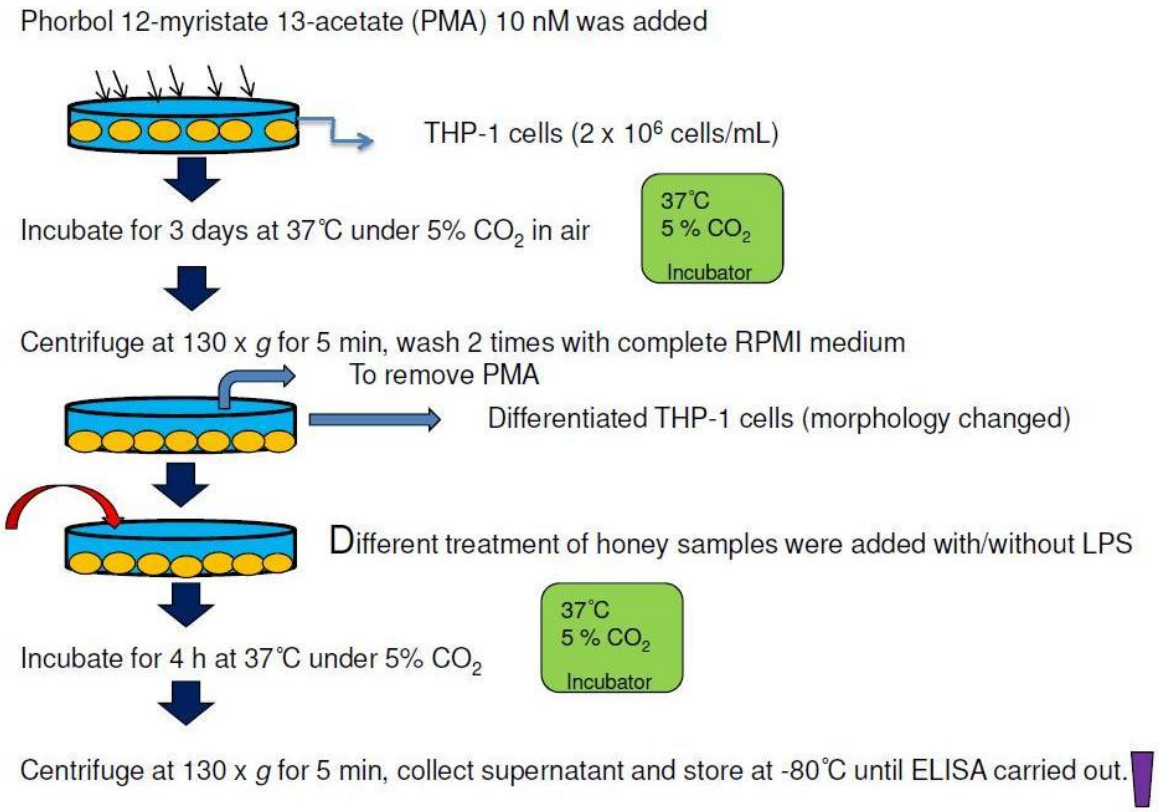

Figure 2.2 Summary of *in vitro* immunostimulation protocol. THP-1 cells were differentiated with phorbol 12-myristate 13-acetate, washed and honey samples were added in the presence or absence of lipopolysaccharide. Tumor necrosis factor (TNF-α) production from the cells were measured in supernatants by ELISA.

2.6.1 THP-1 cells differentiation

THP-1 cells were harvested, centrifuged and resuspended in complete RPMI medium at 2 x 10^6 cells/mL, after counting using a haemocytometer chamber under microscope (Olympus CKX41). The cells were then dispensed (500 µL/well) into a 24-well plate (Greiner) and 20 nM PMA (Sigma Aldrich) was added (500 µL) giving a final concentration of 1 x 10^6

cells/mL and 10 nM PMA. The plates were incubated for 3 days in a 37°C incubator with humidity control and CO_2 maintained at 5% in air to allow them to differentiate (Cheng *et al.*, 2005; Rodiles *et al.*, 1996). After incubation, cells were centrifuged at 130 x *g* for 5 min, washed with fresh RPMI medium, recentrifuged at 130 x *g* for 5 min to remove the PMA, and fresh RPMI medium (500 µL) added for further analysis.

2.6.2 Optimization of lipopolysaccharide (LPS) dose

The response to the dose of LPS was determined using differentiated cells. Different concentrations of LPS (Sigma Aldrich) 1000 ng/mL, 500 ng/mL, 200 ng/mL and 100 ng/mL were prepared from 1 mg/mL stock using complete RPMI medium. Of the range of LPS concentrations, 500 µL were added to the wells that contained 500 µL of differentiated cells to give final concentrations of 500 ng/mL, 250 ng/mL, 100 ng/mL and 50 ng/mL. Each concentration of LPS was tested in duplicate and the experiment was repeated. The plates were incubated for 4 h in a Sanyo Model MCO-20AIC CO_2 incubator at 37°C with humidity control and CO_2 elevated to 5 % in air. After incubation, the plates were removed from the incubator and centrifuged at 130 x *g* for 5 min and the supernatant was collected in eppendorf tubes and stored at –80ºC until required for the ELISA (Section 2.8.1).

2.6.3 Optimization of capture and detection antibodies for ELISA

The capture and detection antibodies were optimized by using different concentrations in a chequor board manner. The capture antibody was prepared for coating, by adding capture antibody (antibody purified mouse anti-human TNF-α) in 10 µL, 20 µL, 40 µL, 60 µL and 80 µL to each 10 mL coating buffer to give final concentrations of 1 µg/mL, 2 µg/mL, 4 µg/mL, 6 µg/mL and 8 µg/mL. The solutions were mixed by a vortex mixer (VELP Scientifica) and 100 µL of each concentration of the coating antibody was added in triplicate for each working concentration to each well of an ELISA high binding plate. The ELISA plate was wrapped in glad wrap and incubated for 18 h in a dark at 4 ^{o}C in humidity chamber.

After incubation, the plate was washed 5 times with 1x PBS-T (PBS containing 0.05 % (v/v) Tween 20 (BDH Chemicals), using a plate washer (ELx 50-Auto strip washer, Bio-tek instruments). Each well was blocked to minimize non-specific binding by the addition of 1xPBS-10% FBS solution (100 µL) and incubated for 1 h in a darkened humidity chamber at

room temperature. The TNF-α standards were prepared by dilution in 1 x PBS containing 1% (w/v) BSA (ICP Bio Ltd) (Table 2.3). The human TNF-α standard was at 0.2 mg/mL, a stock solution (2500 ng/mL) was prepared, aliquoted in small eppendorf tubes and stored at -80°C. The TNF-α standard dilution were prepared according to Table 2.3.

Table 2.3 *Preparation of Tumor Necrosis Factor-α (TNF-α) standard concentrations*

Sample number	Dilution	TNF-α (pg/mL)
1	1/100	25000
2	1/10 of 25000 conc.	2500
3	2/5 of 2500 conc.	1000
4	1/2 of 1000 conc.	500
5-9	Serial ½ of 500 conc.	125-7.8125

After blocking, the plate was washed 5 times with 1 x PBS-Tween using a plate washer. TNF –α standard and controls were added to the wells in duplicate and incubated for 2 h at room temperature in a darkened humidity chamber. After 2 h the plate was further washed 5 times with 1x PBS-Tween solution with the plate washer. The detection antibody was prepared by adding 5 µL, 10 µL 20 µL, 40 µL and 60 µL to each 10 mL of 1 x PBS-10 % FBS to get a final concentration of 0.25 µg/mL, 0.5 µg/mL, 1 µg/mL, 2 µg/mL and 3 µg/mL and 10 µL of streptavidin was added to each diluted concentration of antibody. After mixing, 100 µL was added in triplicate per each ELISA plate well and incubated for 1 h in a dark at room temperature in a humidity chamber.

Substrate was prepared by mixing Substrate Reagent A and B in equal volumes and 100 µL was added to each well and the plates were incubated at room temperature for 15-30 min in the dark. When colour had developed 50 µL of stop solution (2M H_2SO_4) was added to each well. The absorbance of the samples was measured immediately using a multiscan plate reader (Medica Pacifica Ltd, Auckland, New Zealand) at 450 nm with a reference wavelength of 620 nm. Microsoft Excel was used to process the data from the combinations of capture and detection antibodies and for determining TNF-α cytokine levels using a standard curve prepared from 1000 pg/mL – 7.825 pg/mL TNF-α. Graphs were plotted using Sigma plot software.

2.7 Treatment of honey samples for in vitro cell-based assay

The treatment schedule for honey samples used in *in vitro* cell-based assays is shown in Figure 2.3

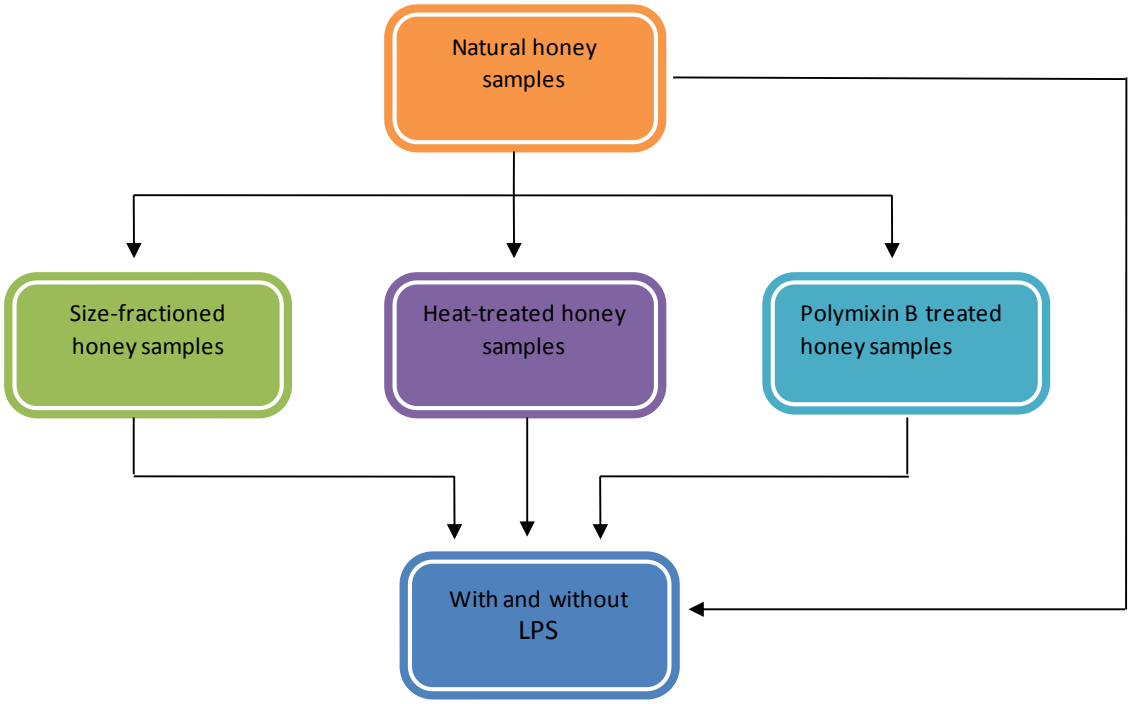

Figure 2.3 Summary of treatments with and without lipopolysaccharide in *in vitro* based assays. The honey samples were treated in three different ways (section 2.4.3.1 to 2.4.3.3). The natural honey samples and three treatment samples were added to the cells in the presence or absence of lipopolysaccharide.

2.7.1 Effect of honey with and without LPS on TNF-α production in THP-1 cells

The honey samples are analyzed for their ability to stimulate TNF-α production from differentiated THP-1 cells with and without added LPS. Stock 5 % (w/v) honey samples were further diluted to 2 % (w/v) and 1 % (w/v) using complete RPMI medium.

Samples and controls

(i) Cells alone: Complete RPMI medium (500 µL) was added to the differentiated cells

(ii) Honey samples alone: The natural honey samples (500 µL) at 1% (w/v) were added directly to the differentiated cells to give a final concentration of 0.5 % (w/v) of honey samples.

(iii) LPS alone: LPS (200 ng/mL) in complete RPMI culture medium was prepared from a stock solution and 500 µL was added to the differentiated cells to give a final concentration of 100 ng/mL of LPS.

(iv) Artificial honey alone: The AH (500 µL) at 1 % (w/v) was added directly to the differentiated cells to give a final concentration of 0.5 % (w/v) of AH that is equal to 0.5 % (w/v) of honey sample.

(v) MGO alone: MGO (500 µL) at 1 % (prepared (Section 2.2.10 (500 mg/kg)) and dilution was carried out with complete RPMI medium) which is equal to 1 % (w/v) of honey sample was added directly to the differentiated cells to give a final concentration of 0.5 % (w/v) of MGO that is equal to 0.5 % (w/v) of MGO in honey sample.

(vi) Honey + MGO: Honey sample INTPH-20 (500 µL) at 2% (w/v) and MGO (500 µL) at 2 % which is equivalent to 2 % (w/v) of honey sample were mixed together to give a concentration of 1 % honey samples and 1 % of MGO (equivalent to 1 % (w/v) of honey sample). These mixed samples are added to the differentiated cells to give a final concentration of 0.5 % honey sample and 0.5 % (w/v) of MGO that is equivalent to 0.5 % (w/v) of honey sample.

(vii) AH+ LPS: The AH (500 µL) at 2 % (w/v) and LPS (500 µL) at 400 ng/mL were mixed together to get a concentration of 1 % AH and 200 ng/mL of LPS. These mixed samples were added directly to the differentiated cells to give a final concentration of 0.5 % (w/v) of AH that is equivalent to 0.5 % (w/v) of honey sample and 100 ng/mL of LPS.

(viii) MGO + LPS: MGO (500 µL) at 2 % (w/v) and LPS (500 µL) at 400 ng/mL were mixed together to get a concentration of 1 % MGO (500 mg/kg) and 200 ng/mL of LPS. These mixed samples were added directly to the differentiated cells to give a final concentration of 0.5 % (w/v) of MGO that is equivalent to 0.5 % (w/v) of honey sample and 100 ng/mL of LPS.

(ix) Honey + LPS: Honey samples (500 µL) of 2% (w/v) and LPS (500 µL) of 400 ng/mL in complete RPMI culture medium were mixed together to get a concentration of 1 % honey samples and 200 ng/mL of LPS. These mixed samples are added to the differentiated cells to give a final concentration of 0.5 % honey samples and 100 ng/mL of LPS.

(x) Honey + MGO + LPS: INTPH-20 (500 µL) at 4% and MGO (500 µL) at 4 % (w/v) were mixed together to give a concentration of 2 % honey sample and 2 % MGO present in honey (500 mg/kg). Then LPS (500 µL) at 400 ng/mL were mixed with the mixed solution of honey and MGO to give a concentration of 1 % honey sample, 1 % MGO and 200 ng/mL of LPS.

This mixed sample was added to the differentiated cells to give a final concentration of 0.5 % honey samples, 0.5% MGO and 100 ng/mL of LPS.

The plates were incubated for 4 h in a 37°C incubator with humidity control and CO_2 at 5% in air. After incubation, the plates were centrifuged at 130 x *g* for 5 min and supernatants were collected in eppendorf tubes and stored at –80 ºC until the ELISA (Section 2.8.1) was carried out to test for TNF-α production.

2.7.2 Effect of PmB treated honey samples with and without added LPS

The PmB treated honey samples (Section 2.4.3.3) were tested for their ability to stimulate TNF-α production from differentiated THP-1 cells with and without added LPS. Natural honey samples and LPS were prepared as described in section 2.7.1

Samples and controls

(i) Cells alone: Complete RPMI medium (500 µL) was added to the differentiated cells
(ii) Honey samples alone: The natural honey samples (500 µL) at 1% (w/v) were added directly to the differentiated cells to give a final concentration of 0.5 % (w/v) of honey sample.
(iii) Cells + PmB: 500 µL of differentiated cells are treated with 500 µL of PmB (20 µg/mL) to give a final concentration of 10 µg/mL PmB.
(iv) LPS alone: The LPS (500 µL) at 200 ng/mL was added directly to the differentiated cells to give a final 100 ng/mL of LPS concentration.
(v) PmB treated LPS: The LPS (500 µL) at 400 ng/mL were added with 500 µL of PmB (20 µg/mL) at room temperature and added together immediately to the differentiated cells to give a final concentration of 100 ng/mL of LPS and 10 µg/mL of PmB.
(vi) PmB treated honey samples alone: 500 µL of PmB (40 µg/mL) were just mixed at room temperature with honey samples (2 % (w/v)) to give a concentration of 20 µg/mL of PmB and 1 % of honey sample. Then 500 µL of this mixed sample was added together immediately to the differentiated cells to give a final concentration of 10 µg/mL PmB and 0.5 % (w/v) honey sample.
(vii) PmB treated honey samples + LPS: 500 µL of PmB (80 µg/mL) were just mixed at room temperature with honey samples 4 % (w/v) to give a concentration of 40 µg/mL PmB and 2

% honey sample and this mixed sample was treated with 500 µL of 400 ng/mL LPS giving a concentration of 1 % honey sample, 20 µg/mL of PmB and 200 ng/mL LPS. These mixed samples were added to 500 µL of differentiated cells to give a final concentration of 0.5 % honey sample, 10 µg/mL PmB and 100 ng/mL LPS.

The plates were incubated for 4 h in a 37 °C incubator with humidity control and CO_2 at 5 % in air. After incubation, the plates were centrifuged at 130 x *g* for 5 min and supernatants were collected and stored at –80 ºC until the ELISA (Section 2.8.1) was carried out to test for TNF-α production.

2.7.3 Effect of heat treated honey samples with and without added lipopolysaccharide (LPS)

The heat treated honey samples (Section 2.4.3.2) were tested for their ability to stimulate TNF-α production from differentiated THP-1 cells with and without added LPS. The samples were cooled down to room temperature and further diluted using complete RPMI medium to result in a 2 % (w/v) and 1 % (w/v) concentration. Natural honey samples and LPS were prepared as described in (Section 2.7.1).

Samples and controls

(i) Cells alone: Complete RPMI medium (500 µL) was added to the differentiated cells
(ii) Honey samples alone: The natural honey samples (500 µL) at 1% (w/v) were added directly to the differentiated cells to give a final concentration of 0.5 % (w/v) of honey samples.
(iii) LPS alone: The LPS (500 µL) at 200 ng/mL was added directly to the differentiated cells to give a final concentration of 100 ng/mL LPS.
(iv) Heat treated honey samples alone: Heat treated honey samples 1% (w/v) (500 µL) was added directly to the differentiated cells to give a final concentration of 0.5 % (w/v)
(v) Heat treated LPS alone: The heat treated LPS (500 µL) at 200 ng/mL was added directly the differentiated cells to give a concentration of 100 ng/mL LPS.
(vi) Heat treated honey samples + LPS: Heat treated honey samples 2% (w/v) (500 µL) was mixed with 500 µL of 400 ng/mL LPS to give a concentration of 1 % heat treated honey

samples and 200 ng/mL of LPS. These mixed samples were added to differentiated cells to get a final concentration of 0.5 % heat treated honey samples and 100 ng/mL LPS.

The plates were incubated for 4 h in a 37 °C incubator with humidity control and CO_2 at 5 % in air. After incubation, the plates were centrifuged at 130 x *g* for 5 min and supernatant were collected and stored at –80 ºC until the ELISA (Section 2.8.1) was carried out to test for TNF-α production.

2.7.4 Effect of combined treatments (heat and PmB) of honey samples with and without LPS

The combined treated honey samples were tested for their ability to stimulate TNF-α production from differentiated THP-1 cells with and without added LPS. The samples are first heat treated (Section 2.4.3.2) and then cooled down to room temperature and further diluted using complete RPMI medium to result in a 4 % (w/v) and 2 % (w/v) concentration and then treated with PmB (Section 2.4.3.3).

Samples and controls

(i) Cells alone: Complete RPMI medium (500 µL) was added to the differentiated cells
(ii) LPS alone: The LPS (500 µL) at 200 ng/mL was added directly to the differentiated cells to give a final concentration of 100 ng/mL of LPS concentration.
(iii) Heat treated honey samples alone: Heat treated honey samples 1% (w/v) (500 µL) was directly added to the differentiated cells to give a final concentration of 0.5 % (w/v)
(iv) Heat treated LPS alone: The heat treated LPS (500 µL) at 200 ng/mL were added directly the differentiated cells to give a final concentration of 100 ng/mL of LPS.
(v) Heat treated honey samples + LPS: Heat treated honey samples 2% (w/v) (500 µL) was mixed with 500 µL of 400 ng/mL LPS giving a concentration of 1 % heat treated honey samples and 200 ng/mL of LPS. These mixed samples were added to differentiated cells to get a final concentration of 0.5 % heat treated honey samples and 100 ng/mL of LPS.
(vi) Cells + PmB: 500 µL of differentiated cells are treated with 500 µL of PmB (20 µg/mL) to give a final concentration of PmB at 10 µg/mL.

(vii) Honey samples alone: The natural honey samples (500 µL) at 1% (w/v) were added directly to the differentiated cells to give a final concentration of 0.5 % (w/v) of honey samples.

(viii) PmB treated LPS: The LPS (500 µL) at 400 ng/mL was added with 500 µL of PmB (20 µg/mL) at room temperature was added together immediately to the differentiated cells to give a final concentration of 100 ng/mL of LPS concentration and 10 µg/mL of PmB.

(ix) PmB + heat treated honey samples alone: 500 µL of PmB (20 µg/mL) was mixed at room temperature with heat treated honey samples (1 % (w/v)) and 500 µL of this mixed sample was added immediately to the differentiated cells to give a final concentration of 10 µg/mL PmB and 0.5 % (w/v) heat treated honey samples.

(x) PmB + heat treated honey samples + LPS: 500 µL of PmB (80 µg/mL) was mixed at room temperature with heat treated honey samples 4 % (w/v) to get a concentration of 40 µg/mL of PmB and 2 % of heat treated honey sample. This mixed sample was treated with 500 µL of 400 ng/mL LPS to give a concentration of 1 % heat treated honey sample, 20 µg/mL PmB and 200 ng/mL LPS. These mixed samples were added to 500 µL of differentiated cells to give a final concentration of 0.5 % heat treated honey sample, 10 µg/mL PmB and 100 ng/mL LPS.

The plates were incubated for 4 h in a 37 °C incubator with humidity control and CO_2 at 5 % in air. After incubation, the plates were centrifuged at 130 x *g* for 5 min and supernatant were collected and stored at –80 ºC until the ELISA (Section 2.8.1) was carried out to test for TNF-α production.

2.7.5 Effect of irradiated honey samples with and without LPS

The various irradiation doses for the honey were achieved via multiple passes through gamma irradiator (Dave Harris, Intervet Schering-Plough Animal Health and Wellington). Gamma irradiation is known as a ‘cold process’ as the temperature of the processed product does not increase by any significant amount during irradiation. Irradiation is achieved by exposure of goods to highly penetrating gamma waves from an ionizing radiation source. The radiation source used at this facility was Cobalt 60. Irradiation achieves sterilization by breaking down bacteria DNA and preventing cell division. The product is exposed to radiation in a specially designed, heavily shielded concrete room known as the cell. Products are moved through the cell automated carriers for controlled durations to achieve specific

doses. The standard irradiation dose is 25-32 kilo Gray (kGy), but higher doses are achieved via multiple passes at 25kGy. One sample Kanuka blend was supplied from Comvita New Zealand Ltd after the gamma irradiation process. The sample was tested for its immunostimulatory activity.

Sample and controls

(i) Cells alone: Complete RPMI medium (500 µL) was added to the differentiated cells
(ii) Irradiated honey sample alone: The irradiated honey sample at different doses (500 µL) at 1% (w/v) was added directly to the differentiated cells to give a final concentration of 0.5 % (w/v) of irradiated honey sample with different doses.
(iii) LPS alone: The LPS (500 µL) at 200 ng/mL were added directly to the differentiated cells to give a final concentration of 100 ng/mL of LPS concentration.
(iv) Irradiated honey + LPS: Irradiated honey sample (500 µL) of 2% (w/v) irradiated at different doses (RH0- 0 kGy, RH26-26 kGy, RH54-54 kGy and RH87-87 kGy) and LPS (500 µL) of 400 ng/mL in complete RPMI culture medium were mixed together to give a concentration of 1 % irradiated at different doses and 200 ng/mL of LPS. These mixed samples are added to the differentiated cells to give a final concentration of 0.5 % irradiated honey sample doses and 100 ng/mL LPS.

The plates were incubated for 4 h in a 37 °C incubator with humidity control and CO_2 at 5 % in air. After incubation, the plates were centrifuged at 130 x *g* for 5 min and supernatant were collected and stored at –80 ºC until the ELISA (Section 2.8.1) was carried out to test for TNF-α production.

2.7.6 Effect of size fractioned honey samples alone or with added LPS

The size fractioned honey samples (section 2.4.3.1) with and without added LPS were tested for their ability to stimulate TNF-α production from differentiated THP-1 cells. The 4 % (w/v) of retentate and filtrate honey samples are further diluted using complete RPMI medium giving 2 % (w/v) and 1 % (w/v) concentrations of both retentate and filtrate samples. The filtrate and retentate samples were tested with and without added LPS. Natural honey samples and LPS are prepared as described above (Section 2.7.1).

Samples and Controls

(i).Cells alone: Complete RPMI medium (500 µL) was added to the differentiated cells
(ii) Honey samples alone: Natural honey samples (500 µL) at 1% (w/v) were added directly to the differentiated cells to give a final concentration of 0.5 % (w/v) of honey samples.
(iii) LPS alone: LPS (500 µL) at 200 ng/mL was added directly to the differentiated cells to give a final concentration of 100 ng/mL LPS.
(iv) Size fractioned honey samples alone: The retentate and filtrate samples 1% (w/v) (500 µL) were added directly to differentiated cells giving a final concentration of 0.5 % (w/v).
(v) Size fractioned honey samples with LPS: The retentate and filtrate samples 2% (w/v) (500 µL) were mixed with 500 µL of 400 ng/mL LPS giving a concentration of 1 % retentate and filtrate honey samples and 200 ng/mL LPS. These mixed samples were added to differentiated cells to give a final concentration of 0.5 % for both retentate and filtrate honey samples and 100 ng/mL LPS.

The plates were incubated for 4 h in a 37°C incubator with humidity control and CO_2 at 5% in air. After incubation, the plates were centrifuged at 130 x *g* for 5 min and supernatant were collected and stored at –80 ºC until the ELISA (Section 2.8.1) was carried to test for TNF-α production.

2.8 Analytical methods

2.8.1 ELISA for the measurement of TNF-α production

Capture antibody (20 µL) was diluted in 10 mL of coating buffer and 100 µL of capture antibody solution was added to the ELISA plate wells. Plates were wrapped in glad wrap and incubated over night for 18 h in a darkened 4°C humidity chamber. After incubation plates were washed 5 times with 1x PBS-T, using a plate washer (ELx 50-Auto strip washer). Each well was blocked to minimize non-specific binding by the addition of 1 x PBS-10% FBS solution (100 µL) and incubated for 1 h in a darkened humidity chamber at room temperature. The TNF-α standards were prepared by dilution in 1 x PBS containing 1% (w/v) BSA. (Table2). Human TNF-α were used at 1000 -7.825 pg/mL in duplicate and a standard curve were calculated for each ELISA plate. After blocking, the plate was washed 5 times with 1x PBS-T using the plate washer. Then 100 µL of honey supernatants (natural or

treated) from the THP-1 cells added to the wells with and without LPS. TNF –α standards and controls were added to the wells in duplicate and incubated for 2 h at room temperature in a darkened humidity chamber. After 2 h incubation the plates were further washed 5 times with 1 x PBS-T solution with the plate washer.

Working Detector (20 µL detection antibody and 10 µL of HRP streptavidin) was diluted and added (100 µL) to each well, and plates were incubated for 1 h at room temperature. The plates were washed again 7 times with 1 x PBS-T solution using the plate washer. TMB substrate was prepared by mixing Substrate Reagent A and B in equal volumes and added (100 µL) to each well and plates were incubated at room temperature for 30 min in the dark until colour formation. Then 50 µL of stop solution (2M H_2SO_4) (50 µL) was added to each well. The absorbance of the samples was measured immediately using a multiscan plate reader (Medica Pacifica Ltd,) at 450 nm with a reference wavelength of 620 nm. Microsoft Excel was used to determine the concentration of TNF-α in the supernatant samples using the standard curve from the known TNF-α concentration.

2.8.2 Limulus Amebocyte Lysate (LAL) assay

An outer cell wall glycolipid component of gram negative bacteria, (LPS) or endotoxin is recognized by the innate immune system (Tobias and Ulevitch, 1999). The LAL assay is extremely sensitive and detects even low levels of endotoxins in biotechnological substances (Guimaraes *et al*., 2006). There are two types of techniques to test endotoxin levels, one is gel-clot method which is based on gel formation and the other one is a photometric method. For this study ESR used the gel-clot method to determine the endotoxin levels in honey samples. The honey samples were diluted using LAL reagent water and the LAL kinetic assay was performed in a 96 well plate with predetermined conditions of reaction temperature (37 ºC ±1 ºC). The assay method was followed according to United States Pharmacopeia (USP 32) Biological Endotoxins Test (1985). In the gel-clot method, the reaction of the endpoint was determined from dilutions of the samples under test in direct comparison with parallel dilutions of a reference endotoxin. The endotoxin levels are expressed in USP Endotoxin Units (USP-EU).

2.8.3 Effect on TNF-α production by arabinogalactan (AG)

Arabinogalactan with and without PmB was tested for its ability to stimulate TNF-α production from differentiated THP-1 cells. The sample was prepared using complete RPMI equivalent to the amount of AG present in a 2 % (w/v) and 1 % (w/v) honey concentration. Thus when % AG is described it relates to the amount present in that percentage of honey. Natural honey samples and LPS were prepared as described in section 2.7.1. One gram of honey contains 0.08 mg of AG (I. Sims, IRL, and Lower Hutt).

Samples and controls

(i) Cells alone: Complete RPMI medium (500 µL) was added to the differentiated cells.
(ii) Honey samples alone: Natural honey samples (500 µL) at 1% (w/v) were added directly to the differentiated cells to give a final concentration of 0.5 % (w/v) of honey samples.
(iii) LPS alone: LPS (500 µL) at 200ng/mL was added directly to the differentiated cells to give a final concentration of 100 ng/mL LPS.
(iv) AH alone: The AH (500 µL) at 1 % (w/v) was added directly to the differentiated cells to give a final concentration of 0.5 % AH concentration.
(v) AG alone: The AG (500 µL) at 1 % (w/v) of honey equivalent amount present (8 µg) was added directly to the differentiated cells to give a final concentration of 0.5 % of AG equivalent to 0.5% honey concentration.
(vi) AH and AG: AH solution 2% equivalent to a honey solution (500 uL) was added to 500 uL of 2% honey equivalent AG (16 µg) to give a concentration of 1% honey equivalent of each sample. Then 500 µL of this solution was added on to the cells which simulating a 0.5% AH solution containing native levels of AG.
(vii) Honey + AG: 500 µL of 2% honey sample was added to 2% AG to give a final concentration of 1% of AG and 1% honey sample. Then 500 µL of this solution was added on to the cells to get a final concentration of 0.5% AG which stimulates a 1% honey solution containing native levels of AG and 0. 5% honey sample.
(viii) PmB treated LPS: The LPS (500 µL) at 400 ng/mL was added to 500 µL of PmB (20 µg/mL) at room temperature and the mixture was immediately added to the differentiated cells to give a final concentration of 100 ng/mL LPS and 10 µg/mL PmB.

(ix) PmB treated AG: 500 µL of 2 % AG was added to 500 µL of 40 µg/mL PmB to obtain a final concentration of 1 % AG which is equal to 1% honey solution containing native levels of AG plus PmB (20 µg/mL).

The plates were incubated for 4 h in a 37°C incubator with humidity control and CO_2 at 5% in air. After incubation, the plates were centrifuged at 130 x *g* for 5 min and supernatant were collected and stored at –80 ºC until the ELISA (Section 2.8.1) was carried to test for TNF-α production.

2.8.4 Carbohydrate analysis

The methods used in this analysis were conducted by Dr Ian Sims from Industrial Research Institute (IRL) Lower Hutt, who kindly allowed me to reproduce them here.

2.8.4.1 Centrifugal concentration

Each honey sample was diluted with distilled water to 50 mL and high molecular weight components separated from the low molecular weight constituents using Vivaspin 15R centrifugal 10,000 molecular weight cut-off ultrafilters. The diluted honey samples were transferred into 15 mL aliquot filters and centrifuged (3000 x *g*, 20 °C) until all of the honey had been filtered. The retentates were then diluted with milliQ water (15 mL) and washed by repeating the centrifugation step five times. The resulting concentrates were freeze-dried and weighed.

2.8.4.2 Monosaccharide analysis

Retained monosaccharides, present in the high molecular weight (HMW) fractions, were analyzed by high-performance anion-exchange chromatography (HPAEC). Samples (10.0 g) dissolved in distilled water (0.5 mg/ mL) were separated on a CarboPac PA-100 (4 x 250 mm) column equilibrated in 150 mM NaOH, eluted with a gradient of sodium acetate and monitored by pulsed amperometric detection. Monosaccharide content (glucose and fructose) was estimated using a standard curve of glucose (0 – 6 µg).

2.8.4.3 Constituent sugar analysis

Neutral constituent sugar compositions of the HMW fractions were determined by gas chromatography-mass spectrometry (GC-MS) of alditol acetate derivatives after hydrolysis of the polysaccharides present to their component monosaccharides. Samples (0.25 mg) were derivatised by reductive hydrolysis using 4-methylmorpholine borane and 3 M TFA followed by acetylation (Stevenson and Furneaux, 1991). Each sample was analyzed in duplicate. The alditol acetate derivatives produced were separated by GC on an Agilent HP-5MS fused silica capillary column and detected by MS using a Hewlett Packard 5973 MSD. Identifications were based on peak retention times and by comparison of electron impact mass spectra with standard spectra. Weight calibration constants were determined from a seven sugar standard mix (derivatised at the same time as the samples) following the TAPPI standard method T 249 cm-85 (1985). Myo-inositol was used as an internal standard. Monosaccharide yields were expressed as weight percent anhydro-sugar because this is the form of sugar present in a polysaccharide.

2.8.4.4 Glycosyl linkage analysis

Glycosyl linkage compositions were determined by GC-MS of partially methylated alditol acetate derivatives. Each sample was analyzed in duplicate. High molecular weight honey fractions (0.25 mg) were methylated using NaOH and CH_3I in DMSO (Ciucanu and Kerek, 1984) as described by McConville *et al*. (1990). The methylated polysaccharides were hydrolyzed with 2.5 M TFA (200 µL) for 1 h at 121°C, concentrated to dryness under an air stream at 40 °C, and then reduced with 1.0 M $NaBD_4$ in 2.0 M NH_4OH (200 µL) overnight at 25 °C. The reaction was stopped by the addition of glacial acetic acid (50 µL). Borate was removed as volatile trimethylborate by addition of 5 % (v/v) acetic acid in MeOH (3 x 0.5 mL), and concentrated under an air stream at 40 °C, followed by addition of MeOH (3 x 0.5 ml) and concentrated to dryness under an air stream at 40 °C. The resulting alditols were acetylated in acetic anhydride (100 µL) and TFA (100 µL) for 30 min at 50 °C and extracted into CH_2Cl_2 for analysis. The partially methylated alditol acetate derivatives were separated by GC on an Agilent HP-5MS fused silica capillary column and analyzed by MS using a Hewlett Packard 5973 MSD. Identifications were based on peak retention times and by comparison of electron impact mass spectra with standard spectra. Linkage compositions were expressed as mole percent of the total linkages detected.

2.8.4.5 Proton nuclear magnetic resonance (NMR) spectroscopy

The HMW fractions were dissolved in D_2O (0.6 mL) and transferred to 5 mm NMR tubes. NMR spectra were recorded on a Bruker 500 MHz spectrometer at 30 °C.

2.8.4.6 Size-exclusion chromatography

The size-exclusion (SEC) system consisted of a Waters 2690 Alliance separations module, a Waters 450 variable wavelength detector set at 280 nm and a Waters 2410 refractive index monitor. Samples (1 mg/mL) were centrifuged (14.1 x g for 5 min) before injection (100 µL) and eluted with 0.1 M $LiNO_3$ containing 0.02 % NaN_3 (0.7 mL/ min) from two columns (TSK-Gel G5000PWXL and G4000PWXL, 300 x 7.8 mm, Tosoh Corp., Tokyo, Japan) connected in series. Molecular weights were estimated by comparison of the peak elution volumes with those of pullulan standards.

2.9 Data Analysis

Data were presented in Microsoft Excel Spreadsheet as means of two replications of two independent experiments. The graph packages used Graph Pad (Prism) software and excel. The results were analyzed using one way ANOVA in Microsoft Office 2007 and multiple comparisons were carried out using Turkey's intervals (Plant and Food Research Institute, 2010).

Chapter 3

The stimulation of tumor necrosis factor alpha (TNF-α) production by active honey

3.0 Introduction

Tumor necrosis factor alpha (TNF-α) is a regulatory cytokine which plays an important role in the defence against intracellular organisms (Ciacci-Woolwine *et al*., 1999) and induces fibroblast collagen synthesis to initiate healing by activated macrophages. Macrophages are immediately activated in response to foreign substances from invading microorganisms (Van den Berg *et al*., 2008). In inflammation, macrophages have three major functions: antigen presentation; phagocytosis; and immunomodulation through production of various growth factors and cytokines. They play a critical role in the initiation, maintenance, and resolution of inflammation (Fujiwara and Kobayashi, 2005). The aim of this study was to investigate the ability of honey to stimulate TNF-α production by a human monocytes cell line differentiated to behave like a macrophage.

This chapter presents results of honey samples (Table 2.1) screened for cytotoxicity, the optimization of protocols for an ELISA of human TNF-α and the establishment of the immunostimulatory effects of five honey samples at non-toxic concentrations.

3.1 Cytotoxic activity of honey samples

3.1.1 Introduction

Fructose and glucose are the major components of honey. However, honey also contains methylglyoxal (MGO), hydrogen peroxide (H_2O_2) and low concentrations of several other chemical compounds (Gheldof *et al*., 2002) thought to function as anti-oxidants, including pinocembrin chrysin, pinobanksin, and vitamin C (Herken *et al*., 2009), all of which may be detrimental to cells. Some mechanisms of cell damage include osmotic stress from the high sugar concentration (Molan, 1998), induction of free radical cytotoxicity by high glucose concentrations (Faure *et al*., 2005), bactericidal concentrations of MGO (>0.6 mM)

(Ferguson *et al*., 1998), and H_2O_2-induced oxidative stress leading to cell death (Zhang *et al*., 2008).

In this study, because an *in vitro* cell-based culture system was used to measure the immunostimulatory effects of honey samples, it was necessary to determine the highest honey concentration that could be used while avoiding these potentially detrimental effects.

3.1.2 Method

The WST-1 cytotoxicity assay was used to measure the cytotoxicity levels of all natural honey samples (Table 2.1, chapter 2) and an artificial honey solution (Section 2.4.2). The method was followed as described in section 2.5.4. The negative control contained THP-1 cells with complete RPMI medium alone, and the positive control was camptothecin used at different concentrations (10 µM, 1 µM and 0.1 µM) to kill the cells. In this assay, there can be a plate to plate variation; to avoid this effect the same dilutions of artificial honey were included with the natural honeys in each plate for standardization purposes.

3.1.3 Results and Discussion

The results show the effect of negative and positive controls (Figure 3.1) and the 16 honey samples (Figure 3.2) on cells after 1 h following the addition of the WST-1 reagent. All data were converted to percentage cell survival relative to negative control (100%).

Figure 3.1 (a) shows the results of the positive control camptothecin demonstrating that 10 µM was more toxic than 0.1 µM. Figure 3.1 (b) shows the cytotoxic effects of the artificial honey from 2 % to 0.0078125 % (w/v) serial dilutions. The data indicates that artificial honey is toxic at 2 %. The non-toxic concentrations (100 % growth) were between 1 % and 0.0078125 % (w/v). The values and error bars particularly for 1 % artificial honey lie within that of the 0.0156 % which is not considered toxic (statistical data was not shown).

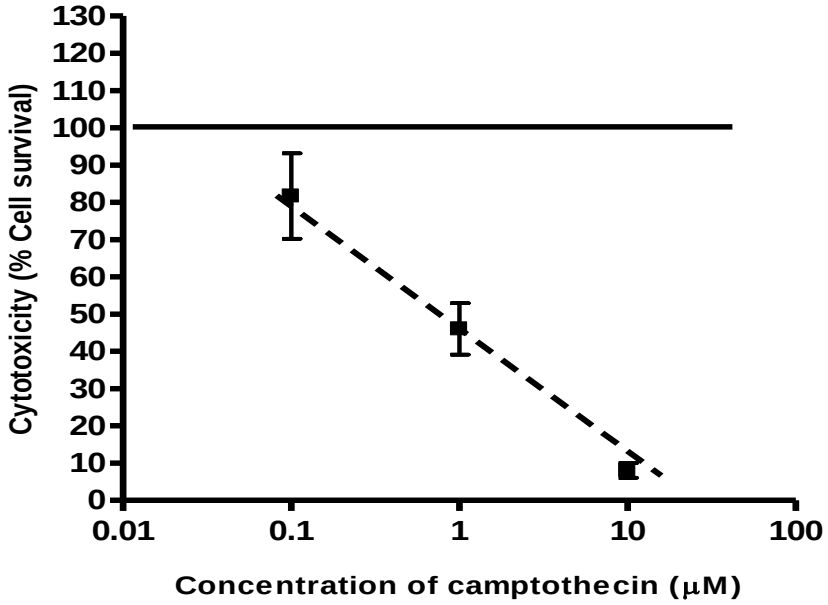

Figure 3.1 (a) Effect of camptothecin on cell viability. THP-1 cells were treated for 24 h with camptothecin doses and incubated at 37°C in a CO_2 incubator. The negative control is shown by the continuous line at 100% cell viability. **Notes:** Data represents the mean of three replicates. Error bars represents the standard deviation.

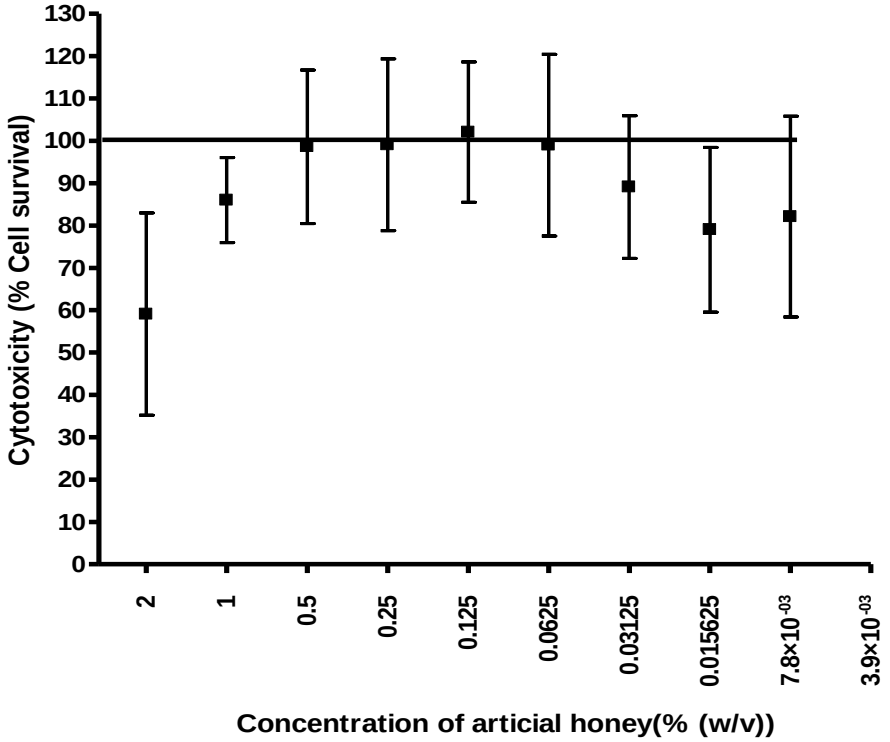

Figure 3.1(b) Effect of artificial honey on cell viability. THP-1 cells were treated for 24 h with artificial honey concentrations (2% (w/v) to 0.0078125 % (w/v) and incubated at 37°C in a CO_2 incubator. The negative control

is shown by the continuous line at 100% cell viability. **Notes:** Data represents the mean of three replicates. Error bars represents the standard deviation.

Figure 3.2 shows the cytotoxicity effects of all honey samples (standardised for plate-to-plate variation). The data were standardized as follows: Plate-to-plate variation was accounted for by standardizing all results against the average artificial honey response from all plates. This was carried out by calculating the conversion factor required to adjust each individual artificial value to the mean value (Equation 3).

$$\text{Conversion factor} = \frac{\text{Calculated mean value}}{\text{Measured absorbance value}} \quad (3)$$

The artificial honey values were used because artificial honey was common to all plates. Each individual sample value was multiplied by the artificial honey conversion factor to give a standardized response. These data show that the highest honey concentrations tested (2 and 1%, w/v) reduced cell metabolic activity by as much as 40% to give 60% cell survival. It was concluded that for some honeys 0.5% (w/v) concentration was the highest non-toxic dose while for others 0.25% concentration was the highest non-toxic dose. This was confirmed by re-testing the honeys at 0.5% (w/v), the highest non-toxic concentration (Figure 3.3). This experiment was carried out using a single plate to avoid plate-to-plate variation. This plate-to-plate variation was thought to be attributable to factors such as loading time and position within the incubator.

The camptothecin positive control, honey samples and artificial honey were tested against the cells only negative control (100%). There was a significant difference between the negative control and the positive control. Whereas there was no significant difference between the honey samples (0.5% , w/v), the non-toxic artificial honey control at 0.5 % (w/v) compared with negative control (100%).

In recent studies, Tonks *et al*. (2001) and Timm *et al*. (2008) used 1% (w/v) honey to study the immunomodulatory effects of honey because they observed that 1 % honey was not toxic to MM6 cells, HL-60 cells and human peripheral monocytic cells. In this study, a THP-1 cell line was used, and for these cells 0.5 % (w/v) honey concentration was not toxic. Therefore, in all subsequent experiments in this study the highest concentration of honey used was 0.5 % (w/v).

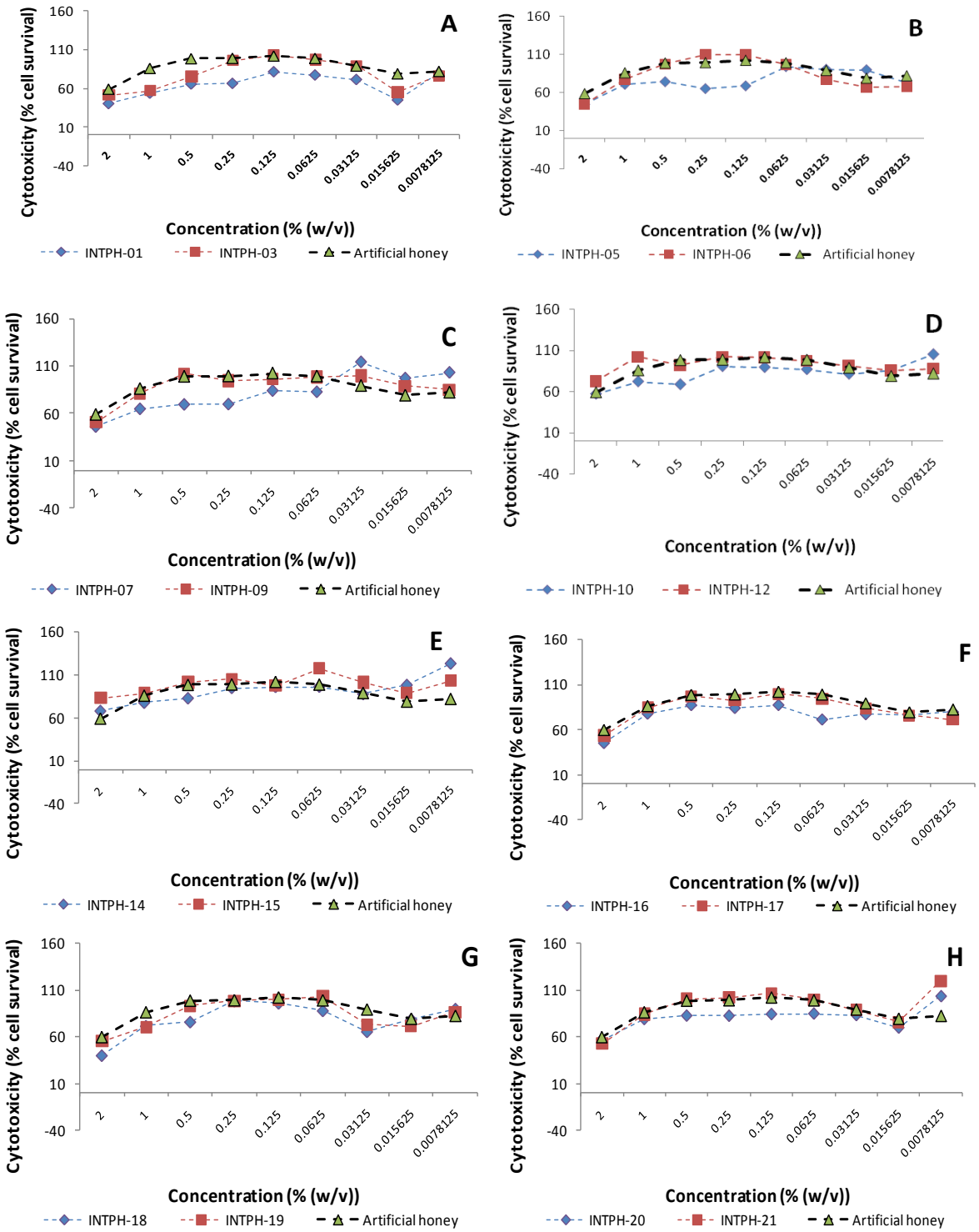

Figure 3.2 Cytotoxicity effects of 16 honey samples on THP-1 cells. A to H contains artificial honey and 16 honey samples (INTPH-01 to INTPH-21), two per graph. Data expressed as cell survival relative to negative control (100%, not shown). **Notes:** Data mean of two replicates. Error bars represents standard deviation.

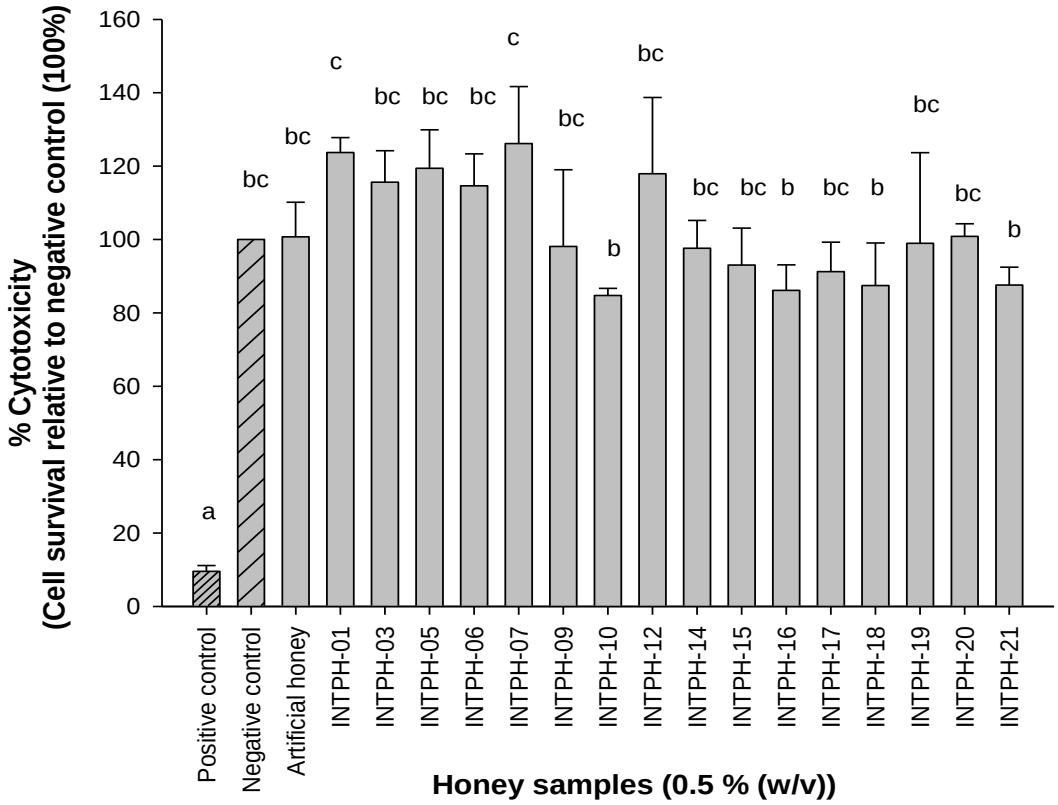

Figure 3.3 Cytoxicity effects of 16 honey samples (0.5% (w/v)) expressed relative to negative control (100%) and positive control (10 µM camptothecin). Data represents the mean of three replicates. Error bars represent standard deviation. **Notes:** Data with the same letter are not significantly different (P <0.05); statistical data are shown in appendices.

3.2 Optimization of TNF-α ELISA

3.2.1 Introduction

As immunostimulation activity was to be measured by stimulation of production of TNF-α from THP-1 cells, it was necessary to optimise the method for determining the concentration of TNF-α produced, which was an ELISA. The assay was optimized for the concentration of capture and detection antibodies to be used.

3.2.2 Method

The capture antibody was prepared by diluting capture antibody in 10 mL of coating buffer at 1, 2, 4, 6 and 8 µg/mL. After mixing, 100 µL was added to each well of an ELISA high

binding plate (BD Biosciences) that was then wrapped in glad wrap and left overnight (18 h) in a dark humidity chamber at 4°C. TNF-α at 500 pg/mL and 15.625 pg/mL were added followed by different concentrations of detection antibody at 0.25, 0.5, 1, 2 and 3 µg/mL and an equal volume of HRP enzyme (Streptavidin – HRP) added as described in section 2.6.3. The range of dilutions used for the capture and detection antibodies when optimizing this assay was followed according to manufacturer's instruction.

3.2.3 Results and Discussion

The results from varying capture and detection antibody concentrations using TNF-α at either 500 pg/mL or 15.6 pg/mL concentration are shown in Figure 3.4.

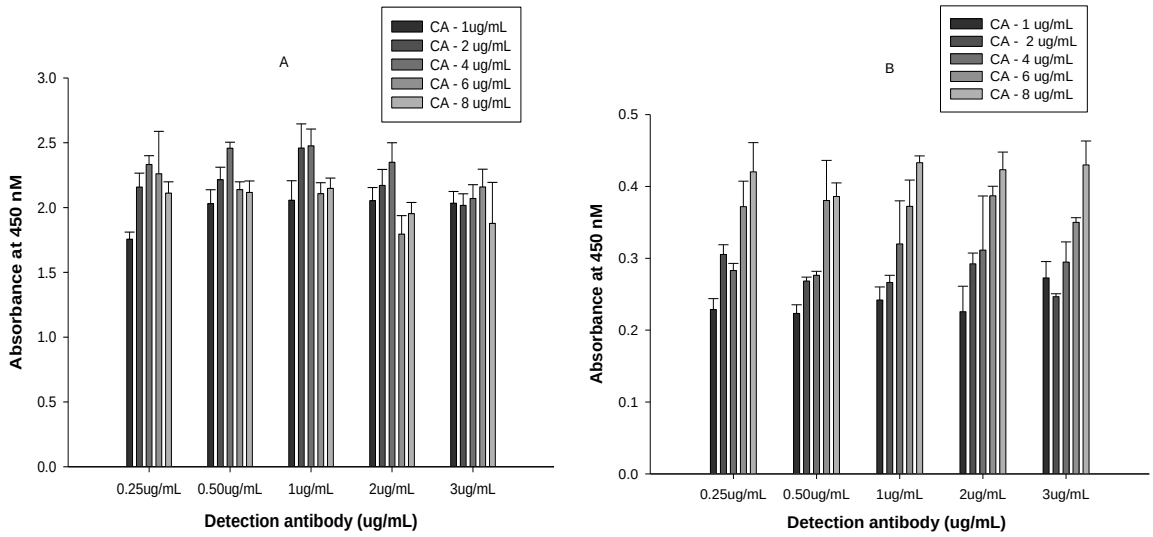

Figure 3.4 Optimising the tumor necrosis factor (TNF)-α ELISA by varying capture and detection antibodies using high (500 pg/mL)(A) and low (15.625 pg/mL)(B) concentrations of tumor necrosis factor (TNF)-α standard. **Notes:** Data represent the mean of three replicates. Error bars represent standard deviation.

When either 500 pg/mL or 15.625 pg/mL TNF-α was used, the optimum combination of capture antibody was 4 µg/mL with 0.5 µg/mL and 1 µg/mL detection antibody. Therefore, 4 µg/mL of capture antibody and 1 µg/mL of detection antibody were used in all further experiments as these were optimal concentrations for the respective experiments and were recommended by the manufacturer. One of the reasons why this experiment was carried out was because sometimes due to environmental conditions the kit does not work. Then the standard curve of TNF-α concentration from 500 pg/mL to 7.825 pg/mL was determined

using 4 µg/mL capture antibody and 1 µg/mL of detection antibody to ensure that a linear dose-response could be detected.

The results shown in Figure 3.5 demonstrate that a linear dose-response could be generated using TNF-α standards and the assay was ready to detect cytokine production by THP-1 cells upon stimulation by honey samples and controls.

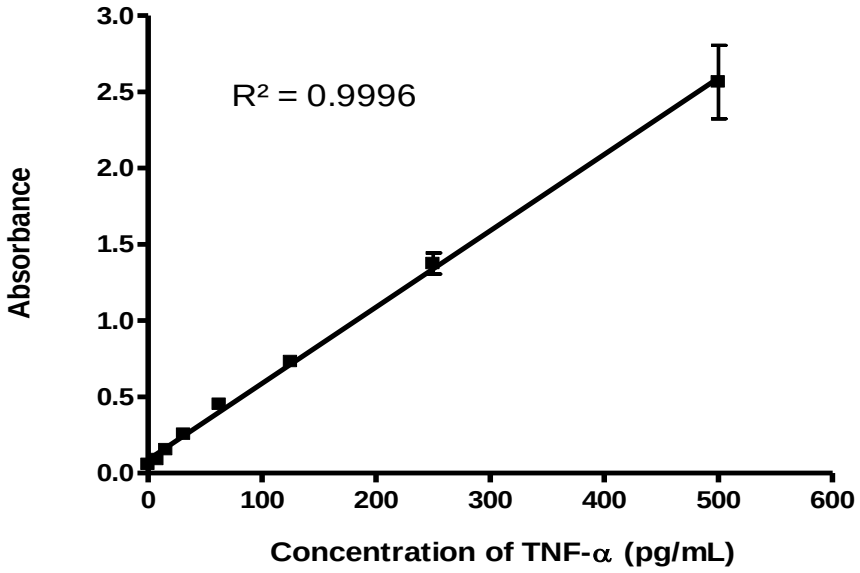

Figure 3.5 The standard curve of tumor necrosis factor (TNF)-α. **Notes:** Data represent the mean of two replicates. Linear curve ($R^2 = 0.9996$)

3.3 Optimization of TNF- α cytokine production from THP-1 cells

One of the aims of this work was to measure TNF-α production from THP-1 cells when they are exposed New Zealand natural honey samples. THP-1 cells were chosen for this study because they are easy to handle and cultivate large numbers of cells, as well as avoid the variability encountered between donors with primary cells (Bocchietto *et al*., 2007; Oliveira *et al*., 2001). It was therefore necessary to determine conditions for optimal TNF-α production by THP-1 cells and also to determine the ideal LPS dose to be used to stimulate TNF-α production. Undifferentiated THP-1 cells produce little TNF-α, but differentiated cells produce significant quantities of TNF-α (Cheng *et al*., 2005; Rodiles *et al*., 1996). Differentiation of the THP-1 cells into macrophage-like cells can be induced by exposure of the cells to a concentration of 10 nM of PMA for three days (Rodiles *et al*., 1996). The PMA-

differentiated THP-1 cells become adherent, stop proliferating, and produce reactive oxygen species (ROS) and cytokines. The method to differentiate THP-1 cells is illustrated in Figure 3.6.

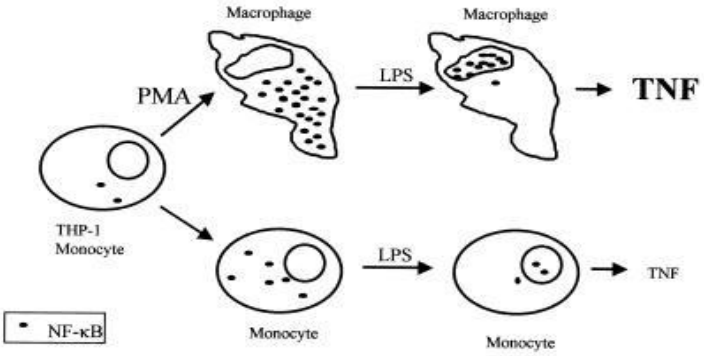

Figure 3.6 Effects of differentiating THP-1 cells with phorbol 12-myristate 13-acetate (PMA) on magnitude of lipopolysaccharide-stimulated tumor necrosis factor (TNF)-α production (from (Takashiba *et al*., 1999) reproduced without permission).

3.3.1 Differentiation of THP-1 cells with PMA

3.3.1.1 Introduction

12-O-Tetradecanoylphorbol-13-acetate (TPA), also commonly known as phorbol 12-myristate 13-acetate (PMA), is a diester of phorbol and a potent tumor promoter often employed in biomedical research to activate the signal of transduction enzyme protein kinase C(PKC). The structure of PMA shown in Figure 3.7.

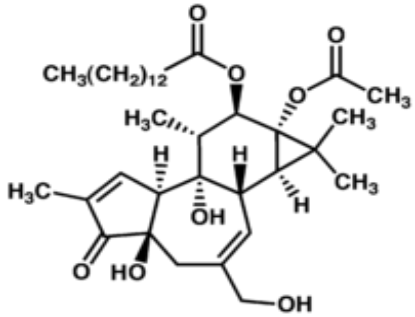

Figure 3.7 Structure of phorbol 12 myristate 13-acetate. www.lclabs.com/PRODFILE/P-R/P-8880.php4

During infection, circulating blood monocytes migrate from the vasculature to the extravascular compartments where they mature into tissue macrophages. The maturation process prepares the cell to actively participate in the inflammatory and the immune responses (Takashiba *et. al*., 1999). The accumulation of NFkB complexes in the cytoplasm

of differentiated macrophages and their translocation to the nucleus is responsible for the enhanced ability of the cell to respond to LPS stimulation, as determined by TNF-α secretion.

3.3.1.2 Method

The cells were differentiated in the presence of 10 nM or 50 nM PMA for up to 3 days as described in section 2.6.1. A final concentration of 1 x 10^6 cells/mL was incubated with PMA for 3 days at 37°C with humidity control and CO_2 maintained at 5%. After incubation the cells were centrifuged at 130 x *g* for 5 min and then washed with fresh complete RPMI medium, re-centrifuged at 130 x *g* for 5 min to remove the PMA, and then fresh complete RPMI medium (500 µL) was added. The THP-1 cells are round and floating before differentiation whereas when differentiated they are attached to the wells and changed shape. They were viewed under phase a contrast microscope (Olympus CKX4., Japan) using 40 X magnifications and their image (Coolpix 995, Nikon, Japan) was taken.

3.3.1.3 Results and Discussion

The THP-1 cells were differentiated after 3 days exposure to PMA as shown in Figure 3.8. Undifferentiated cells were unattached and rounded (A) whereas differentiated the cells adhered and changed shape (B). THP-1 cells differentiate into macrophages after PMA-treatment (Lai et al., 2006; Tsuchiya et al., 1982) and the results from this study are in agreement with these reports. THP-1 cells can be differentiated using PMA and adhere to the bottom of the plate with morphological characteristics of macrophages (Takashiba et al., 1999).

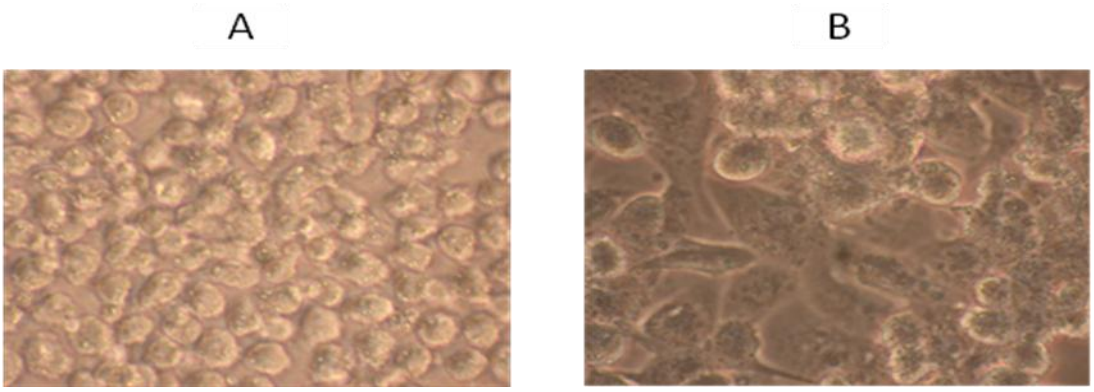

Figure 3.8 THP-1 cells before (A) and after (B) differentiation with 10 nM phorbol 12-myristate 13-acetate (PMA) (40 X magnification)

3.3.2 Response of differentiated THP-1 cells to LPS

3.3.2.1 Introduction

Lipopolysaccharide (LPS), an endotoxin, is a compound in which a lipid molecule (Lipid A) is bound to a polysaccharide (O-antigen) by a covalent bond, with a structure as shown in Figure 3.9. Andre Boivin first purified endotoxin in 1932 by using a trichloroacetic acid (TCA)-based method. Later, Walter T. J. Morgan and Walther F. Goebel used organic solvents and water to purify endotoxin. Both groups found endotoxin to be composed of lipid and polysaccharide with very little if any associated protein (Raetz *et. al*., 2002).

Lipopolysaccharide is commonly found in outer cell wall membranes of certain microorganisms, particularly the gram negative bacteria, many of which are human and plant pathogens (Naik et al., 2001; Raetz *et al*., 2002; Lis and Zhang, 2009). It is a highly immunogenic compound that stimulates the production of cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6) and TNF-α by macrophages (Li *et al*., 2003). In macrophages the lipid A portion of LPS activates TLR4 receptors (Naik *et al*., 2001) and CD14 (Tapping *et al*., 1999) and stimulates the production of cytokines that are the mediators of inflammation and also activates the expression of co-stimulatory molecules that are required for the adaptive immune response.

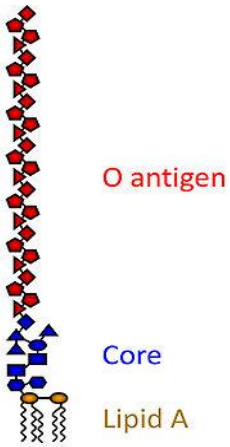

Figure 3.9 Structure of lipopolysaccharide (LPS)

3.3.2.2 Method

THP-1 cells were differentiated either with 10 or 50 nM PMA and then incubated with 50 - 500 ng/mL LPS at 37^{o}C for 4h (Section 2.6.1 & 2.6.2) and TNF-α production was determined using the ELISA (Section 2.8.1).

3.3.2.3 Results and Discussion

The data (Figure 3.10) demonstrated that both concentrations of PMA used induced differentiation as shown by their ability to produce TNF-α, but the higher concentration of PMA (50 nM) also induced a cytokine response in the absence of LPS. To avoid this potentially high background result, the lower concentration of PMA (10 nM) was used for subsequent THP-1 culture differentiation.

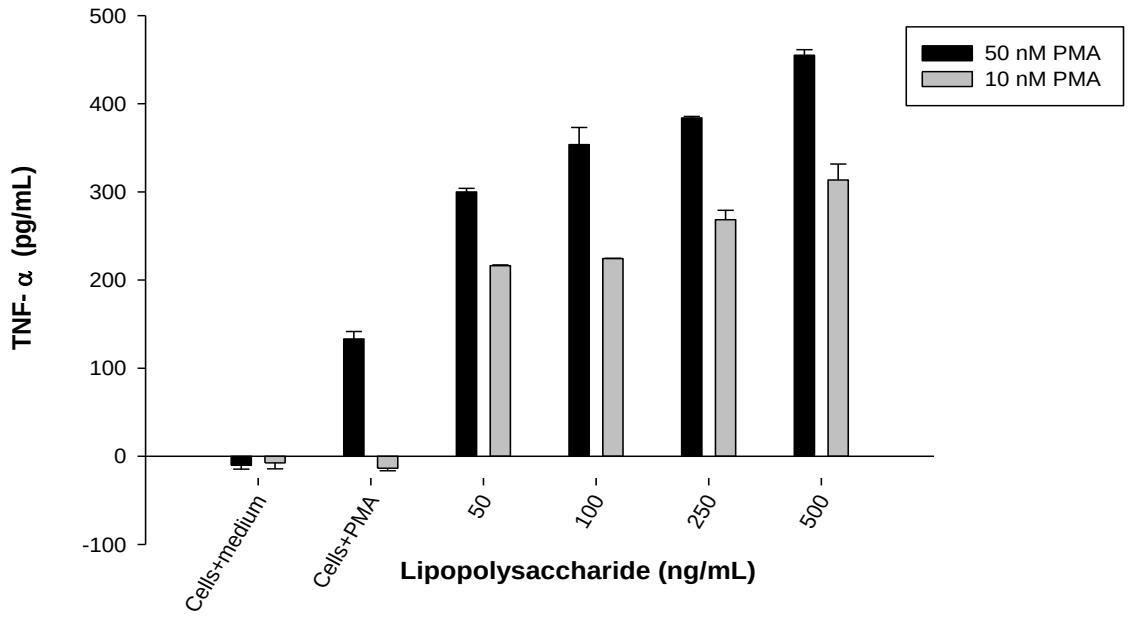

Figure 3.10 Tumor necrosis factor (TNF)-α production by THP-1 cells in response to lipopolysaccharide dose (50 -500 ng/mL) after differentiation by 3 days exposure to 10 nM or 50 nM phorbol 12-myristate 13-acetate (PMA). **Notes:** Data represents the mean of two replicates. Error bar represents the standard deviation.

As honey is reported to contain low concentration of LPS (Tonks *et al.*, 2007) the effect of lower doses of LPS on TNF-α production by differentiated THP-1 was investigated. The results are shown in Figure (3.11).

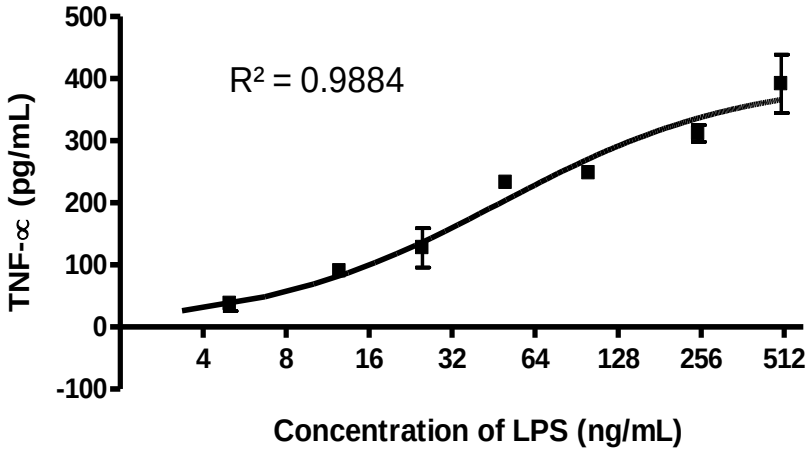

Figure 3.11 Tumor necrosis factor (TNF)-α production by THP-1 cells in response to varying concentrations of lipopolysaccharide (LPS) after differentiation by 3 days exposure to 10 nM phorbol 12-myristate 13-acetate (PMA). **Notes:** Data represents the mean of two replicates. Error bar represents the standard deviation

It is evident from the results that the differentiated THP-1 cells will respond to a dose of LPS as low as 5 ng/mL. Varying the concentration of LPS resulted in a dose-dependent increase in TNF-α production (R^2 value = 0.9884). Takashiba *et al*. (1999) suggested that the peak levels of TNF-α production in LPS stimulation from differentiated cells were higher than the levels secreted from undifferentiated cells.

Overall, these results confirm that 10 nM PMA with 3 days incubation could be used in the subsequent experiments, and LPS at 100 ng/mL was chosen as a suitable single concentration for subsequent work. The dose of LPS that is used to stimulate TNF-α production depends on cell type and culture conditions (Tonks *et al*., 2003). A dose of 100 ng/mL LPS was chosen because it gave a good TNF-α response which fell within the middle of the TNF-α standard curve of the ELISA.

3.4 Effect of honeys on TNF-α production by THP-1 cells

3.4.1 Introduction

Honey is a known antimicrobial agent, which has been used since ancient times as a medicine for the treatment of wounds and burns. Recently, the medicinal use of honey has re-emerged,

due to clinical studies indicating that honey accelerates wound healing activity (Efem, 1988). While the antimicrobial activity of honey is well studied, and has been shown to be due to its osmolarity, pH, hydrogen peroxide (Molan, 2005; Cooper *et al*., 1999; Willix *et al*., 1992) and MGO (Mavric *et al*., 2008), examination of other therapeutic effects remain incomplete. Honey reduces inflammation and scarring. However, the mechanism of wound healing stimulated by honey and the compounds in the honey that are involved in this process are both still unclear. One of the cytokines involved in the relevant immune response is TNF-α. Honey has been shown to stimulate TNF-α cytokine production from monocytes and macrophages and further examination suggested that the TNF-α production did not correlate with LPS levels in honey (Tonks *et al*., 2007). However (Timm *et al*., 2008) speculated that the TNF-α production stimulated by honey was due to endotoxin content. The main objective of the work described in this section was to investigate the ability of the Comvita honey samples to elicit a TNF-α cytokine response from THP-1 cells so that the component responsible for inducing TNF-α production could be investigated.

3.4.2 Method

The THP-1 cells were isolated, purified and cultured as described in sections 2.5.1 to 2.5.3 and differentiated using PMA as described in section 2.6.1. The following five honey samples (INTPH-01, INTPH-03, INTPH-15, INTPH-16 and INTPH-20) were selected for the study based on MGO concentration, age, floral and geographical origin (Tables 2.1 and 2.2). One of the honey samples (INTPH-20) was supplemented with MGO (500 mg/kg) as this was a non-Manuka honey, and had no detectable MGO. The honey samples and controls were prepared according to Section 2.7.1 and incubated with THP-1 cells for 4 h at 37°C in a 5% CO_2 humidified atmosphere with and without LPS. After incubation, the cells were centrifuged at 130 x *g* and supernatants were collected and stored at -80°C until assayed for TNF-α production by ELISA (Section 2.8.1.). The whole assay was repeated on a separate occasion to confirm the reproducibility of the results.

3.4.3 Results and Discussion

The data shown in Figure 3.12 (a) suggest that in the absence of LPS all five honey samples have pro-inflammatory activity. The old (INTPH-16) and new (INTPH-15) Kanuka honeys

stimulated a high level of TNF- α production at 250 and 350 pg/mL respectively. The next level of TNF-α production was stimulated by Clover honey (INTPH-20), while the lowest level of TNF- α production was stimulated by the old Manuka honey (INTPH-03) in the absence of LPS. Neither the artificial honey nor the MGO had any effect when LPS was absent. In the presence of LPS (Figure 3.12 (b)), the honey samples as well as the artificial honey, the MGO and the INTPH-20 honey sample supplemented with MGO had no effect on LPS stimulated TNF- α production. The TNF-α cytokine production was measured after 4 h incubation at 35°C because cytokine production reaches maximum levels between 4 and 6 h; thereafter, it starts decreasing rapidly to undetectable levels after 12 h (Tonks *et al*., 2003; Rodiles *et al*., 1996). The results suggested that all the natural honey samples tested have pro-inflammatory activity and also indicates that the stimulation effect is not due to the high sugar concentration or the presence of MGO in any of the honeys.

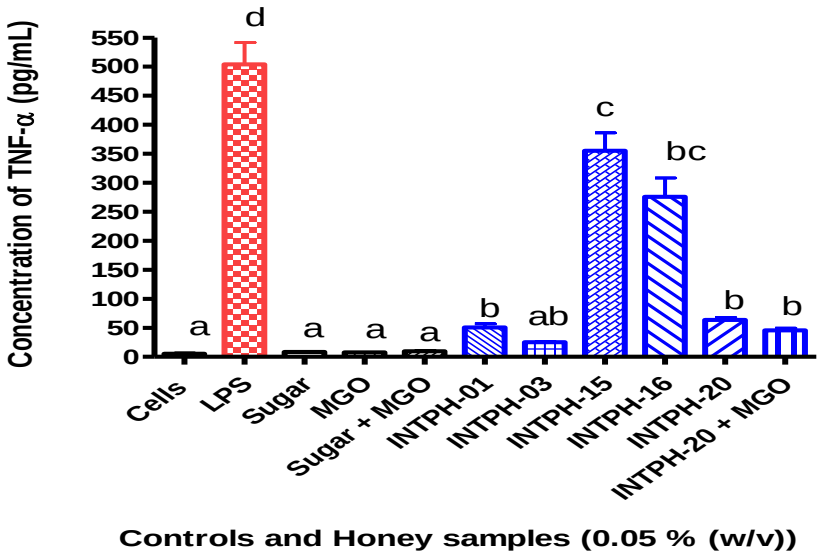

Figure 3.12(a) Tumor necrosis factor (TNF)-α production from differentiated THP-1 cells stimulated by different honey samples (■), lipopolysaccharide (■) and controls (■). **Notes:** Data represents the mean of two replicates. Error bars represent the standard deviation. There is a significant difference between the samples ($p<0.05$) that have different letters. There is no significant difference between the samples that have same letter. Statistical data are shown in appendices.

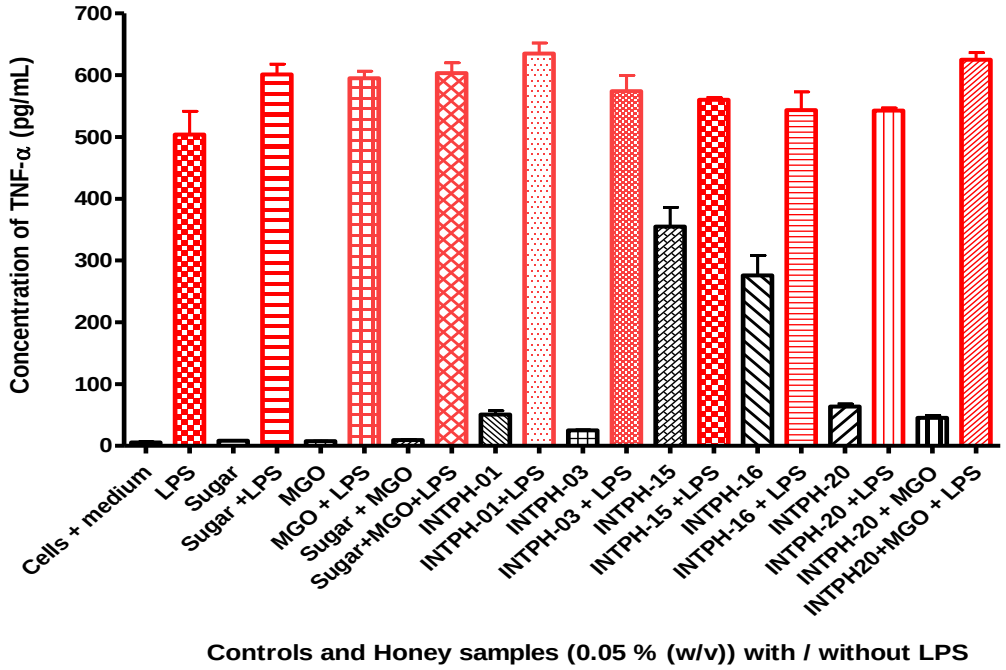

Figure 3.12(b) Tumor necrosis factor **(**TNF)-α production from differentiated THP-1 cells stimulated by different honey samples in the absence (■) and presence of lipopolysaccharide (■). Data mean of two replicates. Error bars represent the standard deviation.

3.5 Conclusion

In summary five honey samples after 4 hour incubation significantly stimulated the release of TNF-α cytokine from differentiated THP-1 cells when compared with untreated, artificial-honey or MGO treated cells. Kanuka honey (INTPH-15) significantly induced the maximal release of TNF-α cytokine compared with Manuka or Clover honeys (Figure 3.12 (a)).

This study confirms that natural honey samples modulate the production of the pro-inflammatory cytokine TNF-α as reported by Timm *et al*. (2008) and Tonks *et al*. (2003). The production of TNF-α from THP-1 cells strongly suggests the presence of an immunomodulatory substance in honey. It is known that endotoxin (LPS) is a most potent immunomodulatory substance and it has been claimed that it is the LPS in honey that is stimulating monocytes and macrophages (Timm *et al*., 2008). However, glycoprotein in honey may have various biological effects, whereas deproteinized honey has no stimulatory activity on the release of TNF-α (Majtan *et al*., 2006). In the next chapter honey samples will be further tested to establish the component responsible for this immunostimulation.

Chapter 4

Characterization of active component(s) in honey that stimulate tumor necrosis factor (TNF)-α production

4.0 Introduction

Although the immunostimulatory effect of natural honeys have been reported previously in this thesis, it is still not clear which component or components may be responsible for this activity. Therefore, the possibility that honey-stimulated TNF-α production may be initiated by the presence of microorganisms or LPS as contaminants in honey was investigated. Natural honeys contain substantial amounts of endotoxin and the work of Timm *et al*. (2008) demonstrated that the cytokine response to honey in a cell- based assay system using human peripheral monocytes was characteristic of a response induced by LPS alone. However, the work of Tonks *et al*. (2007) indicated that the cytokine response to natural honeys in human peripheral blood monocytes was different to the cytokine response to LPS and did not correlate with LPS levels in the honeys.

Most bacteria do not grow in honey due to its acidic nature, high sugar content and low water activity (Olaitan *et al*., 2007; Henriques *et al*., 2006). Even so, a number of studies have reported bacterial and fungal contamination of honey (Snowdon, 1996; Nakono et al., 1991; Sackelt, 1919) suggesting that the contaminated honey may act as a potential source of infection. In support of this, Tonks and co-workers (2007) reported in their study that no vegetative bacteria were cultured but bacterial spores were isolated and results correlated well with Snowdon, (1996) on bacterial contamination. Isolation of bacterial spores raises the possibility that honeys may be contaminated with bacteria components like endotoxin and the biological activity of endotoxin is associated with the LPS.

To eliminate LPS as an agent responsible for stimulating TNF-α production, it is normal to assess the effect of heat treatment or inhibition of responses by PmB (Timm *et al*., 2008; Tonks *et al*., 2007; Tsan and Gao, 2004).

The aims of the experiments described in this chapter were to determine the component or components responsible for inducing immunostimulation via a pro-inflammatory cytokine response. The honeys were treated in two ways, either with PmB or with heat (80°C), to determine whether the immunostimulation effect produced by the honeys was decreased by blocking LPS stimulation, or by heat treatment. Then an irradiated honey sample were investigated to determine whether irradiation of honey effects immunostimulatory activity and also to determine whether the ability of honey to stimulate the production of TNF-α is not due to live microorganisms. The molecular weight range of the active component(s) was then determined by size fractionation.

4.1 Effect of pre-treatment with PmB on activity of honey samples

4.1.1Introduction

Polymixin B (PmB) is a cyclic cationic antibiotic that acts as an LPS antagonist (Shimomura *et al*., 2003). It binds to the lipid A-ketodeoxy-octonate portion of LPS and neutralizes the biological effects of LPS *in vitro (*Bannatyne *et al.*, 1977, Hideyuki *et al.*, 2001*)* and its toxic effects in *vivo* (Cooperstock and Riegle., 1981).

LPSs are complex amphiphilic molecules with a molecular weight of about 10 kDa, (Todar, 2008). The cell wall antigens (O antigens) of gram-negative bacteria are components of LPS. LPS elicits a variety of inflammatory responses (Losa Garcia *et al*., 1999). The immunogenicity of LPS is associated with the polysaccharide components, while toxicity is associated with the Lipid A component.

The objective of the work described in this section was to investigate the ability of PmB to interfere with elicitation of a cytokine response stimulated by honey, thus illustrating the role played by LPS and/or LPS-like compounds. For this work (PmB treatment), two honey samples, Manuka (INTPH-01), and Kanuka (INTPH-15) were chosen. Manuka honey stimulated low levels of TNF-α production whereas Kanuka stimulated the highest levels of TNF- α cytokine production from the THP1 cells.

4.1.2 Method

TNF- α production was measured as described earlier (Section 2.7.1) after differentiation of cells (Section 2.6.1) using both Manuka and Kanuka honey samples at final concentration of 0.5 % (w/v). PmB (10 µg/mL) was used to pre-treat the honeys and neutralize any LPS-like stimulation. Positive and negative controls were also treated with PmB. The samples and controls prepared as previously described (Section 2.7.2) were incubated with THP-1 cells for 4 h at 37°C in a 5% CO_2 humidified atmosphere with and without LPS. After incubation, the cells were centrifuged at 130 x *g* and supernatants were collected and stored at -80°C until assayed for TNF-α production by ELISA (Section 2.8.1.). The whole assay was repeated on a separate occasion to confirm the reproducibility of the results.

4.1.3 Results and Discussion

The data in Figure 4.1 show that when Kanuka honey (INTPH-15) was pre-treated with PmB, its ability to stimulate TNF-α production from THP-1 cells was significantly ($p<0.05$) reduced compared to the untreated honey sample, while Manuka honey (INTPH-01) completely lost its ability to stimulate TNF-α production. When the positive control (LPS) (100 ng/mL) was pre-treated with PmB (10 µg/mL) (Tonks *et al*., 2007; Timm *et al*., 2008), it lost its ability to stimulate TNF-α production from THP-1 cells when compared to the untreated LPS, demonstrating that the dose of PmB used was effective for a dose of 100 ng/mL of LPS.

These data demonstrated that the PmB-treated Kanuka honey sample lost 80% of its immunostimulatory activity and the PmB-treated Manuka honey completely lost its immunostimulatory activity. This suggests that the source of the activity may be LPS. This result is supported by the observation that the addition of PmB completely abrogated the ability of LPS to stimulate TNF-α production. This somewhat agrees with the work of Timm et al. (2008) who demonstrated that natural honeys contain substantial amount of endotoxin (amount not specified) and the response detected in their cell-based assays were similar to the responses induced by endotoxin alone. However, unlike the Manuka results, immunostimulatory activity remaining in the Kanuka honey (INTPH-15) was significantly

different to the negative control after treatment with PmB. This suggests the activity may be due to other components present in the honey.

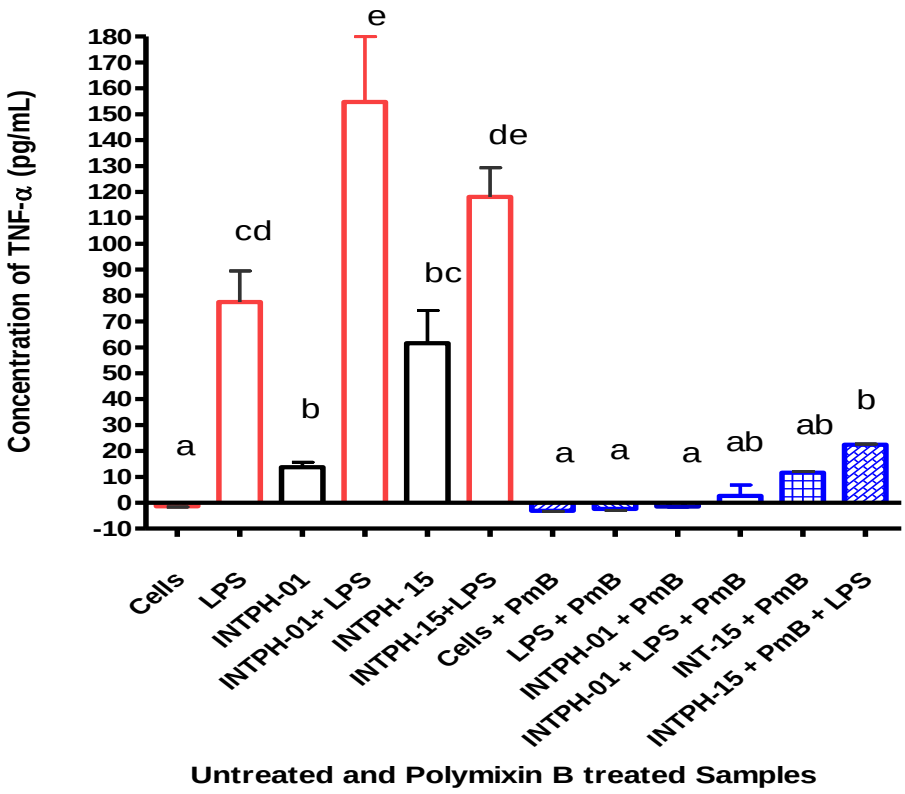

Figure 4.1 Tumor necrosis factor **(**TNF)-α production from differentiated THP-1 cells after treatment with 100 ng/mL of lipopolysaccharide (□) and honey samples (0.5 % (w/v)) with (■) and without pre-treatment with 10 µg/mL of polymixinB (□). Notes: Data represent the mean of two replicates. Error bars represent the standard deviation. There is a significant difference between the samples ($p<0.05$) that have different letters. There is no significant difference between the samples that have the same letter; Statistical data are shown in appendices.

Interestingly when the honey in combination with LPS was pre-treated with PmB, the immunostimulatory activity remained in Kanuka and Manuka is significantly ($P<0.05$) different to the negative control suggesting that there were components other than LPS responsible for the remaining immunostimulatory activity. Future work should be carried out to understand the mechanism responsible for the observation that Manuka honey alone is completely inhibited and LPS alone is completely inhibited but Manuka with the addition of LPS is not completely inhibited.

Another interesting observation was that when LPS (100 ng/mL) was added to the Manuka honey the TNF-α produced was more than an additive effect. With Manuka honey alone the TNF-α produced was only approximately 15 pg/mL and with LPS alone it was 80 pg/mL. This was increased to more than 150 pg/mL instead of an additive amount of 95 pg/mL by

the addition of LPS to Manuka honey, suggesting that Manuka honey contains some other component(s) that synergize with LPS to generate this immunostimulatory activity. When the same amount of LPS (100 ng/mL) was added to the Kanuka honey there was an additive effect suggesting that the Kanuka honey does not contain synergistic components or components that inhibit LPS induced TNF-α production.

4.2 Effect of heat treatment on activity of honey samples

4.2.1 Introduction

LPS is heat stable (Tsuzuki *et al*., 2001; Timm *et al*., 2008; Todar, 2008) due to its ubiquitous presence and potent pyrogenic effect, but in a contradictory study the immunostimulatory activity was shown to be reduced by heat treatment (Gao et al., 2006). The protein component of LPS has been shown to be heat labile (Todar, 2008). It is important to determine whether the honey component(s) that are responsible for the immunostimulatory effect is heat stable or labile, as this will further indicate whether the immunostimulatory activity of honey is due to LPS or not . Therefore the next aim of the study was to determine whether the heat treated honeys were able to elicit a cytokine response from monocytic (THP-1) cells.

4.2.2 Method

Four honey samples, Manuka (INTPH-01), Kanuka young (INTPH-15), Kanuka old INTPH-16 and Clover (INTPH-20) were selected for study based on their ability to stimulate the lowest and highest concentrations of TNF-α from THP1 cells. Honey samples (5% (w/v)) and LPS (1000 ng/mL) were prepared in distilled water. Then the samples were heated at 80ºC for 30 min using a shaking water bath. The combination of this temperature and time was selected because the active components could be proteins and more than 60°C temperature and 30 min time is sufficient to denature most of the proteins (Fagain, 1997). After heat processing, further dilutions were carried out using complete RPMI medium. The samples and controls prepared as described earlier (Section 2.7.3) were incubated with THP-1 cells for 4 h at 37°C in a 5% CO_2 humidified atmosphere with and without LPS. After incubation, the cells were centrifuged at 130 x *g* and supernatants were collected and stored at -80°C

until assayed for TNF-α production by ELISA (Section 2.8.1.). The whole assay was repeated on a separate occasion to confirm the reproducibility of the results.

4.2.3 Results and Discussion

The results of heat treatment on the immunostimulatory effects of the four honeys (INTPH-01, INTPH-15, INTPH-16 and INTPH-20) are shown in Figure 4.2 (a). The results of heat treatment on the immunostimulatory effects of the four honeys with LPS are shown in Figure 4.2 (b). After heat-treatment, the immunostimulatory activity of all the honey samples was significantly ($p<0.05$) reduced when compared to untreated honey sample controls, indicating an immunostimulatory factor that is heat labile, possibly containing a protein structure with a peptide back bone. Abu-Jdayil *et al*. (2002) have previously reported that heat treated honeys have decreased water content, which may contribute to protein denaturation.

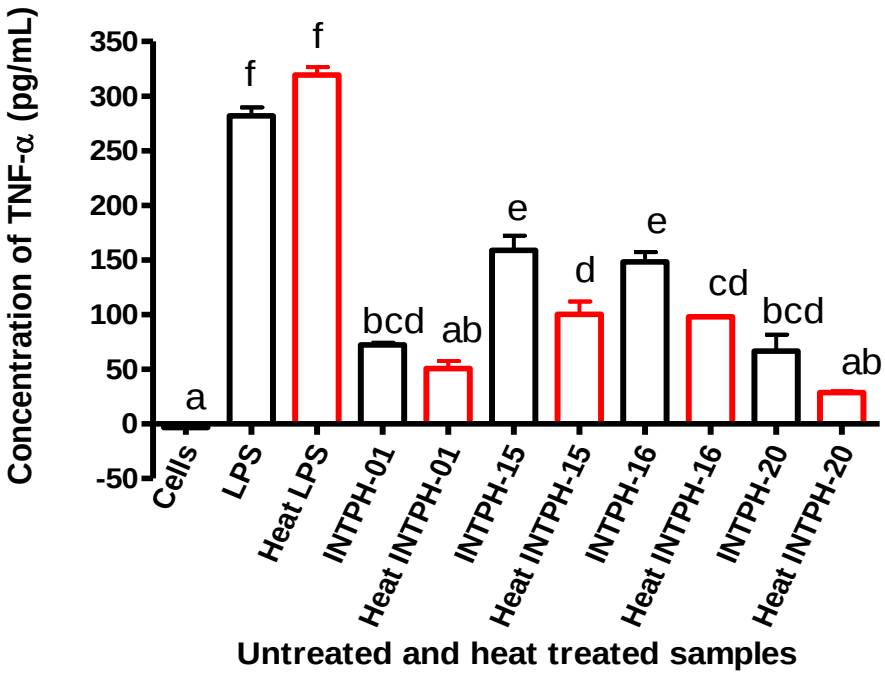

Figure 4.2(a) Tumor necrosis factor (TNF)-α production from differentiated THP-1 cells after treatment with lipopolysaccharide (100 ng/mL) and final concentration of 0.5 5(w/v) of various honeys before (□) and after heat treatment at 80°C for 30 min (□). Notes: Data mean of two replicates of two separate experiments. Error bars represent the standard deviation. There is a significant difference between the samples ($p<0.05$) that have different letters. Statistical data are shown in appendices.

These data shows that heat treatment of honey samples, Manuka (INTPH-01), Kanuka young (INTPH-15) and Kanuka old (INTPH-16) reduced their immunostimulatory activity by 30 %

whereas heat treatment of clover honey (INTPH-20) reduced its activity by 70 % compared to untreated honey sample controls. Heat treated LPS was heat stable and its activity was not reduced compared to the untreated LPS in its ability to stimulate TNF-α production. The results indicate that honey samples INTPH-01, INTPH-15 and INTPH-16 may consist of a mixture of LPS giving 70 % of the response and other active components giving 30% of the response. In contrast, the immunostimulatory ability of Clover honey (INTPH-20) may be due to a mixture of LPS giving 30% of the response and other active components giving 70% of the response. The heat instability of the non-LPS component(s) suggests that it may be a protein, a mixture containing protein(s) or have a protein or peptide contained in its structure (Abu-Jdayil *et al*., 2002). The heat stability activity of honey may be from components of bacterial or fungal origin, as these microorganisms have been previously isolated in honey (Snowdon and Cliver, 1996; Olaitan *et al*., 2007).

These results are in agreement with those reported by (Tonks *et al*., 2007) who showed that immunostimulatory activity was reduced significantly after heat treatment indicating that one or more components other than LPS may be responsible for stimulating cytokine production.

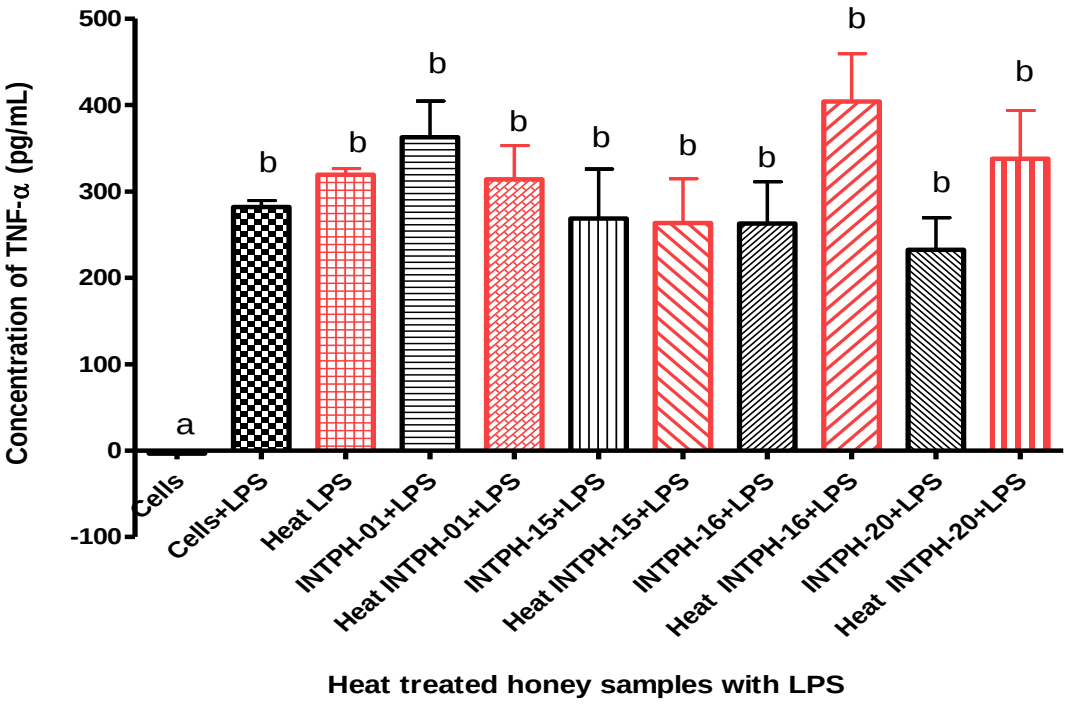

Figure 4.2(b) Tumor necrosis factor (TNF)-α production from differentiated THP-1 cells after heat treatment of honey samples with (■) and without (■) lipopolysaccharide. Notes: Data mean of two replicates of two separate experiments. Error bars represent the standard deviation. There is a no significant difference between the samples ($p<0.05$) that have the same letter. Statistical data are shown in appendices.

Data in Figure 4.2 (b) shows the addition of LPS (100 ng/mL) to all honey samples. The data suggests that there is no significant difference in the production of TNF-stimulation by heated compared with native honey samples when LPS is present.

4.3 Effect of combined treatments (heat and PmB) on activity of honey samples

4.3.1 Introduction

LPS is a heat stable component, whereas the component(s) present in the honey samples are partially heat sensitive, and most of the non-Clover honey activity was heat stable as demonstrated in the previous section. Consequently, the effect of both heat and PmB treatments were investigated together to determine whether the immunostimulatory activity remaining after heat treatment can be inhibited with PmB.

4.3.2 Method

The method was followed as described in Sections 2.4.3.2 and 2.4.3.3 but the honey samples (5% (w/v)) and LPS (1000 ng/mL) were first heat treated and diluted in complete RPMI medium then preincubated with PmB (10 µg/mL). They were then added to the differentiated cells to give a final concentration of 0.5% (w/v) of honey samples and 100 ng/mL LPS. The samples and controls prepared as described earlier (Section 2.7.4) were incubated with THP-1 cells for 4 h at 37°C in a 5% CO_2 humidified atmosphere with and without LPS. After incubation, the cells were centrifuged at 130 x *g* and supernatants were collected and stored at -80°C until assayed for TNF-α production by ELISA (Section 2.8.1.). The whole assay was repeated on a separate occasion to confirm the reproducibility of the results.

4.3.3 Results and Discussion

The results of both heat treatment of the two Kanuka honeys (INTPH-15 and INTPH-16) and PmB treatment of Kanuka young honey (INTPH-15) on the immunostimulatory effects are shown in Figure 4.3. After both treatments, the immunostimulatory activity of the two honey samples was completely inhibited compared to heat treatment alone. Similarly, the

immunostimulatory activity of heat treated LPS was completely inhibited, as was unheated LPS after PmB treatment.

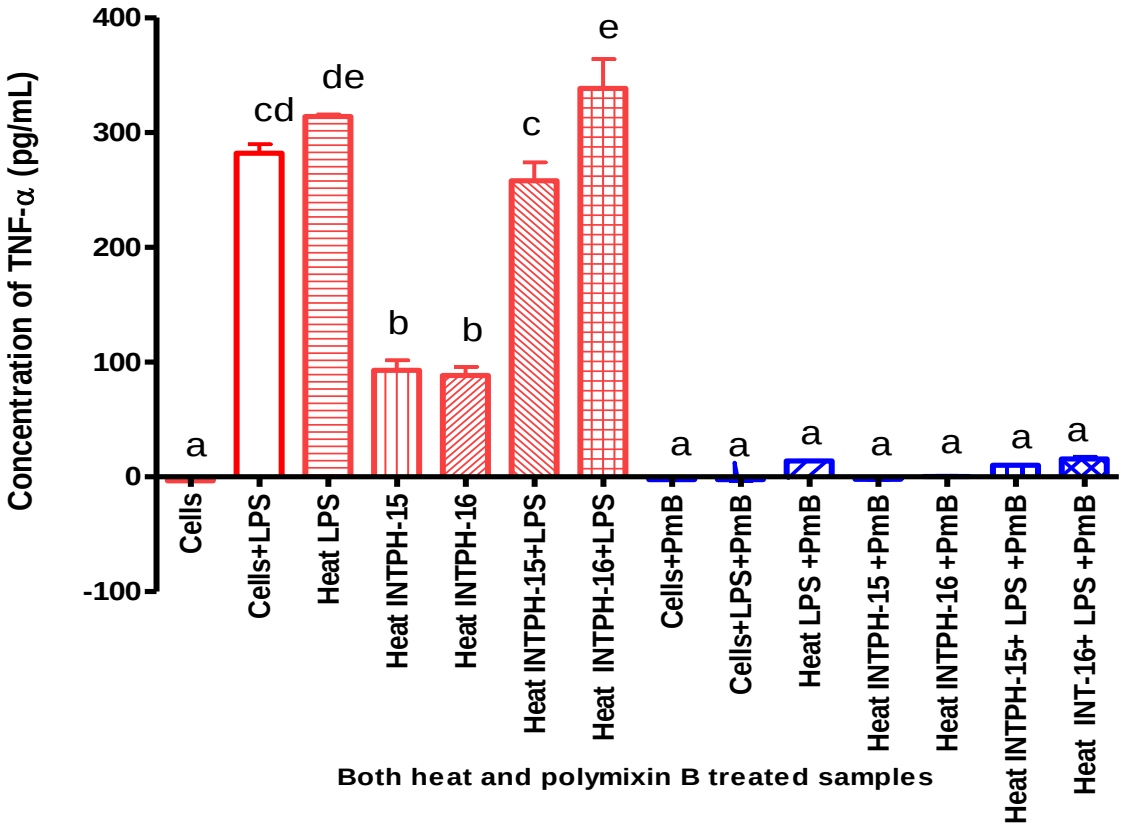

Figure.4.3 Effect of tumor necrosis factor (TNF)-α production from differentiated THP-1 cells stimulated with lipopolysaccharide (100 ng/mL) alone (□) heat treated lipopolysaccharide (100 ng/mL) and the final concentration of 0.5 % (w/v) of two heat treated honey samples before (■) and after polymixin B (PmB) treatment (■). Notes: Data represent the mean of two replicates. Error bars represent the standard deviation. There is a significant difference between the samples ($p<0.05$) that have a different letter. Statistical data are shown in appendices.

These results indicated that the immunostimulatory activity of the honey samples may be due to a mixture of both LPS and one or more other components, or alternatively, that the component(s) in the honey is partially heat sensitive but generates its effects via an LPS-like structure. The reasons for this conclusion was because most of the stimulatory activity in honey was heat stable and inhibited by PmB but still a small amount of activity was heat labile and not inhibited by PmB. However, with combined treatments the activity was lost completely.

4.4 Effect on TNF-α production of irradiated honey sample

4.4.1 Introduction

Natural unprocessed honey can be used directly on wounds (Blaser *et al*., 2007) but for clinical purposes it is submitted to gamma irradiation for sterilization because it may be contaminated with various microorganisms (Jo *et al*., 2005). Most bacteria cannot grow in honey due to its acidic nature, high sugar content and low water activity (Olaitan *et al*., 2007; Henriques *et al*., 2006). Nevertheless, a number of studies have reported the bacterial and fungal contamination of honey (Snowdon, 1996; Nakono et al., 1991; Sackett, 1919) suggesting that the contaminated honey may act as a potential source of infection. Tonks *et al*. (2007) reported that in their study no vegetative bacteria were cultured but bacterial spores were isolated and it is suggested that this correlates with bacterial contamination (Snowdon, 1996). Isolation of bacterial spores raises the possibility that honeys may be contaminated with bacteria components like LPS. The objective of this section is to investigate whether irradiation of honey effects immunostimulatory activity, thus examining whether the ability of honey to stimulate TNF-α production could be due to live microorganisms.

4.4.2 Method

The method followed is described in Section 2.7.5 but costs limited irradiation to only one honey sample; Kanuka blend was supplied from Comvita that had been sterilized by gamma irradiation. Honey and corresponding radiation doses used were RH0, 0 kGy; RH26, 26 kGy; RH54, 54 kGy and RH87, 87 kGy. The samples and controls prepared as described earlier (Section 2.7.5) were incubated with THP-1 cells for 4 h at 37°C in a 5% CO_2 humidified atmosphere with and without LPS. After incubation, the cells were centrifuged at 130 x *g* and supernatants were collected and stored at -80°C until assayed for TNF-α production by ELISA (Section 2.8.1.). The whole assay was repeated on a separate occasion to confirm the reproducibility of the results.

4.4.3 Results and Discussion

The results in Figure 4.4 (a) shows that when the Kanuka blend honey was sterilized by increasing doses of gamma irradiation, it still stimulated the production of similar levels of TNF-α from THP-1 cells as the untreated honey sample (RH0). This suggests that stimulation of TNF-α production was not due to vegetative bacteria, because even after sterilization with gamma irradiation, the stimulation of TNF-α production was neither inhibited nor reduced. There was an insignificant ($p<0.1$) decrease in immunostimulatory activity across the increasing radiation treatment of this honey, indicating that neither the LPS fraction nor an uncharacterized fraction was diminished by radiation.

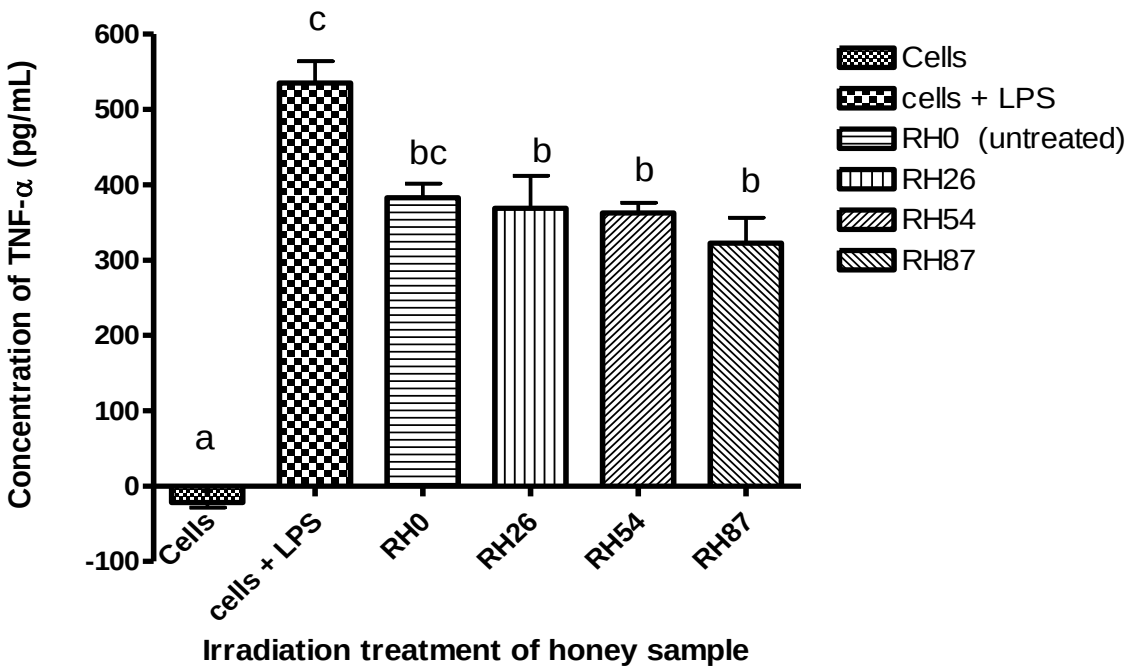

Figure 4.4 (a) Effect on tumor necrosis factor (TNF)-α production from differentiated THP-1 cells after Kanuka blend honey sample (0.5% (w/v)) was treated with increasing doses of gamma irradiation. Note: The data represents the mean concentration of tumor necrosis factor (TNF)-α production in culture supernatants of two replicates. Error bars represent the standard deviation. Treatments with the same letter are not significantly different from each other ($p<0.05$). Statistical data are shown in appendices.

The results shown in Figure 4.4 (b) indicates that the LPS (100 ng/mL) by itself stimulated the production of 550 pg/mL TNF-α. The irradiated Kanuka blend honey by itself stimulated approximately 350 pg/mL of TNF-α production after even the highest irradiation dose. Interestingly, when the same amount of LPS (100 ng/mL) was added to irradiated honey samples, the combined LPS and honey stimulated only 450 pg/mL of TNF-α production: no

additive effect was detected, suggesting that the immunostimulatory activity of this sterilized honey did not combine with added LPS, as endogenous LPS would be expected to, and the stimulatory component was therefore unlikely to be due to LPS.

The sterilization of Kanuka blend by gamma irradiation did not influence the immunostimulatory activity, clearly suggesting that the TNF-α stimulating activity in the honey was not due to vegetative microorganisms. There is no evidence in the literature that radiation can abrogate LPS activity, so the immunostimulatory activity may be due to LPS or not. To investigate these further experiments should be carried out.

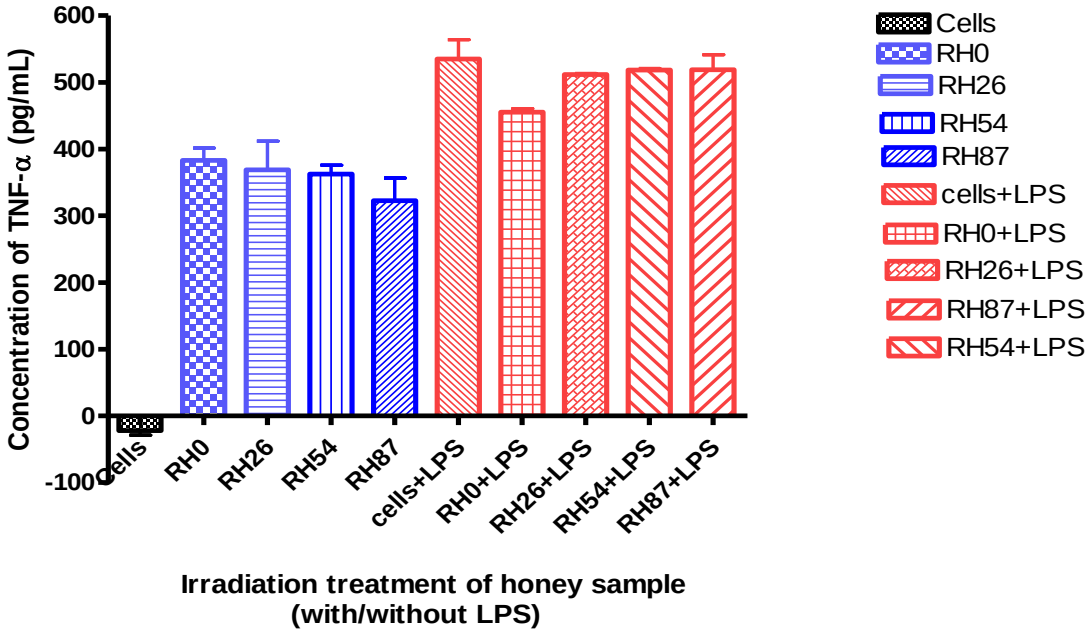

Figure 4.4(b) Effect on tumor necrosis factor (TNF)-α production from differentiated THP-1 cells of Kanuka blend honey sample treated with different doses of gamma irradiation in the absence (■) and presence (■) of lipopolysaccharide. Note: Data represent the mean concentration of tumor necrosis factor (TNF)-α production in culture supernatants of two replicates. Error bars represent the standard deviation.

4.5 Effect of fractioned honey samples

4.5.1 Introduction

Further work was carried out to determine the active component(s) responsible for the immunostimulatory effects of the honeys. Fractionation of honey was carried out to

determine the molecular weight of the active component(s) responsible for immunostimulatory activity because the above results suggested that the component(s) responsible for stimulating cytokine production may not be LPS, despite acting in an LPS-like manner. The molar mass of the LPS monomer is 10 kDa to 30 kDa (Jang *et al*., 2009), while the molecular weight of most of the enzymes is 50-1000 kDa (Todar, 2008). Therefore, a filtration process could be used to separate compounds according to molecular weight, and thus establish whether the immunostimulatory compound(s) were in the LPS-like weight range, or possess a more protein-like molecular weight.

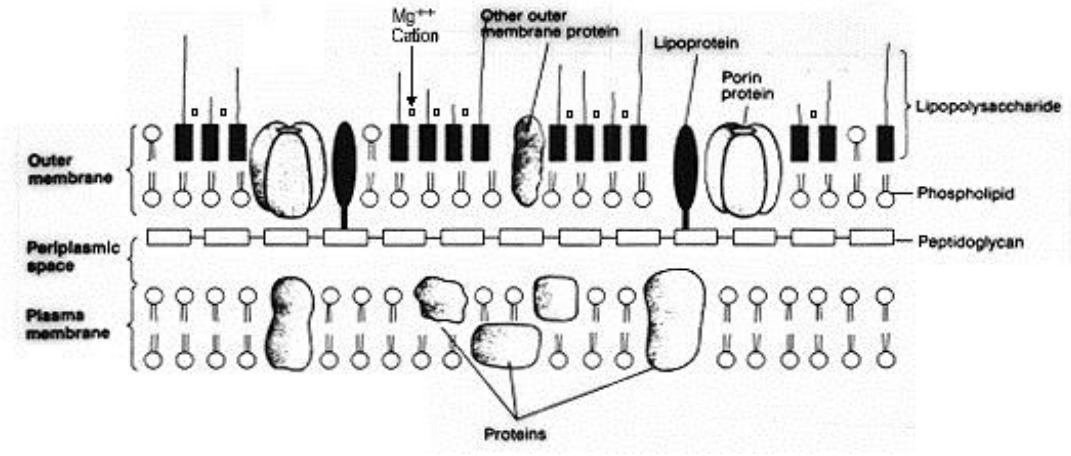

Figure 4.5 Structure of lipopolysaccharide and proteins (from Todar, 2008)

Thus, the objective of this section was to determine the molecular weight of the component that could be responsible for inducing cytokine release, by using a filtration process. Molecular weight 30 kDa and 3 kDa cut off filters were used to fractionate honey that selectively excluded less than 3 kDa, between 3 to 30 kDa and greater than 30 kDa molecules respectively.

4.5.2 Method

Two honey samples Kanuka young (INTPH-15) and Kanuka old (INTPH-16) were selected for the filtration process because they stimulated high TNF-α production, was expected that the filtration process could cause loss of material which may impact on the immunostimulatory assay. The filtration process was followed as described in Section 2.4.3.1 The samples and controls were prepared as described earlier (Section 2.7.6) and incubated with THP-1 cells for 4 h at 37°C in a 5% CO_2 humidified atmosphere with and without LPS. After incubation, the cells were centrifuged at 130 x *g* and supernatants were collected and

stored at -80°C until assayed for TNF-α production by ELISA (Section 2.8.1.). The whole assay was repeated on a separate occasion to confirm the reproducibility of the results.

4.5.3 Results and Discussion

The data shown in Figure 4.6(a) and Figure 4.6 (b) demonstrate the effect of filtered young Kanuka (INTPH-15) and old Kanuka (INTPH-16) honey samples on TNF-α production. These data indicate that the INTPH-15 and INTPH-16 honey samples activity was associated with fractions with an apparent molecular weight greater than 30 kDa. The filtration process caused a significant reduction in TNF-α cytokine production from THP-1 cells relative to unfiltered honey. There was no immunostimulatory activity associated with less than 3 kDa and between 3 kDa and 30 kDa fractions. The LPS itself stimulated TNF-α production, and both honeys stimulated TNF-α production before fractionation. Interestingly there was no activity observed in the 3 -30 kDa fraction that would be expected of the 10 - 30 kDa molecular weight LPS.

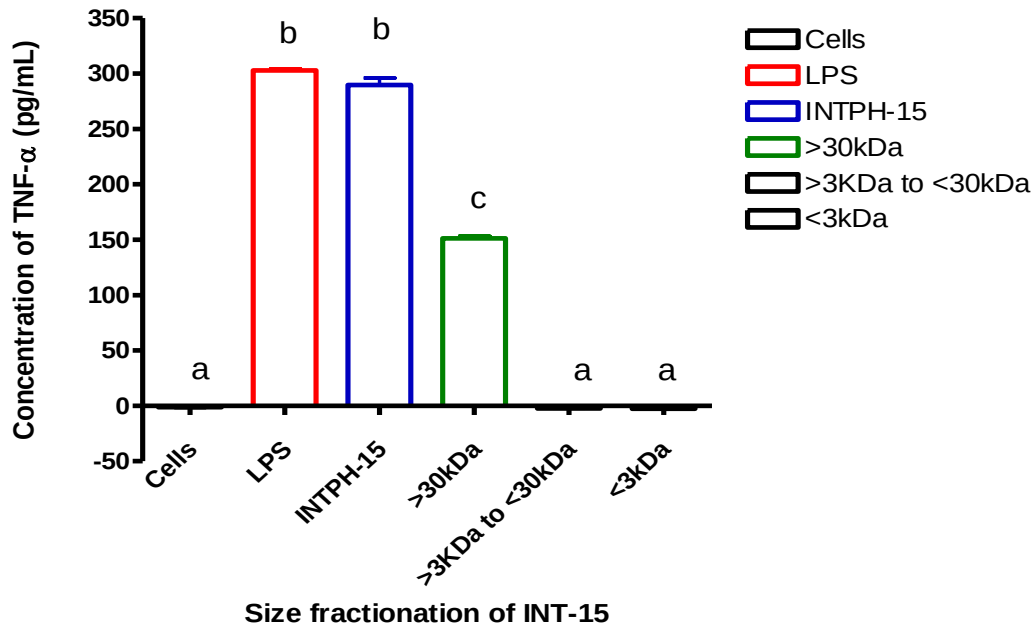

Figure 4.6 (a) Effect on tumor necrosis factor (TNF)-α production from differentiated THP-1 cells after treatment with lipopolysaccharide (100 ng/mL) alone (□), THP-1 cells alone (□) and Kanuka young honey (INTPH-15) without fractionation (□) and after the fractionation of INTPH-15 as above 30kDa (□); between 3 kDa and 30 kDa (□) and less than 3 kDa (□). Note: Data represent the mean of two replicates. Error bars represent the standard deviation. There was a significant ($p<0.05$) difference between the honey and its filtered fractions. Statistical data are shown in appendices.

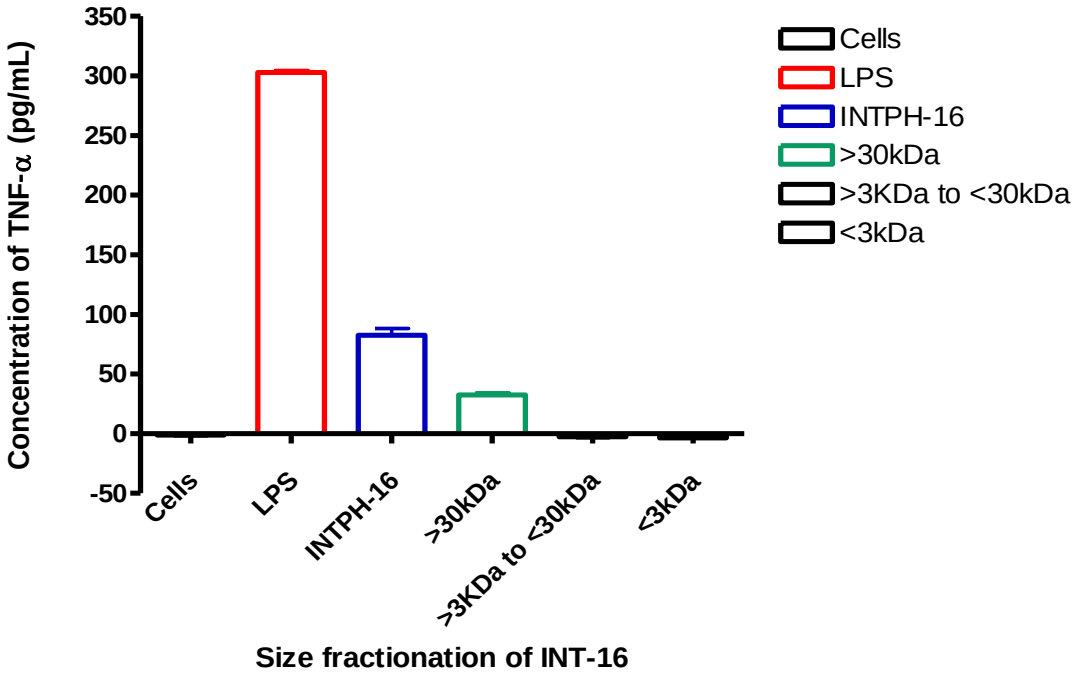

Figure 4.6 (b) Effect on tumor necrosis factor (TNF)-α production from differentiated THP-1 cells after the treatment with lipopolysaccharide (100 ng/mL) alone (□).THP-1 cells alone (□) and with Kanuka old honey (INTPH-16) without fractionation (□) and after the fractionation of INTPH-16, above 30kDa (□); between 3kDa to 30kDa (□); and less than 3kDa (□). **Note**: Data is the mean of two replicates. Error bars represent the standard deviation. There was a significant ($p<0.05$) difference between the honey and its filtered fractions

The data shown in Figure 4.6 (a1) and Figure 4.6 (b1) demonstrate the effect of filtered Kanuka young (INTPH-15) and Kanuka old (INTPH-16) honey samples with and without LPS on TNF-α production. The data indicate that the INTPH-15 and INTPH-16 activity was associated with fractions with an apparent molecular weight greater than 30 kDa, without LPS. There was no immunostimulatory activity associated with less than 3 kDa and between 3 kDa and 30 kDa fractions, without LPS. After the addition of LPS to all fractions, immunostimulatory activity was observed in all the fractions. This showed that no LPS-inhibiting compounds were present.

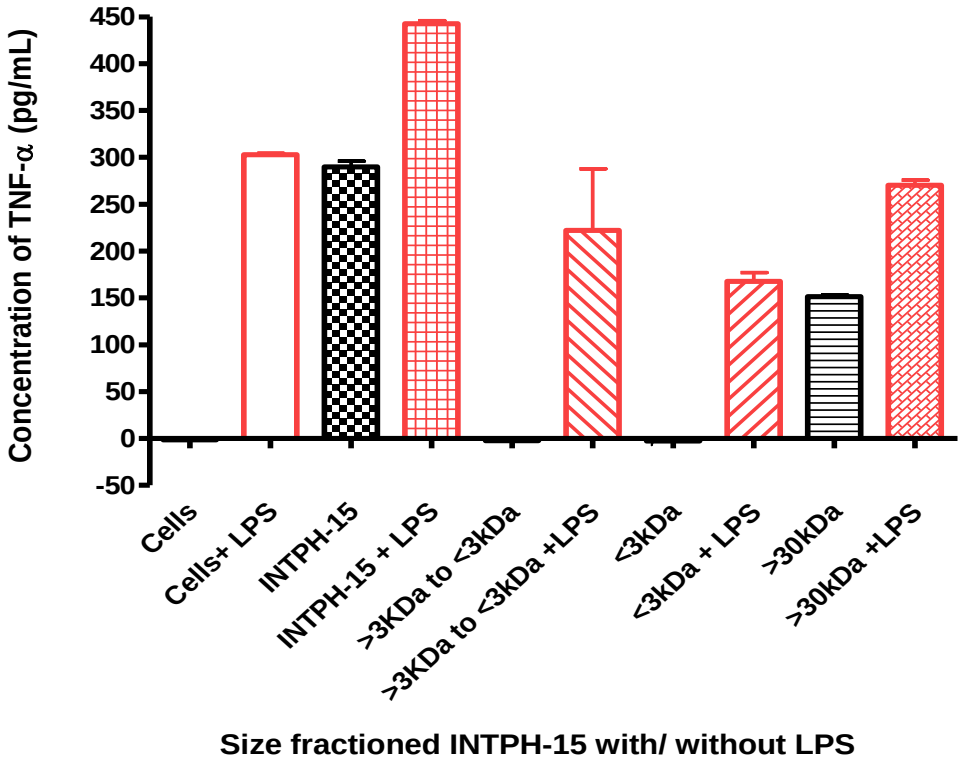

Figure 4.6 (a1) Effect on tumor necrosis factor (TNF)-α production of fractions from Kanuka young honey (INTPH-15) with (■) and without lipopolysaccharide (LPS) (■) of different molecular weight fractions i.e. above 30kDa, between 3kDa to 30kDa and less than 3kDa. Lipopolysaccharide (100 ng/mL) alone (□), THP-1 cells alone (□). **Note**: Data is the mean of two replicates. Error bars represent the standard deviation. There were significant ($p<0.05$) differences between the honey and its filtered fractions.

In the work described by Tonks *et al.* (2007), the majority of the immunostimulatory activity was shown to be associated with material of molecular weight greater than 30 kDa. Minor stimulatory activity was associated with material of less than 30 kDa molecular weight fractions, and this was suggested to be due to the binding of the lower molecular weight (<30 kDa) material to a larger molecule weight (>30 kDa) one. Timm *et al.* (2008) reported that the majority of the immunostimulatory activity was associated with material of molecular weight greater than 20 kDa. Won *et al*. (2008) described that molecular weight of the proteins in honey may differ according to their bee origin.

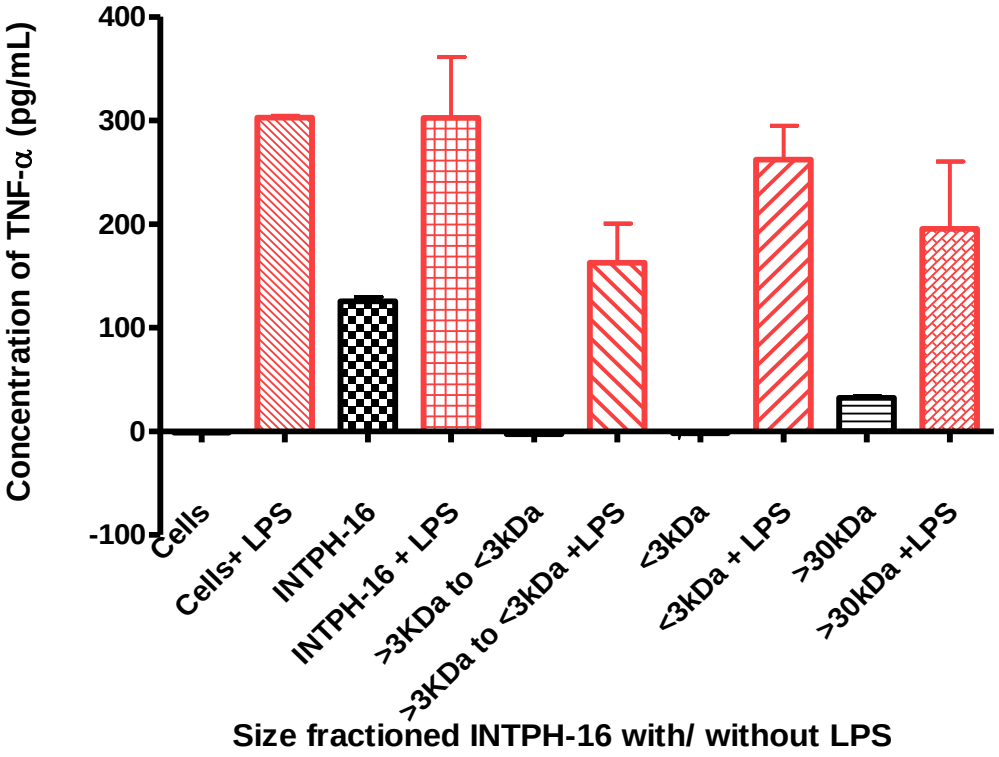

Figure 4.6 (b1) Effect on tumor necrosis factor (TNF)-α production of fractionation Kanuka old honey (INTPH-16) with (■) and without lipopolysaccharide (LPS) (■) of different molecular weight fractions: above 30kDa; between 3kDa to 30kDa; and less than 3kDa. Lipopolysaccharide (100 ng/mL) alone (□), differentiated THP-1 cells alone (□). **Note**: Data is the mean of two replicates. Error bar represents the standard deviation. There were significant ($p<0.05$) differences between the honey and its filtered fractions.

As LPS has a molecular weight of less than 30 kDa and the observed immunostimulatory activity described here was obtained from a fraction with a molecular weight of above 30 kDa molecular weight, these results are therefore in agreement with Tonks *et al*, (2007). But, in aqueous liquids LPS can be 1000 kDa because it can arrange itself into a micellar structure.

4.6 Conclusion

The data obtained from this chapter suggested that the majority of the immunostimulatory activity from active component(s) in honey samples was heat stable and inhibited by the addition of PmB, strongly indicating that the component may be LPS. However, its activity was associated with a molecular weight of above 30 kDa, indicating that the active component may not be an LPS but could be larger while having similar properties to LPS or

enzymes (Todar, 2008). Furthermore, some of the immunostimulatory activity still remained after the addition of PmB and decreased after heat treatment, indicating that minor immunostimulatory activity may be due to proteins or other large complex structures.

To further characterize this component or components in the honeys that were inhibited by PmB, were mostly heat stable, and possessed a molecular weight above 30kDa, the honey was further tested by measuring LPS levels using the LAL assay, and analyzed for carbohydrates as some carbohydrates such as β-glucans have been reported to possess immunomodulatory activity (Guo *et al*., 2009; Tada *et al*., 2009; Cooper and Turcasso, 1999). This work forms the basis of the following chapter.

Chapter 5

Chemical characterization and identification of immunostimulatory component of honey

5.0 Introduction

The work described in the previous chapter explores whether the role of LPS in the immunostimulatory property of honey can be eliminated by studying the inhibitory effect of PmB (Bannatyne *et al.*, 1977, Tsuzuki *et al.*, 2001) and whether heat treatment can reduce the stimulatory activity. Recently, a study showed that heat treatment can cause reduction in the cytokine-stimulatory effect of LPS (Gao *et al*., 2006). Many other polysaccharides obtained from natural sources are considered to be biological response modifiers, and also have been shown to enhance various immune responses (Choi *et al*., 2005). Polysaccharides are important biological molecules (Stafferton, 2007). Polysaccharides are probably the most abundant amongst the natural products produced by plants (e.g. pectin, guar gum, and mannan), algae (e.g. alginates), microorganisms (e.g. dextran, xanthan gum) and animals (e.g. chitosan, chondroitin). Various plants have been used for treating wounds of different types, both internally and externally, and bioassay guided isolation of active compounds in these plants showed that in many cases, polysaccharides were responsible for the biological activity (Paulsen, 2004). Monosaccharide polymers have many favourable properties such as high stability, nontoxicity, hydrophilicity, biodegradability, gel forming properties and ease of chemical modification (Matricardi et al., 2009; Sinha and Kumria, 2001). The complexity and variety of polysaccharides can be explained by two unique structural features: firstly, monosaccharides can be linked together in different ways (1→2, 1→3, 1→4, 1→5 and 1→6, in a α- or β-configuration) and secondly, due to the presence of branched side-chains (Hamman, 2008).

To identify the component(s) responsible for the immunostimulatory activity in honey, further examinations were carried out. First, the LPS levels in honey were quantified, secondly, the high molecular weight material of honey was characterized, and then thirdly, the effects of an identified active component on the stimulation of TNF-α production was determined.

5.1 Measurement of lipopolysaccharide in honey

5.1.1 Introduction

In chapter 4 it was demonstrated that the majority of immunostimulatory activity of honey was inhibited by PmB whereas heat treatment resulted in a small reduction in the immunostimulatory activity assessed by TNF-α production. The objective of this experiment was to measure the LPS levels in honey and further explore the potential role of the LPS and/or LPS-like compounds in the immunostimulatory activity.

5.1.2 Method

Five honey samples; Manuka young (INTPH-01), Manuka old (INTPH-03), Kanuka young (INTPH-15), Kanuka old (INTPH-16) and Clover (INTPH-20), were assayed for endotoxin (LPS) content using the kinetic LAL assay. The honeys were chosen because their various degrees of stimulation of TNF-α production has been shown earlier in this thesis. The assay was carried out by Paul Loong at ESR, Wellington, using a standard protocol (USP 32, 1985) as described earlier (Section 2.8.2). The development of a stable solid clot was considered positive. The specificity and sensitivity of the test was calculated according to the standard protocol (USP 32, 1985). The test for endotoxin content in the honey samples was carried out in 0.5% (w/v) diluted honey samples after removal of β-glucan activity according to a standard protocol (USP 32, 1985), that had been made up in distilled and de-ionized water which would not have been contaminated with LPS.

5.1.3 Results and Discussion

The results from LAL assays are presented in Table 5.1. These data indicated that the LPS level in 4 honey samples was less than 0.5 ng/mL, except for honey sample INTPH-16 (Kanuka old) which possessed 1 ng/mL. The results in Figure 5.1 show that there was a significant difference between the endotoxin concentration and the amount of stimulatory effects in the TNF-α production from honey samples.

The results of the recorded LPS in the honeys did not correlate with TNF-α production (Figure 5.1). Figure 5.2 shows that the recorded LPS in the honeys did not correlate with the stimulation of TNF-α production after heat treatment. In Section 3.3.3 (Figure 3.9) a minimum dose of purified LPS (5 ng/mL) was shown to be required to stimulate TNF-α production from THP1 cells. As none of the honey samples contained more than 1 ng/mL at a 0.5 % (w/v) it is unlikely that LPS is the only constituent in the honeys responsible for its ability to stimulate TNF-α production from THP1 cells. For example, incubation of sample INTPH-16 with THP1 cells at 0.5% (w/v) resulted in 250 pg/mL TNF-α in culture supernatants, while a concentration of 50-100 ng LPS was required to stimulate this level of TNF-α production. Similarly, INTPH-01 which contained less than 1 ng/mL (the lowest level of LPS (0.03ng/mL)) in a 0.5% (w/v) honey solution stimulated 45 pg/mL of TNF-α production. To generate this level of TNF-α from THP1 cells, this honey should have carried 8 ng/mL LPS.

Table 5.1 *Quantification of endotoxin (lipopolysaccharide) levels in honey samples*

Honey samples	**LPS levels (EU/g of honey)**	**LPS levels (ng/g of honey)** 1 EU = 10 Nanograms	**LPS levels in 0.5% (w/v) honey solution. (ng/mL)**
INTPH-01	60	6	0.03
INTPH-03	250	25	0.125
INTPH-15	1000	100	0.5
INTPH-16	2000	200	1
INTPH-20	750	75	0.375

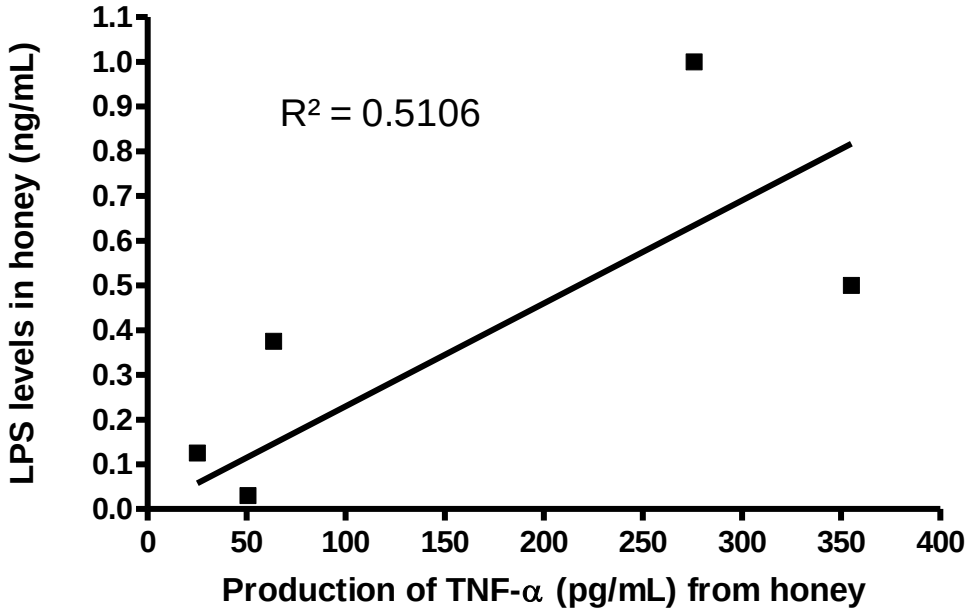

Figure 5.1(a) The correlation between stimulated tumor necrosis factor (TNF)-α production by original honey samples and LPS levels in honey. **Notes:** The data represents the mean concentration of tumor necrosis factor (TNF)-α production in culture supernatants of two replicates. Linear curve (R^2 = 0.5106)

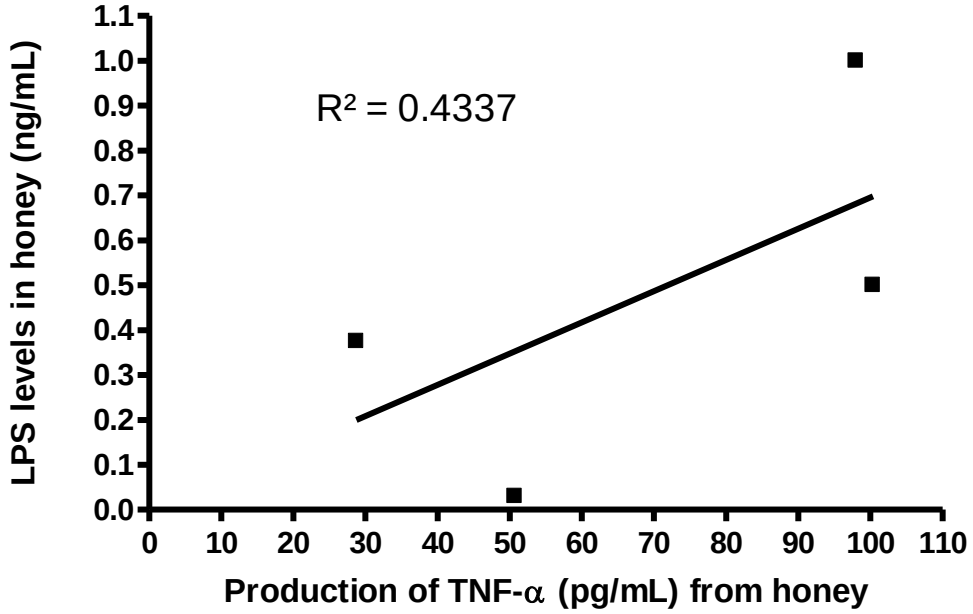

Figure 5.1(b) The correlation between stimulation of tumor necrosis factor (TNF)-α production by heat treated honey samples and lipopolysaccharide (LPS) levels in honey. **Notes:** The data represent the mean concentration of tumor necrosis factor (TNF)-α production in culture supernatants of two replicates. Linear curve (R^2 = 0.5106)

The comparisons in Figure 5.1 (a) and 5.1 (b) suggests that the LPS levels in honey do not correlate with the amount of TNF-α production stimulated by the honey samples because

INTPH-16 (old kanuka) gave the highest levels of LPS, whereas INTPH-15 (young kanuka) gave the highest TNF-α cytokine production. INTPH-03 (old manuka) also showed highest level of LPS but INTPH-01 (young manuka) gave more TNF-α cytokine stimulation than old manuka. These data agrees with the reports of Tonks *et al*. (2003 and 2007) who reported that the LPS levels were less than 0.7 ng/mL in their honey samples and suggested that immunostimulation effects did not correlate with LPS levels in honey. As the immunostimulatory effects of the honey samples in work described here did not correlate with LPS levels in the honey either, it is probable that other component(s) in the honeys are acting to stimulate the production of TNF-α. As described previously (Chapter 4) the component is partially sensitive to heat and has a molecular weight >30kDa.

Interestingly, Dr Paul Loong at ESR discovered that when the LAL assay was initially completed with non-diluted honey samples or with low dilutions of honey in water, the expected positive result that is quantified by the development of a stable solid clot was absent. However, a further dilution of the honey (>1:100) produced a positive stable solid clot, which then disappeared as the LPS activity was diluted out (P. Loong, ESR, pers. comm., 2010). It was suggested by Dr Loong that honey may contain β-glucans that interfere with the LAL assay due to an LPS like structure and mode of action. Subsequently, β-glucans were removed (United States Pharmacopeia 32, 1985) and the LPS results are presented above in Table 5.1 were obtained. This serendipitous observation arising from the LAL assay led to a proposal that the honey may contain β-glucans or some other carbohydrate component(s) that may be responsible for the immunostimulatory activity of honey. It has been reported that β-glucans can influence on TNF-α production (Guo *et al*., 2009, Tada *et al*., 2009, Cooper and Turcasso, 1999)

To further analyze the component(s) that were responsible for stimulating the TNF-α cytokine release, the honey samples were submitted to the Carbohydrate Chemistry Group at Industrial Research Ltd for carbohydrate analysis.

5.3 Carbohydrate (polysaccharide) analysis

5.3.1 Introduction

Beta glucans (β-glucans) are potent biological response modifiers (BRM) that activate the immune response via macrophage cells to yield various therapeutic effects relating to defense reactions against infection (Novak and Vetvicka, 2008). β-glucans are polysaccharides of D-glucose monomers linked by β-glycosidic bonds. They are a diverse group of molecules that can vary with respect to molecular mass, solubility, viscosity, and three-dimensional configuration. They occur most commonly as cellulose in plants, the bran of cereal grains, the cell wall of fungi (Kankkunen *et al*., 2010), mushrooms (Ooi and Liu, 2000) and within the cell wall of bacteria. β-Glucans act on several immune receptors including Dectin-1, complement receptor (CR3), and TLR-2/6, and trigger a group of immune cells including macrophages, neutrophils, monocytes, natural killer cells and dendritic cells (Chan *et al*., 2009). However, β-glucan and many other naturally occurring compounds are thought to contribute to the effects related to treatment of different types of diseases treated by various plants used in traditional medicine in different parts of the world. (BY who ref please). Various plants have been used for treating wounds both internally and externally, and bioassay-guided isolation of active compounds in these plants showed that in many cases, polysaccharides were responsible for the biological activity (Paulsen, 2004). The objective described in this section was to analyse and characterize an active polysaccharide component in honey that might be responsible for the immunostimulatory activity.

The amount and composition of the high molecular-weight carbohydrates present in the above five honey samples were analysed (Dr Ian Sims, Carbohydrate Chemistry Group, IRL, Lower Hutt).

5.3.2 Method

Initially, centrifugal analysis was carried out followed by constituent sugar analysis, glycosyl linkage analysis, and proton NMR spectroscopy and size exclusion chromatography. Methods followed are outlined in sections 2.5.3.1 to 2.5.3.6. The developed methods were carried out

and the results were analyzed by Dr I. Sims, IRL (pers. comm., 2010) who kindly allowed us to reproduce them in this thesis.

5.3.3 Results and Discussion

Figure 5.2 summarises the various treatments carried out at IRL to identify arabinogalactan (AG) and arabinogalactan-protein (AGP) active component in honey.

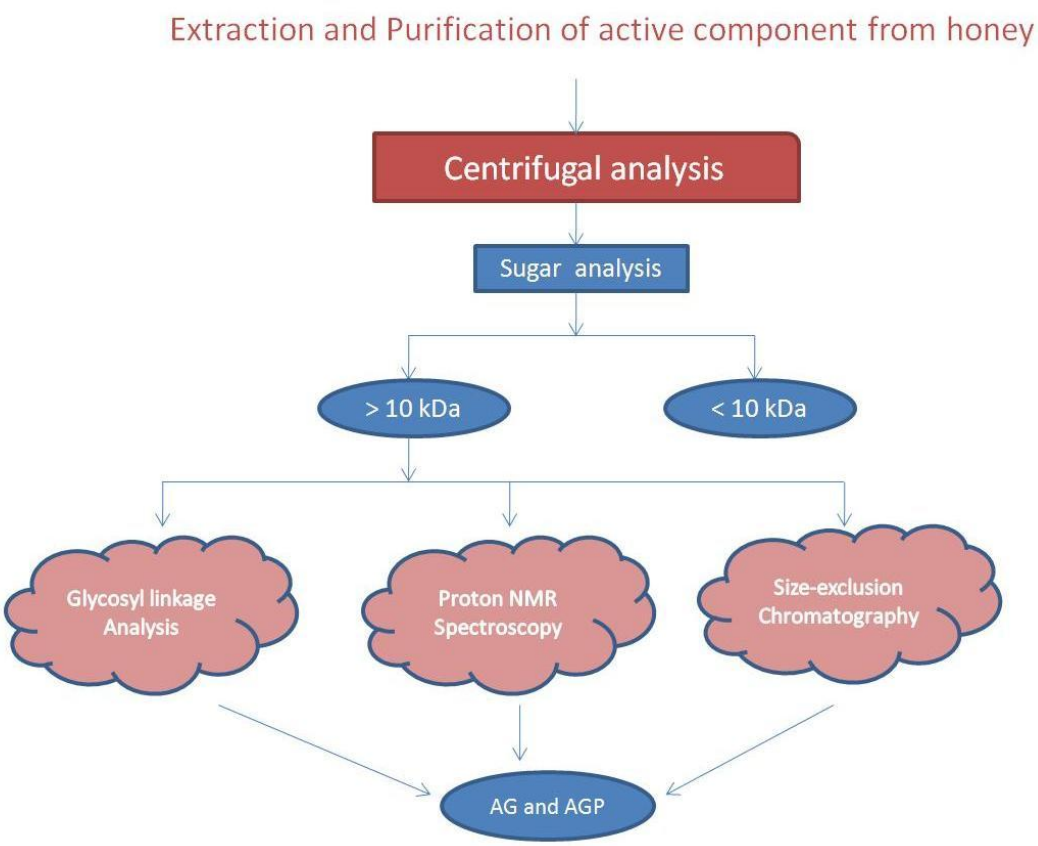

Figure 5.2 Summary of various treatments carried out at Industrial Research Limited to identify the arabinogalactan active component in honey that has immunomodulatory effects. The methods (Sections 2.8.5.1 to 2.8.5.6) and results (Sections 5.3.3.1 to 5.3.3.5) were described briefly in the relevant sections.

5.3.3.1 Centrifugal concentration

Fractionation of the honey samples resulted in yields of the >10000 molecular weight material of between 1.5 and 4.5 mg, representing 0.03 and 0.06 % of the original honeys (Table 5.2).

Table 5.2 *Yield of high molecular weight material from honey samples put through Vivaspin 15R cartridges and the >10000 molecular weight fraction collected and freeze-dried.*

Sample	**Dry weight (mg)**	**% Weight honey**
INTPH-01	2.1	0.04
INTPH-03	4.5	0.09
INTPH-15	3.1	0.06
INTPH-16	1.5	0.03
INTPH-20	3.1	0.06

5.3.3.2 Constituent sugar analysis

Neutral constituent sugar analysis showed that the high molecular weight fractions of the honey samples contained between 14 and 27 % (w/w) carbohydrate. The presence of glucose probably reflects the presence of free glucose, as determined by HPAEC (Table 5.3). Detection of mannose (as mannitol hexaacetate) in the samples may be from glycoproteins present in the high molecular weight fractions of the honeys, or could result from the reduction of free fructose in the samples to mannitol and glucitol during the preparation of the alditol acetates. The constituent sugar analysis also showed the presence of arabinose and galactose in the fractionated honey samples. These sugars were present in higher amounts in the Manuka and Kanuka honeys than in the Clover honey.

Table 5.3 *Constituent sugars composition high molecular weight fractions of honey samples analyzed using the reductive hydrolysis method.*

Sample	**Constituent sugars (%, Dry weight)***				
	Arabinose	**Mannose**	**Glucose**	**Galactose**	**Total**
INTPH-01	7.4	2.8	1.4	10.8	22.4
INTPH-03	4.1	1.5	2.0	6.4	14.0
INTPH-15	4.5	1.5	0.5	8.4	14.9
INTPH-16	4.9	2.4	10.7	9.1	27.1
INTPH-20	0.9	3.2	10.3	2.6	17.0

* Values are the averages of duplicate analyses

5.3.3.3 Glycosyl linkage analysis

The presence of arabinose and galactose in the fractions indicated the presence of arabinogalactan (AG). Type 1 AGs are usually found as neutral side-chains on plant cell wall pectic polysaccharides, while Type 2 AGs are often present as AG-proteins (AGPs), rich in hydroxyproline. In order to determine whether Type 1 or Type 2 AGs were present in the honeys, the glycosyl linkage compositions of the samples were determined (Table 5. 4).

The glycosyl linkage analyses of the Manuka, Kanuka and Clover honeys (INTPH-01, INTPH-03, INTPH-15 and INTPH-16) were consistent with polymers comprised of a highly branched backbone of 1, 3-linked Gal*p* residues, with side-chains made up of Ara*f* containing oligosaccharides, typical of Type 2 AGs. These linkages represented 7 – 10 % of the total weight of the HMW fractions from the Manuka and Kanuka honeys, which were consistent with, but slightly lower than, that calculated from the sum of arabinose and galactose in the constituent sugars analyses (Table 5.3). The glycosyl linkage analysis of the Clover honey also showed the presence of Type 2 AGs, but in much lower amounts (1.3 % of the weight of the HMW fraction, compared with 3.5 % from the sum of arabinose and galactose).

Table 5.4 *Glycosyl linkage composition high molecular weight fractions of honey samples analyzed by GC-MS of partially methylated alditol acetates.*

Sugar	**Linkage**	**Linkage Composition (Mol%)[a]**				
		INTPH-01 (9.8%[b])	**INTPH-03 (10.0%[b])**	**INTPH-15 (12.0%[b])**	**INTPH-16 (12.0%[b])**	**INTPH-20 (4.0%[b])**
Ara *p*	Terminal	-	1.7	0.1	0.3	-
Ara *f*	Terminal	27.5	24.6	30.8	31.1	12.9
	2-	3.4	4.2	2.1	2.3	-
	3-	1.2	1.9	1.7	2.3	-
	5-	4.5	6.6	5.4	8.5	6.7
Gal *p*	Terminal	4.2	1.4	6.8	3.3	2.1
	3-	8.0	4.8	9.9	7.1	4.7
	6-	5.1	5.9	4.1	2.4	2.1
	3,6-	27.5	27.1	23.8	22.5	7.6
	3,4,6-	1.3	-	5.4	3.1	-
Glc *p*	Terminal	7.5	6.7	4.7	7.2	35.9
Man *p*	2-	4.8	3.3	3.2	3.7	15.3
	3,6-	1.4	1.0	0.9	0.7	5.4
Other		3.6	10.8	1.1	5.8	7.3
Total		100	100	100	100	100`

[a] Values are the averages of duplicate analyses
[b] Values in parentheses are % carbohydrate content calculated from sum of all linkages, compared to internal standard of myo-inositol (10 µg)

5.3.3.4 Proton NMR spectroscopy

The ^{1}H NMR spectra of high molecular weight honey fractions are shown in Figures 5.3.3.4. The spectra for INTPH-16 (Figure 5.3.3.4D) and INTPH-20 (Figure 5.3.3.4E), that contained high amounts of free sugars showed sharp signals at about 5.21 and 4.61 ppm, that were attributed to H-1 of α- and β- Glc, respectively. Samples INTPH-01 (Figure 5.3.3.4A), INTPH-03 (Figure 5.3.3.4B), INTPH-15 (Figure 5.3.3.4C) and INTPH-16 showed H-1 signals at 5.23 and 5.08 ppm, with INTPH-15 and INTPH-16 showing an addition H-1 signal at 5.43 ppm. These H-1 signals were consistent with NMR assignments of α-L-Ara*f* residues of AGs (Gane *et al*., 1995; Sims and Furneaux, 2003). Several other H-1 signals were observed in 4.4-4.6 ppm ranges that were assigned to H-1 of β-D-Gal*p* residues. These data provide further evidence for the presence of Type 2 AGs, probably present as AGPs.

Figure 5.3 Proton nuclear magnetic resonance (NMR) spectra of five honey samples. (A) INTPH-01, (B) INTPH-03, (C) INTPH-15, (D) INTPH-16 and (E)INTP H-20.

A

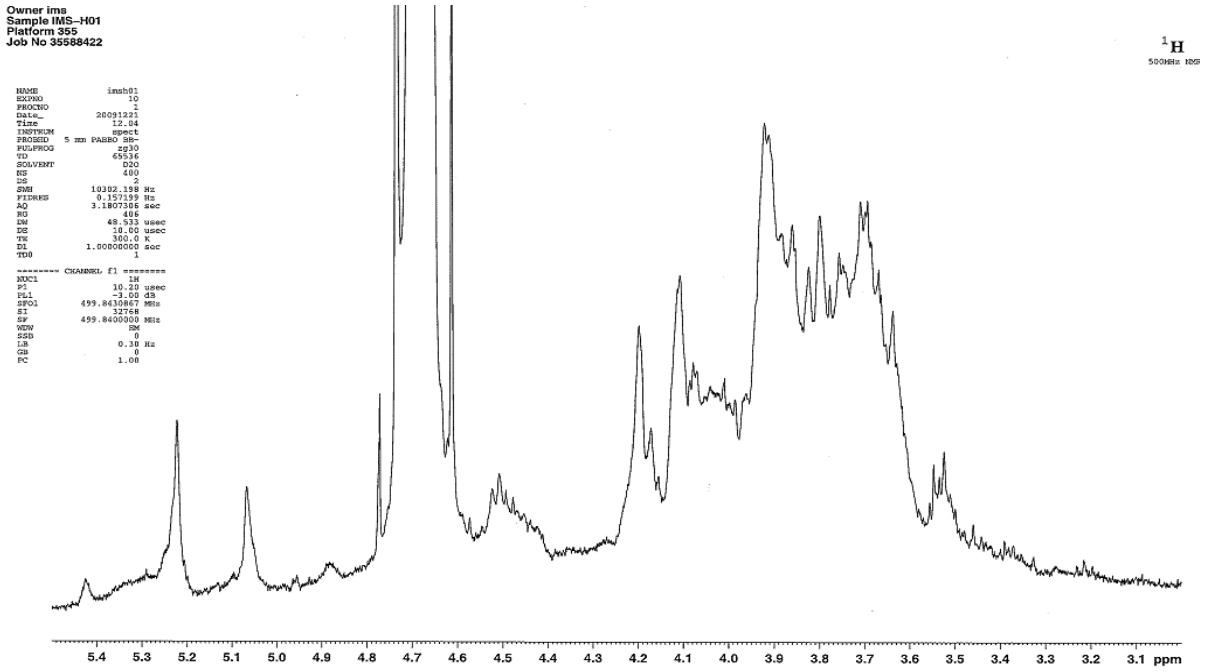

Figure 5.3 Proton nuclear magnetic resonance (NMR) spectra of (A) INTPH-01

B

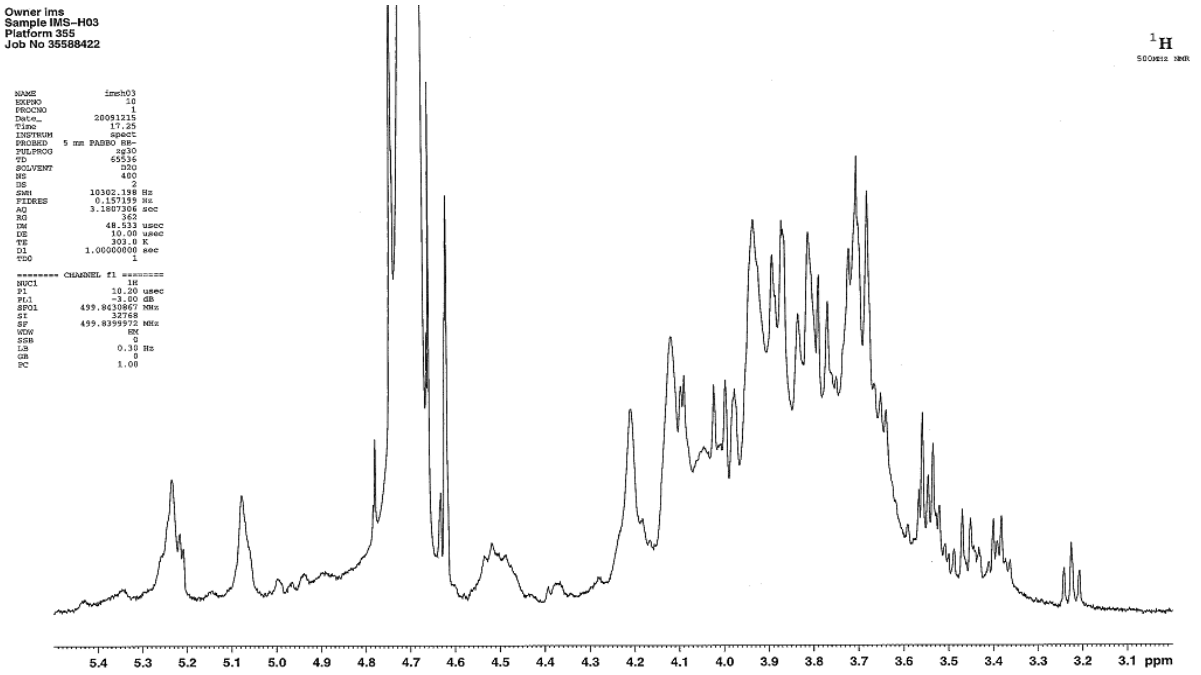

Figure 5.3 continued Proton nuclear magnetic resonance (NMR) spectra of (B) INTPH-03

C

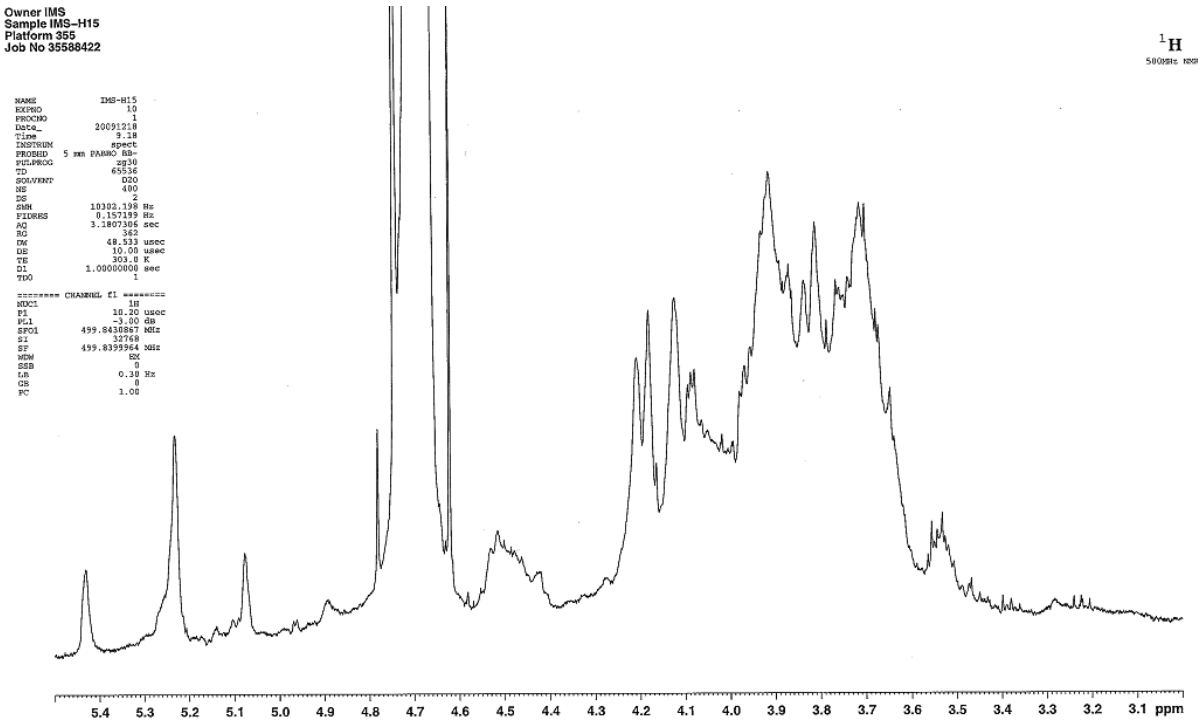

Figure 5.3 continued Proton nuclear magnetic resonance (NMR) spectra of (C) INTPH-15

D

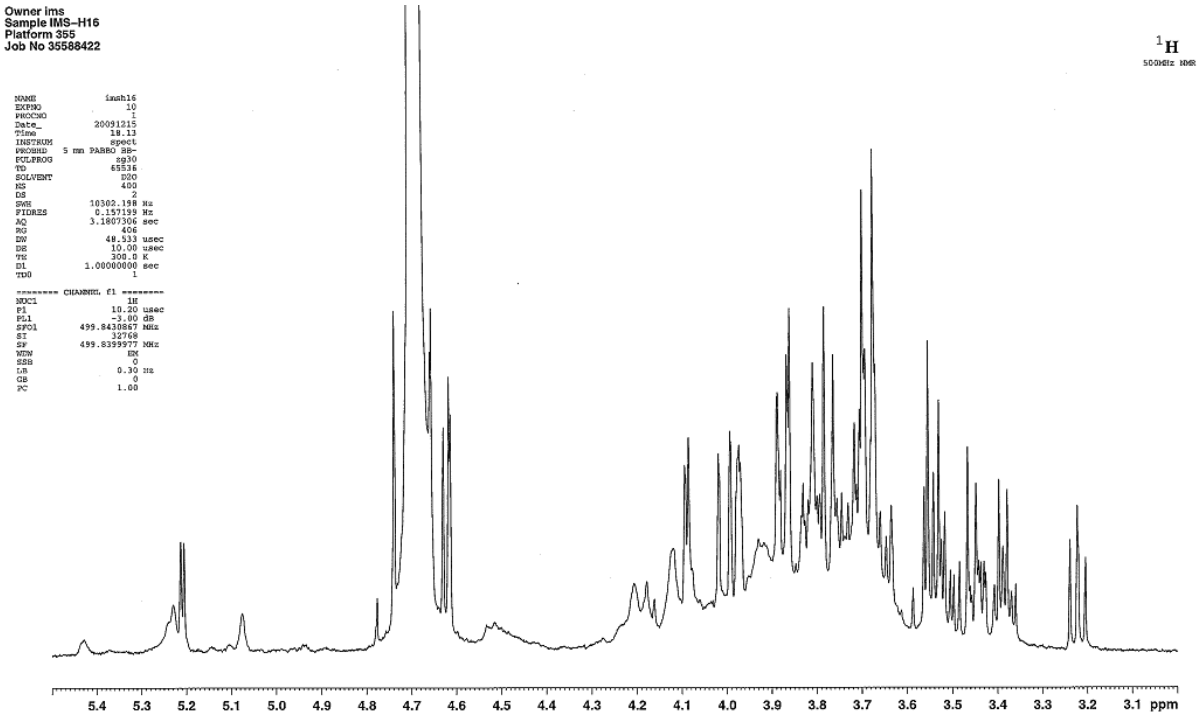

Figure 5.3 continued Proton nuclear magnetic resonance (NMR) spectra of (D) INTPH-16

E

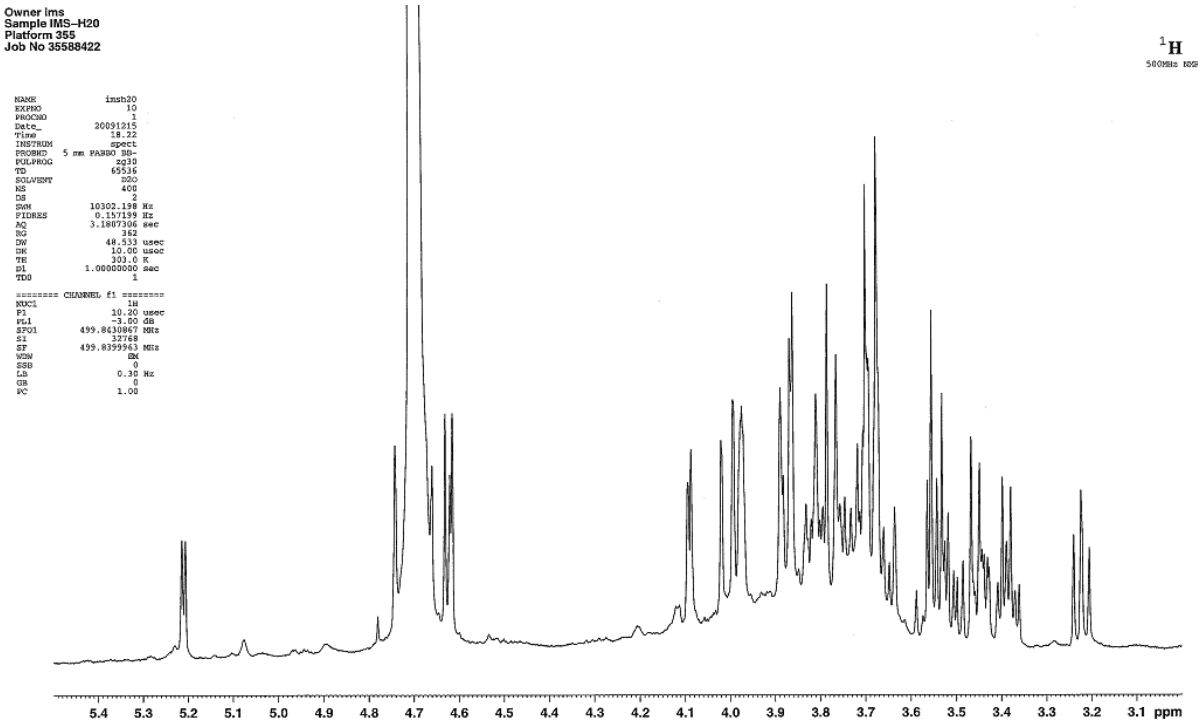

Figure 5.3 continued Proton nuclear magnetic resonance (NMR) spectra of (E) INTP H-20.

5.3.3.5 Size-exclusion chromatography

The HMW fractions of the honeys showed complex chromatograms on SEC eluting from the V_0 (Void volume) to the V_t (total volume) of the columns (not shown). Compounds elute at the void volume if they are not retained, salts elute at the total column volume as they are retained to the greatest extent. INTPH-01 showed a predominat peak (~18.5 mL) eluting just before the 24 kDa pullulan standard and a second smaller peak (~16.7 mL) eluting just after the 100 kDa pullulan. Similar peaks were also observed for INTPH-03, but in smaller amounts. INTPH-15, INTPH-16 and INTPH-20 also showed peaks eluting at ~18.5 and ~16.7 mL (24 kDa and 100 kDa, respectively), but INTPH-15 also showed a peak at 20 mL, eluting after the 24 kDa standard. Additionally INTPH-16 and INTPH-20 showed peaks at 21-22 mL, assumed to be due to low molecular weight sugars present in these samples (see Table 5. 4).

The work carried out with IRL showed that the molecular weight of the fractions at 24 kDa and 100 kDa contained arabinogalactans in the honey samples. The molecular weight of the LPS ranges from >10 kDa to 30 kDa suggesting that the LPS may not be responsible for immunostimulatory activity. The filtered honey samples of >30 kDa fractions showed the immunostimulatory activities indicating that the arabinogalactans rather than LPS may be responsible for the immunostimulatory activity in honey.

In this study, the detection peak of arabinogalactan was observed before 24 kDa and after 100 kDa fractions, and the immunostimulatory property of honey was observed in the fraction above 30 kDa. Tonks *et al*. (2007) reported that most of the immunostimulatory activity was observed in the fraction above 30 kDa. Whereas, Timm *et al*. (2008) reported that the immunostimulatory activity was observed in the fraction above 20 kDa. This study showed that the activity was associated with the fraction above 30 kDa suggesting that the component responsible for the immunostimulatory activity could be the 100 kDa fraction of arabinogalactans. Results of the INTPH-01 and INTPH-03 honey samples showed similar peaks but a smaller peak was observed at the 100 kDa fraction, indicating that they contain very small amounts of arabinogalactans; this is probably the reason for the low immunostimulatory activity observed in the two honey samples.

The results of the work carried out at IRL shows that AG and AGPs are present in the immunostimulatory honey samples and there is the possibility that these molecules could contribute to the immunomodulatory effect. The work described in this study was not carried with any non-immunostimulatory honey samples. In future it will be important to determine whether this component is present only in immunostimulatory honeys or in all honeys, immunostimulatory and non-immunostimulatory alike. To confirm that the AG components of the honey has immunomodulatory effects, further work has been carried out using AG which has been purified and freeze dried at IRL.

5.5 Effect on TNF-α production of the arabinogalactan component of Kanuka honey

5.5.1 Introduction

Arabinogalactans (AG) are essential polymers in the cell wall of plants and are found in all higher plants as structural polysaccharides (Cipriani *et al*., 2004). AG polysaccharides were isolated mostly from the traditional medicinal plants including cell-culture exudates of *Echinacea* purpurea (Classen *et al*., 2000), leaves of *Maytenus ilicifolia* (Cipriani *et al*., 2004), *Diospyros kaki* (Duan *et al*., 2003), *Nerium Indicum* (Dong and Fang, 2001), roots of Curcuma longa (Kundu *et al*., 2005) and arabinogalactan proteins from Baptisia. They are also found in edible and inedible plants including carrots, radish, wheat, black gram, pears, beans, tomatoes etc. (Peter Dadamo, 1996). Among the plant tissue proteins the arabinogalactan proteins are the most widely-spread representatives in nature and immunologically active (Classen *et al*., 2006; Duan *et al*., 2003). It has been shown by Wagner *et al*. (1988) that AG specifically stimulates macrophages to produce TNF-α *in vitro*. Siddiqui (1965) found arabinogalactomannan in honey and discussed the structural features of it. No subsequent work was carried out on its activities in honey. The exact structures of the active compounds remain unknown. The objective of this section was to investigate whether the arabinogalactan purified from honey possesses immunostimulatory effects.

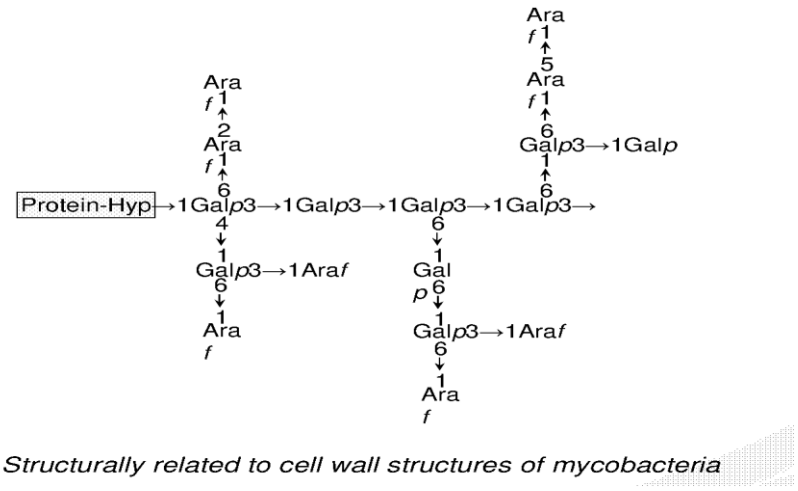

Figure 5.4 The structural model of β-arabinogalactan-protein from honey

5.5.2 Method

The arabinogalactan component from Kanuka honey (INTPH-15) (0.08 mg AG per g honey) was exposed to THP1 cells and TNF-α production measured as described in Section 2.8.3. The concentration of the AG component used was calculated to be the concentration that would be present in 0.5% (w/v) honey.

5.5.3 Results and Discussion

The result in Figure 5.5 clearly show that the AG derived from honey INTPH-15 stimulated the production of TNF-α from differentiated THP1 cells, and was inhibited by PmB. The amount of TNF-α produced after stimulation with AG was significantly less than that stimulated by the original honey. When AG was added to the original honey an additive effect was observed. With artificial honey no TNF-α was produced, but when AG was added to the artificial honey the amount of TNF-α produced was similar to when AG was used alone. These results suggest that an AG component may be responsible for immunostimulation effects of honey via mechanisms similar to those used by LPS, and that its efficacy may rely on the presence of other honey monosaccharides.

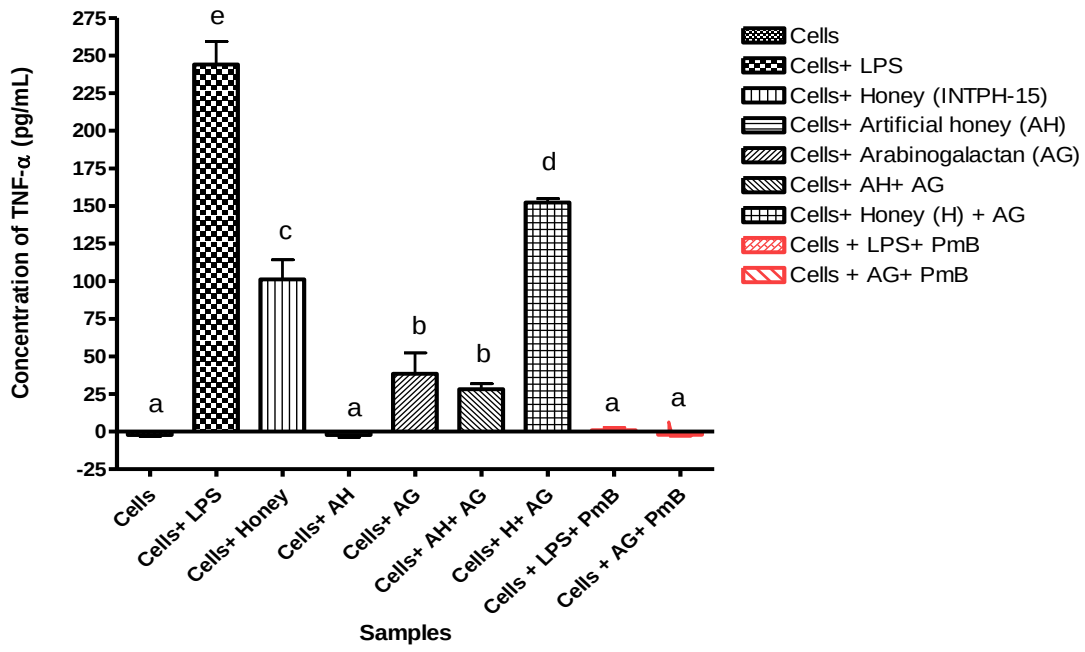

Figure 5.5 Effect on tumor necrosis factor (TNF)-α production from differentiated THP-1 cells from purified arabinogalactan (AG) of honey. Notes: The data is the mean of two replicates. Error bars represent the standard deviation. The treatments with the same letter are not significantly different from each other ($p<0.05$). There is a significant ($p<0.05$) difference between the samples that have different letters. Statistical data are shown in appendices.

These data are supported by the literature. AGs have been reported to possess immunomodulatory activities (Wagner *et al*., 1988; Classen *et al*., 2006). Recently Kundu *et al*., (2005) reported that *Curcuma longa* (turmeric) and honey show similar wound healing properties and he also demonstrated that immune modulation property of turmeric is largely mediated by arabinogalactone core structure and Ikonan C, an acidic polysaccharide in the rhizome of *C. longa*. Recently, Scheptkin *et al*., (2005) reported that type II AGPs have significant numbers of 1,6-linked galactosyl chains, and this side-chain has been reported to be important for the immune activity by inducing both inflammatory (IL-1, IL-6, TNF-α and IL-12) and anti-inflammatory (IL-10) cytokines. One of the polysaccharides (pectic arabinogalactan) from *Vernonia kotschyana* modulated the immune responses through CD19, TLR4, TLR2 and CD79b receptors, therefore suggesting possession of broader receptor specificity than LPS (Nergard *et al*., 2005). Significantly, Tonks *et al*. (2007) showed that a mechanism by which a honey component stimulates immune cells was via the TLR4 receptors, in agreement with the Nergard paper.

5.6 Conclusions

The results described in this chapter suggest that the TNF-α stimulatory activity in honey is due to component(s) other than LPS. The high molecular weight complex carbohydrate (arabinogalactan) was identified and is likely to be partially responsible for this stimulatory effect.

In the future, the dose response of arabinogalactans should be determined. Further, additional work could involve an analysis of other cytokines stimulated by AG, for example IL-1, -6, -12, and -10 and comparing these to those stimulated by honey.

CHAPTER 6

Summary

6.1 Summary

The aim of this study was to investigate the ability of honey to elicit a cytokine response from acute monocytic leukemia cell line (THP-1) cells and to identify the component(s) responsible for inducing cytokine release. In the present project, five different types of New Zealand honeys were assessed for their ability to stimulate TNF-α production using a cell model system. This was achieved by using the honey samples after heat treatment, PmB treatment, an irradiation process, fractionation methods and investigation of the effects of the fractions on TNF-α production. Heat and PmB treatment showed that the activity was predominantly LPS-like, but partially heat labile. Fractionation showed the activity was associated with a molecular weight fraction larger than 30 kDa and LPS ranges from 10 kDa to 30 kDa but in aqueous liquids LPS can be 1000 kDa because it can arrange itself into a micellar structure. However, levels of LPS naturally occurring in the honey were shown to be too low to account for the observed activity. Carbohydrate analysis revealed a complex arabinogalactan carbohydrate structure that was likely to be responsible for immunostimulation in honey.

Natural honey has been used as a wound healing substance since ancient times due to its antimicrobial properties (Dustmann, 1979; Molan, 1992a), and these effects are most probably due to inflammatory (Tonks *et al*., 2001 and 2003; Abuharfeil *et al*., 1999) and anti-inflammatory properties (Molan, 2002; Timm *et al*., 2008). Antimicrobial properties are due to the high sugar content, osmolarity, pH, hydrogen peroxide (Cooper *et al*., 1999), flavonoids and the phenolic acids (Wahdan, 1998), antibacterial peptides (Lee *et al*., 2008; Kamran Azim and Sajid, 2009) and presence of MGO (Mavric *et al*., 2008). In addition to its antibacterial activity, honey possesses anti-inflammatory effects that result in reduction of pain and pressure in tissues, which in turn reduces oedema and down regulates the amount of wound exudate. The component(s) responsible for anti-inflammatory effects of honey have not yet been clearly identified and the mechanisms of action are poorly established.

Recently, studies have been reported that honey stimulates inflammatory responses in leukocytes that give rise in the production of growth factors (Tonks *et al*., 2001 and 2003;

Timm *et al*., 2008; Abuharfeil *et al*., 1999). Tonks *et al*. (2007) demonstrated that Manuka honey contains an unidentified 5.8 kDa component that stimulated the production of TNF-α in macrophages using TLR-4 receptors. In contrast, Timm *et al*. (2008) disagreed with the claims of Tonks and co-workers, and maintained that LPS was responsible. The findings presented in this thesis provides a better understanding of the component that is responsible for the ability of immunostimulatory activity in honey, and the results appears to have other component in honey rather than LPS is responsible for immunostimulatory activity.

The data presented here suggests that arabinogalactans in honey stimulate inflammatory responses and the release of cytokines that are crucial in regulating wound healing, significantly contributing to the understanding of how honey influences wound healing, and how other complex carbohydrates may play important biomedical roles.

Information from this study will contribute to the development of novel therapeutics to improve the healing of acute and chronic wounds. Arabinogalactans and also many other complex carbohydrates obtained from plants including glucomannan, β-glucans, pectins, rhamnogalacturonan, and sulphated polysaccharides may be involved in potential therapeutic effects and have shown diverse biological activities such as wound healing, enhancement of the reticuloendothelial system, stimulation of the immune system, treatment of tumours and effects on the hematopoietic system (Jung *et al*., 2008; Paulsen *et al*., 2004).

CHAPTER 7

Conclusions and Future study

7.1 Conclusions

This study has demonstrated the presence of a complex arabinogalactan carbohydrate component of immunomodulatory honeys of New Zealand origin, which has a high molecular weight, may be acts in an LPS-like manner, and may be partially responsible for stimulating TNF-α production. If it is confirmed that this unique component gives honey the ability to modulate the immune responses, it gives honey an exclusive property in addition to its antibacterial effect in wound healing.

7.2 Future study

The work described in this thesis has provided some interesting and valuable information about the arabinogalactan in honey that may be responsible for immunostimulatory activity on the innate immune cells involved in the wound healing process. The precise mechanisms of action, other cytokines involved and arabinogalactan activity on different cells that are involved in wound healing still require investigation.

Further investigations are also required to determine whether the immunostimulatory activity is mainly due to arabinogalactan, or whether another component(s) combines with the arabinogalactan in honey to stimulate cytokine production.

Further investigations should be carried out on pure arabinogalactans purified from honey and its immunostimulatory activity.

Furthermore, additional work on the characterization of the arabinogalactan and structure-function relationships by which this component stimulates innate immune cells in the wound healing process is still required.

Collectively, these findings reveal some of the components involved in honey stimulation of wound healing, begin to explain the mechanism of action, and open new avenues for both honey research and wound healing.

Appendices

Appendix I: Publication

Poster and Oral presentation (Same conference)

The New Zealand Postgraduate Conference (NZPGC), 2009 **"Breaking the mold-pushing the boundaries of science and technology in NZ"** Conference, 20-21 November, 2009, Wellington, New Zealand

INVESTIGATING NEW ZEALAND INDIGENOUS HONEY WITH IMMUNOSTIMULATORY PROPERTIES

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Poster Abstract

The use of honey has re-emerged as a therapy to aid wound healing. Clinical studies indicate that honey accelerates the wound healing process. This process is believed to be related to cytokine release. It is still not known whether the presence of LPS or other active components in the honey is responsible for the release of inflammatory cytokines from monocytic cells

The main objective of this study was to investigate the ability of honey to elicit a cytokine response from acute monocytic leukemia cell line (THP-1) cells and to identify the component which is responsible for inducing cytokine release.

Eight honey samples were used together with sugar and methylglyoxal controls. The samples (0.5 %, w/v) were incubated for 4 h at 37ºC in 5% CO_2 atmosphere with THP-1 cells, with and without LPS. The cell culture supernatants were collected after centrifuging at 130 *g* for 5 minutes and were assayed for TNF-α by ELISA. Further, the most active honey samples were investigated by heat treating and by treating with PmB, with and without LPS, with

incubation at 37ºC in 5% CO_2 atmosphere for 1 hour. In addition, samples were filtered by molecular weight centrifugal filters and the fractions were assessed for immunostimulatory activity.

All eight honey samples stimulated the TNF-α cytokine from THP-1 cells when compared with untreated, artificial-honey and MGO treated cells. Cytokine production was partially inhibited by heating, and was inhibited by PmB. In filtered honey samples the activity was observed in the >30 kDa fraction. This suggests LPS from the honey, possibly in conjunction with other honey constituents, stimulates cytokine release.

Preliminary results from this study suggest that the stimulation of inflammatory cytokines may contribute to the effect of honey on wound healing. Information from this study will contribute to the development of novel therapeutics to improve the healing of acute and chronic wounds.

Appendix II: Data analysis

Chapter 3

3.3 Cytoxicity effects of 16 honey samples (0.5% (w/v)) expressed relative to negative control (100%) and positive control (10 µM camptothecin)

Analysis of variance

Variate: %_toxic

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample	18	5.33430	0.29635	13.80	<.001
Residual	38	0.81608	0.02148		
Total	56	6.15039			

Percentage variance accounted (adjusted R-sq) for 80.4
Standard error of observations is estimated to be 0.147.

Standard errors of differences of means

Table	Sample
rep.	3
d.f.	38
s.e.d.	0.1197

Least significant differences of means (5% level)

Table	Sample
rep.	3
d.f.	38
l.s.d.	0.2422

Tukey's 95% confidence intervals

Sample	Mean	
Pos control	0.235	a
Neg control	1.367	bc
INTPH-10	1.176	b
INTPH-16	1.194	b
INTPH-18	1.210	b
INTPH-21	1.212	b
INTPH-17	1.258	bc
INTPH-15	1.280	bc
INTPH-14	1.337	bc
INTPH-09	1.344	bc

INTPH-19	1.355	bc
A H	1.376	bc
INTPH-20	1.378	bc
INTPH-05	1.550	bc
INTPH-02	1.563	bc
INTPH-12	1.592	bc
INTPH-03	1.611	bc
INTPH-01	1.664	c
INTPH-07	1.695	c

3.12 (a)TNF-α from differentiated THP-1 cells stimulated by different honey samples

Analysis of variance

Variate: TNF

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Samples	10	599832.5	59983.3	94.21	<.001
Residual	11	7003.8	636.7		
Total	21	606836.3			

Percentage variance accounted (adjusted R-sq) for 97.8
Standard error of observations is estimated to be 25.2.

Standard errors of differences of means

Table	Samples
rep.	2
d.f.	11
s.e.d.	25.23

Least significant differences of means (5% level)

Table	Samples
rep.	2
d.f.	11
l.s.d.	55.54

Tukey's 95% confidence intervals

Samples

	Mean	
Cells	5.4	a
MGO	7.6	a
Sugar	8.4	a
Sugar + MGO	9.4	a
INT-03	25.1	ab
INT-20 + MGO	45.6	b
INT-01	50.7	b
INT-20	63.6	b
INT-16	275.9	bc
INT-15	355.1	c
LPS	504.0	d

Chapter 4

4.1 TNF-α production from differentiated THP-1 cells after treatment with 100 ng/mL of lipopolysaccharide and honey samples (0.5 % (w/v)) with and without pre-treatment with 10 µg/mL of PolymixinB

Analysis of variance

Variate: TNF

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Samples	11	65621.8	5965.6	33.03	<.001
Residual	12	2167.5	180.6		
Total	23	67789.3			

Percentage variance accounted (adjusted R-sq) for 93.9
Standard error of observations is estimated to be 13.4.

Standard errors of differences of means

Table	Samples
rep.	2
d.f.	12
s.e.d.	13.44

Least significant differences of means (5% level)

Table	Samples
rep.	2
d.f.	12
l.s.d.	29.28

Tukey's 95% confidence intervals

Samples

	Mean	
INTPH-01 + PmB	-3.97	a
Cells + PmB	-3.09	a
LPS + PmB	-2.35	a
Cells	-1.39	a
INTPH-01 + LPS + PmB	2.69	ab
INT-15 + PmB	11.58	ab
INTPH-01	13.69	b
INTPH-15 + PmB + LPS	22.38	b
INTPH- 15	61.61	bc
LPS	77.47	cd
INTPH-15+LPS	118.11	de
INTPH-01+ LPS	154.76	e

4.2(a) TNF-α production from differentiated THP-1 cells after treatment with LPS (100 ng/mL) and final concentration of 0.5 5(w/v) of various honeys before and after heat treatment at 80°C for 30 min

Analysis of variance

Variate: TNF

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Samples	10	205173.9	20517.4	143.44	<.001
Residual	11	1573.5	143.0		
Total	21	206747.4			

Percentage variance accounted (adjusted R-sq) for 98.5
Standard error of observations is estimated to be 12.0.

Standard errors of differences of means

Table	Samples
rep.	2
d.f.	11
s.e.d.	11.96

Least significant differences of means (5% level)

Table	Samples
rep.	2
d.f.	11
l.s.d.	26.32

Tukey's 95% confidence intervals

Samples

	Mean	
Cells	-3.5	a
Heat INTPH-20	28.8	ab
Heat INTPH-01	50.7	ab
INTPH-20	66.6	bcd
INTPH-01	72.5	bcd
Heat INTPH-16	98.0	cd
Heat INTPH-15	100.4	d
INTPH-16	148.2	e
INTPH-15	158.7	e
LPS	281.8	f
Heat LPS	319.4	f

4.2(b) TNF-α production from differentiated THP-1 cells after heat treatment of honey samples with and without lipopolysaccharide.

Analysis of variance

Variate: TNF

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Samples	10	222934.	22293.	6.41	0.003
Residual	11	38281.	3480.		
Total	21	261215.			

Percentage variance accounted (adjusted R-sq) for 72.0
Standard error of observations is estimated to be 59.0.

Standard errors of differences of means

Table	Samples
rep.	2
d.f.	11
s.e.d.	59.0

Least significant differences of means (5% level)

Table	Samples
rep.	2
d.f.	11
l.s.d.	129.8

Tukey's 95% confidence intervals

Samples

	Mean	
Cells	-3.5	a
INT-20+LPS	232.6	b
INT-16+LPS	262.9	b
Heat INT-15+LPS	263.5	b
INT-15+LPS	268.6	b
Cells+LPS	281.8	b
Heat INT-01+LPS	314.1	b
Heat LPS	319.4	b
Heat INT-20+LPS	337.7	b
INT-01+LPS	362.8	b
Heat INT-16+LPS	404.1	b

4.3 Effect on TNF-α production from differentiated THP-1 cells after treatment with LPS (100 ng/mL) alone, heat treated lipopolysaccharide (100 ng/mL) and the final concentration of 0.5 % (w/v) of two heat treated honey samples before and after Polymixin B treatment

Analysis of variance

Variate: TNF

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Samples	13	471103.5	36238.7	231.25	<.001
Residual	14	2194.0	156.7		
Total	27	473297.4			

Percentage variance accounted (adjusted R-sq) for 99.1
Standard error of observations is estimated to be 12.5.

Standard errors of differences of means

Table	Samples
rep.	2
d.f.	14
s.e.d.	12.52

Least significant differences of means (5% level)

Table	Samples
rep.	2
d.f.	14
l.s.d.	26.85

Tukey's 95% confidence intervals

Samples

	Mean	
Cells	-3.5	a
Cells+LPS+PmB	-2.5	a
Cells+PmB	-2.3	a
Heat INTPH-15 +PmB	-2.2	a
Heat INTPH-16 +PmB	0.4	a
Heat INTPH-15+ LPS +PmB	10.0	a
Heat LPS +PmB	13.7	a
Heat INT-16+ LPS +PmB	15.6	a
Heat INTPH-16	88.2	b
Heat INTPH-15	92.9	b
Heat INTPH-15+LPS	257.9	c
Cells+LPS	281.8	cd
Heat LPS	313.9	de
Heat INTPH-16+LPS	338.5	e

4.4 (a) Effect on TNF-α production from differentiated THP-1 cells after Kanuka (INTPH-15) honey sample (0.5% (w/v)) was treated with increasing doses of gamma irradiation.

Analysis of variance

Variate: TNF_

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Samples	5	342550.	68510.	46.71	<.001
Residual	6	8800.	1467.		
Total	11	351350.			

Percentage variance accounted (adjusted R-sq) for 95.4
Standard error of observations is estimated to be 38.3.

Standard errors of differences of means

Table	Samples
rep.	2
d.f.	6
s.e.d.	38.3

Least significant differences of means (5% level)

Table	Samples
rep.	2
d.f.	6
l.s.d.	93.7

Tukey's 95% confidence intervals

Samples

	Mean	
Cells	-22.0	a
RH87	322.6	b
RH54	362.4	b
RH26	368.9	b
RH0	383.0	bc
cells + LPS	535.2	c

Chapter 5

5.5 Effect of TNF-α production from differentiated THP-1 cells with purified arabinogalactan (AG) from honey

Analysis of variance

Variate: TNF

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Samples	8	121256.1	15157.0	109.93	<.001
Residual	9	1240.9	137.9		
Total	17	122497.1			

Percentage variance accounted (adjusted R-sq) for 98.1
Standard error of observations is estimated to be 11.7.

Standard errors of differences of means

Table	Samples
rep.	2
d.f.	9
s.e.d.	11.74

Least significant differences of means (5% level)

Table	Samples
rep.	2
d.f.	9
l.s.d.	26.56

Tukey's 95% confidence intervals

Samples

	Mean	
Cells	-2.30	a
Cells+ AH	-2.30	a
Cells + AG+ PmB	-2.12	a
Cells + LPS+ PmB	0.90	a
Cells+ AH+ AG	28.11	b
Cells+ AG	38.47	b
Cells+ Honey	101.21	c
Cells+ H+ AG	152.31	d
Cells+ LPS	243.99	e

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