MALAYSIAN TUALANG HONEY AND ITS IMMUNOMODULATORY PROPERTIES

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Thesis submitted to the University of Nottingham Malaysia Campus for the degree of Doctor of Philosophy

May 2017

ABSTRACT

Discoveries of antibiotic resistant pathogens and failure in some conventional modern cancer treatments have led to the re-evaluation of ancient therapeutic remedies such as honey. In recent years, Malaysian Tualang honey (MTH) had been proven scientifically to possess many beneficial properties such as antimicrobial, antioxidant, anticancer, anti-inflammatory and wound healing potential. However, little scientific evidence about its immunomodulatory property has been published, therefore this study aimed to investigate the immunomodulatory effect of MTH.

Initially, human monocytic cell lines (THP-1 and U-937) were first cultured and tested against MTH as part of methodology optimization before proceeding to peripheral blood mononuclear cells (PBMCs) isolated from healthy donors. PBMCs were used in this study because these cells can mimic the *in vivo* system of immune responses more closely. The cytotoxic effect of MTH on THP-1, U-937 and PBMCs was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyltetrazolim bromide (MTT) assay. It was found that the combination of 0.125 % to 2 % MTH and incubation durations 16, 24 and 48 hours yielded at least 90 % cell viability in PBMCs (except 2 % MTH for 48 hours of incubation). Hence, 0.125 % MTH was selected to treat the PBMCs in subsequent experiments.

Using microarray approach, the gene expression profile in MTH-treated PBMCs were studied and it was found that 361 genes were significantly regulated by at least two fold changes ($p < 0.05$). Among these genes, the expression of immuno-related genes such as *IFNG*, *IL10*, *IL20*, *IL24*, *CXCL1*, *CXCL3*, *CXCL9*, *IL2* and *IL4* were validated using reverse transcription quantitative real time polymerase chain reaction (RT-qPCR). Functionally, these genes play crucial role in wound healing by facilitating the recruitment of leukocytes to sites of infection, supporting the wound healing activity in honey. Using flow cytometry, the immunomodulatory effect of MTH in activating PBMCs subpopulations was also determined using cell surface marker cluster of differentiation (CD) 69. It was found that MTH did not possessed

any immunosuppressive effect in regulating cell activation in helper T cells (CD3+ CD4+), cytotoxic T cells (CD3+ CD4-) and B cells (CD3- CD19+).

The production of interferon gamma (IFN-γ) and interleukin (IL)-10 from mitogen-stimulated and non-stimulated PBMCs after MTH treatment were also quantified using enzyme-linked immunosorbent assay (ELISA). IL-10 (immunoregulatory cytokine) and IFN-γ (pro-inflammatory cytokine) production were found elevated significantly ($p < 0.05$). These results suggested that MTH involved in early immunoregulation and late pro-inflammatory responses, supporting the antimicrobial activity of honey.

This is the first study ever conducted to investigate the gene expression profile in PBMCs treated with MTH. Overall, present findings showed that MTH possessed immunomodulatory effect by regulating the expression of immune related genes, leading to significant increase in the production of type 1 cytokine (IFN-γ) and type 2 cytokine (IL-10) in PBMCs. This suggested that MTH possessed immunomodulatory effect that can potentially contribute to the antimicrobial and wound healing activities in honey. These findings can further justify the application of MTH as topical dressing especially in wound management potentially by eliminating wound infection as well as promoting wound healing process.

ACKNOWLEDGEMENT

First, I would like to express my sincere gratitude to my supervisors, Dr Kok Wai Ling, Dr Fang Chee Mun and Dr Maha Abdullah for all their guidance, encouragement and advice throughout the progress of this project.

I would like to thank my colleagues, Dr Lim Su Wen, Ms Sree Vaneesa A/P Nagalingam, Mrs Suraya Binti Abdul Sani, Ms Chai Li Chin and late Ms Wong Quin Nee for sharing helpful discussion and suggestions.

I would also like to express my gratitude to Malaysia Ministry of Higher Education for FRGS Grant and MyBrain15 scholarship as well as to the University of Nottingham Malaysia Campus for the PumpPriming Grant.

Lastly, thanks to my family for their support and encouragement.

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%	Percentage
<	Less than
±	Plus-minus
°	Degree Celsius
α	Alpha
γ	Gamma
β	Beta
UMF	Unique Manuka Factor
rpm	Revolutions per minute
w/v	Weight/Volume
v/v	Volume/Volume
v	Volume
w	Weight
M	Molar
mg	Milligram
g	Gram
ml	Millilitre
µl	Microlitre
kGy	Kilogray
AU	Absorbance Unit

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

The relationship between honey bees and human started since the stone age when honey was initially discovered as food source for both nutritional and medicinal purposes (Bogdanov, 2011a). In fact, the medicinal importance and applications of honey have been well documented since the ancient time. These documentations can be found in the world's oldest medicinal literatures such as the first compendium of ancient Chinese Medicine Shen Nang (200 AD) and the ancient Indian Ayruveda classic Ashtanga Hridaya (500 AD) (Bogdanov, 2011b; Mandal & Mandal, 2011). From these ancient literatures, it was stated that honey possessed antimicrobial and wound healing properties and hence honey was used to treat infected wounds even before the discovery of bacteria as the cause of infection (Mandal & Mandal, 2011; Molan, 1992). Traditionally, honey was widely used to enhance general health by boosting the immune system as well as to treat diseases related to pollen hypersensitivity and reduce allergic reactions such as hay fever (Bogdanov, 2011a).

Nowadays, the discoveries of antibiotic resistant pathogens and failure in some conventional modern therapeutic treatments have posed a very serious threat to human health (Mandal & Mandal, 2011; Molan, 1992). These situations have led to the re-evaluation of ancient therapeutic remedies such as honey. At present, honey is probably one of the last untreated natural food in the world (Bogdanov, 2011a). Over the years, several new discoveries and scientific studies had been conducted and reported that honey possessed beneficial properties such as antibacterial, antiviral, antifungal, anti-parasitic, anti-lipid, anti-ulcerous, anticancer, antioxidant, anti-inflammatory and immunological functions (Ghashm *et al*., 2010; Manyi-Loh *et al*., 2011). This has led to the development of an alternative medicine branch, known as apitherapy (Mandal & Mandal, 2011). With more scientific evidence supporting the beneficial properties of honey, it is believed that honey can potentially treat various

diseases in human by naturally boosting the immune system (Mandal & Mandal, 2011).

1.2 Immune system

The immune system is a complex system which protects an organism against pathogenic microbes and infectious agents such as bacteria, viruses, fungi, parasitic worms, cancerous cells and toxic molecules through a series of physical, chemical and biological processes (Doan *et al.*, 2008a). In general, the immune system consists of 3 layers of defense. The first layer of defense is a series of physical, chemical and biological barriers that prevent the entry of pathogens and infectious agents into the host's body. When the first layer of defense is breached, the second layer of defense (innate immune response) and the third layer of defense (acquired immune response) will be activated to eliminate the invading pathogenic microbes from the host's body without damaging its own tissue (Delves & Roitt, 2000).

1.2.1 Innate immune response

Innate immune response is rapidly activated when the pathogens successfully breached the first layer of defense (Doan *et al.*, 2008b). Granular leukocytes and agranular phagocytic cells are activated in the presence of pathogens to induce non-specific immune responses where these immune cells non-selectively kill the pathogens without recognizing their specific identities. These non-specific immune responses include the secretion of cytokines and chemokines by specific immune cells to regulate the function of other immune cells and attract inflammatory leukocytes to the site of infection, production of reactive free radical species and enzymes which promote tissue inflammation, the activation of macrophages, monocytes and neutrophils to perform phagocytosis as well as the activation of complement system (Parkin & Cohen, 2001).

Activated neutrophils can carry out phagocytosis to engulf and destroy the invading pathogens using lysosomal enzymes. Besides phagocytosis, activated neutrophils also have the ability to trigger oxidative burst by secreting free radicals and reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2) and nitric oxide (NO) to kill the pathogens directly. In addition, neutrophils also secrete cytokines such as IL-8 and IL-12 in order to attract other monocytes, macrophages and neutrophils to the site of infection (Martini *et al*., 2005).

Mononuclear cells such as monocytes and macrophages also play a crucial role in innate immune response by performing janitorial and surveillance functions. Generally, monocytes circulate the bloodstream while macrophages, which also derived from monocytes, can cross the endothelium and reside in tissues and organs (Doan *et al*., 2008a). Functionally, both monocytes and macrophages are phagocytic cells that remove cell debris and invasive pathogens through phagocytosis and enzymatic degradation. Monocytes and macrophages also serve as antigen presenting cells (APC) by presenting the degraded pathogen peptides on the major histocompatibility complex class II protein (MHC class II) in order to activate CD4+ helper T cells. In addition, activated monocytes and macrophages have the ability to secrete various cytokines and chemokines such as tumor necrosis factor (TNF)-α, IL-8 and IL-12 to attract other monocytes, macrophages and neutrophils to the site of infection, increase the permeability of vascular endothelium, activate natural killer cells (NK) and promote differentiation of lymphocytes (Martini *et al*., 2005).

NK cells also contribute to innate immune response by recognizing and eliminating abnormal cells such as tumor cells and virus-infected cells (Delves & Roitt, 2000). The role of NK cells in innate immune response is crucial because some viruses such as herpes virus have developed mechanisms to downregulate the expression of major histocompatibility complex class I protein (MHC class I) molecules in virus-infected cells to avoid being killed by CD8+ cytotoxic T cells (Chaplin, 2010). The immunoglobulin receptors (FcR) expressed on the surface of NK

cells can bind to antibody-coated targets which eventually lead to antibody dependent cellular cytotoxicity (Parkin & Cohen, 2001). NK cells also rely on killer-activating receptors (KAR) and killer-inhibitory receptors (KIR) which recognize MHC class I molecules commonly expressed on the surface of all nucleated cells. Upon the activation of KAR receptors, NK cells are ready to kill the targeted cell unless an inhibitory signal from the KIR receptor is activated upon binding with sufficient MHC class I molecules on the surface of targeted cells to prevent the killing process. In tumor cells and virus infected cells, the expression of MHC class I molecules is downregulated and hence these cells are susceptible to NK cells (Delves & Roitt, 2000). During the killing process, activated NK cells secrete perforin to puncture the membrane of targeted cells and granzymes are injected through these pores to induce apoptosis in the targeted cells (Parkin & Cohen, 2001).

1.2.2 Acquired immune response

When the first and second layers of defense are breached, acquired immune responses are activated which involve the recognition of specific antigen by receptors on T and B lymphocytes to generate specific immune responses such as the production of antibodies and direct physical or chemical killing towards that particular antigen (Chaplin, 2010; Delves & Roitt, 2000). In fact, all the immune cells in both innate and acquired immune responses interact together to provide an adequate and effective immune system protection for the host against various pathogenic microbes and infectious agents. This interaction is mediated through immunomodulatory proteins such as cytokines and chemokines (Doan *et al.*, 2008c). In general, acquired immune responses can be categorized into cell-mediated immunity and humoral immunity (Martini *et al*., 2005).

1.2.2.1 Cell-mediated immunity

T lymphocytes such as CD8+ cytotoxic T cells and CD4+ helper T cells play a key role in cell-mediated immunity to fight against abnormal cells and pathogens inside the cells. During antigen recognition process, T cell receptors (TCR) on the surface of CD8+ cytotoxic T cells and CD8+ suppressor T cells recognize antigen presented on MHC class I protein commonly expressed in all nucleated cells. On the other hand, TCR on the surface of CD4+ helper T cells recognize antigen presented on MHC class II protein expressed in APC such as B cells, dendritic cells and macrophages (Martini *et al*., 2005).

CD8+ cytotoxic T cells are involved in antitumor and antiviral activities. Upon activation, CD8+ cytotoxic T cells immediately destroy the targeted cells by inject granzymes into the targeted cells through the cell membrane. These granzymes can activate caspase enzyme to induce deoxyribonucleic acid (DNA) fragmentation and cell apoptosis in the targeted cells (Parkin & Cohen, 2001). Besides, CD8+ cytotoxic T cells also secrete poisonous lymphokines to disrupt cell metabolism in killing the targeted cells (Martini *et al*., 2005).

CD4+ helper T cells are involved in stimulating and coordinating immune responses in both cell-mediated and humoral immunities (Martini *et al*., 2005). Upon activation, helper T cells can be subdivided into type 1 (Th1) cells and type 2 (Th2) cells based on the types of cytokine they produce (Parkin & Cohen, 2001). For example, Th1 cells produce IL-2 which can induce T cell proliferation in an autocrine manner (Gaffen & Liu, 2004). Th1 cells also produce IFN-γ which can activate macrophages and NK cells to kill intracellular pathogens. Generally, the cytokines produced by Th1 cells favour cell-mediated inflammatory responses. Conversely, Th2 cells produce IL-4, IL-5, IL-6 and IL-10 which favour antibody production in B cells (Parkin & Cohen, 2001). For example, IL-10 can regulate the growth and differentiation of B cells while IL-4 can stimulate immunoglobulin (lg) class

switching to lgE and lgG in B cells during the development of humoral immunity (**Table 1.1**) (Moore *et al*., 2001; Pouliot *et al.*, 2005).

Table 1.1 Immunological functions of IFN-γ, IL-2, IL-4 and IL-10 in human

Cytokine	Function
IFN-γ	➢ Induce antimicrobial and antitumor activities in macrophages by up-regulating antigen processing and presentation ➢ Enhance NK cells activity ➢ Promote leukocytes migration to sites of inflammation by up-regulating the expression of adhesion molecules and chemokines (Schroder *et al.*, 2004)
IL-2	➢ Regulate the growth, proliferation and differentiation of T cells ➢ Promote proliferation of B cells and NK cells ➢ Enhance antibody secretion in B cells ➢ Enhance cytolytic activity and cytokine production in NK cells (Gaffen & Liu, 2004)
IL-4	➢ Stimulate proliferation of activated B cells and T cells ➢ Regulate B cells differentiation into plasma cells ➢ Decrease the proliferation of Th1 cells (Zamorano & Rivas, 2003)
IL-10	➢ Inhibit the production of cytokines and chemokines in activated monocytes and macrophages ➢ Regulate the growth and differentiation of B cells ➢ Enhance the activity of NK cells (Pestka *et al.*, 2004)

In addition, cytokines produced by Th1 cells can regulate the immune responses in Th2 cells and vice versa. For example, IFN-γ produced by Th1 cells can inhibit IL-4 production in Th2 cells and hence reduce Th2 cells proliferation. On the other hand, IL-10 produced by Th2 cells can inhibit the production of IFN-γ and IL-2

in Th1 cells and hence reduce Th1 cells proliferation. The acquired immune responses and regulation of Th1 and Th2 cytokines are depicted in **Figure 1.1** below (Wong, 2003).

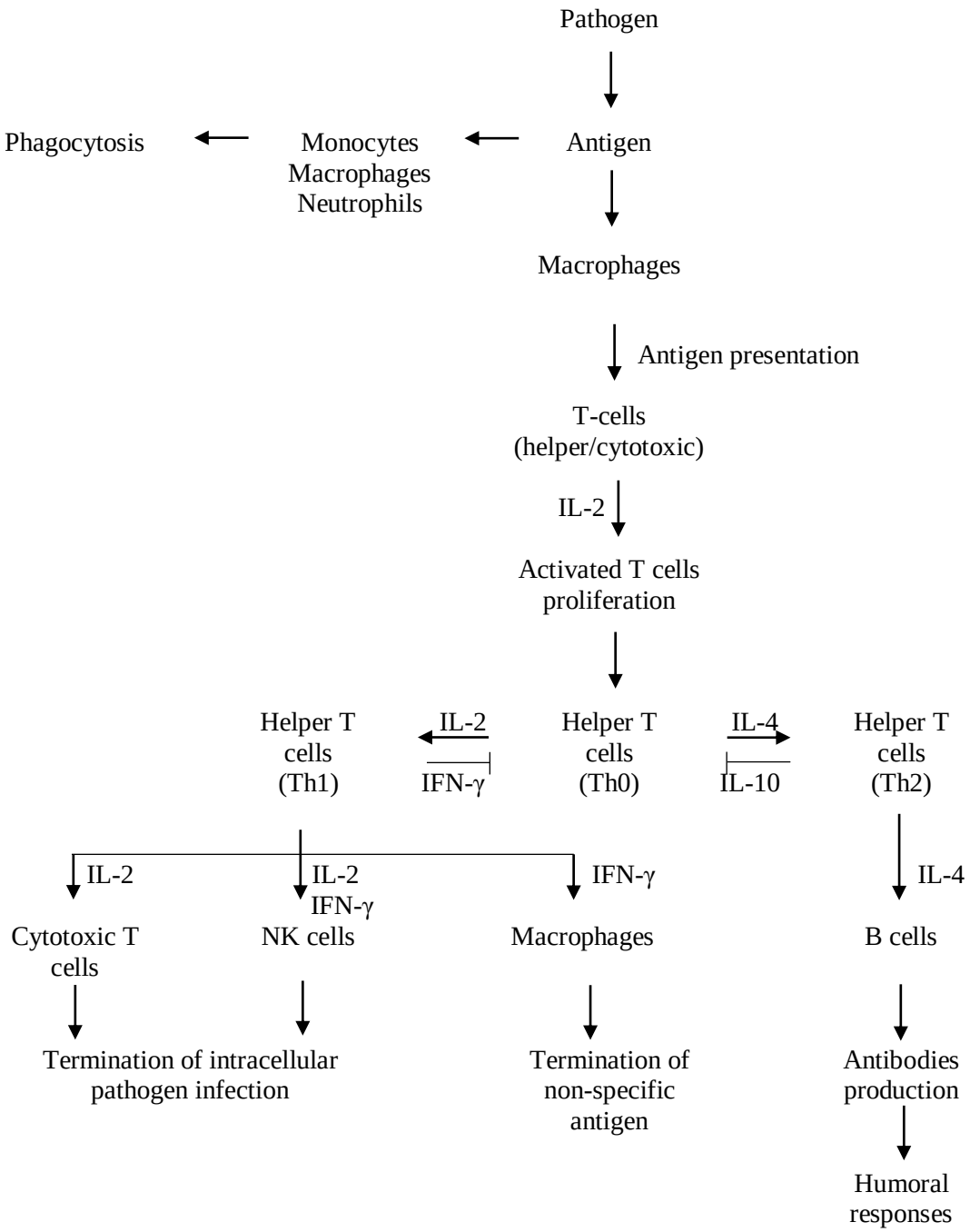


Figure 1.1 Acquired immune responses and regulation of Th1 and Th2 cytokines. (⟶) represents stimulatory effect and (⟞) represents inhibitory effect (Wong, 2003).

1.2.2.2 Humoral immunity

B lymphocytes play a key role in humoral immunity to fight against antigens and pathogens in body fluid. In the presence of antigens, antigens are recognized by the surface lgM of B cells, internalized, processed and re-expressed on MHC class II molecules to be presented to CD4+ helper T cells (Parkin & Cohen, 2001). Upon activation, activated CD4+ helper T cells will produce various cytokines such as IL-2, IL-4 and IL-10 to stimulate the proliferation and differentiation of B cells into plasma cells in order to produce more specific antibodies for that particular antigen (Gaffen & Liu, 2004; Moore *et al*., 2001; Pouliot *et al*., 2005; Schroder *et al*., 2004). These antibodies serve to neutralize toxin, opsonise bacteria for phagocytosis as well as sensitize tumor cells for antibody dependent cytotoxic killing by NK cells (Parkin & Cohen, 2001).

1.3 General overview of honey

Traditionally, many natural foods such as honey, green tea and ginseng are found to be beneficial to human health by boosting the immune system (Christian *et al*., 2000). In recent years, honey has been proven scientifically to possess several beneficial properties such as immunomodulatory, antioxidant, antimicrobial, anti-inflammatory, anticancer and wound healing (Bashkaran *et al*., 2011; Fauzi *et al*., 2011; Mohamed *et al*., 2010; Rodzaian *et al*., 2011; Sulaiman *et al*., 2011; Tan *et al*., 2009).

Nowadays, various types of honey are available in the market such as Tualang, Manuka, Blossom, Honeydew, Chestnut, Buckwheat and many more which showed some variation in their common properties (Bogdanov, 2011a). In this research project, MTH was used as the study material. The MTH has been proven to possess several beneficial properties such as antimicrobial, anti-inflammatory, antioxidant, stimulating immune system and wound healing potential (Ghashm *et al*., 2010). However, the immunomodulatory role of MTH in regulating immune system in

human has not been studied extensively and hence there is still considerable interest to investigate its immunomodulatory aspect.

1.3.1 Types of honey

Honey is a collection of nectar and sweet deposits from plants by honeybees of the genera *Apis* and *Meliponini* (Hussein *et al*., 2011; Manyi-Loh *et al*., 2011). Honey can be categorized into floral honey and non-floral (honeydew) honey depending on the source of nectar (Manyi-Loh *et al*., 2011). Floral honey can be further categorized into unifloral honey or multifloral honey depending on whether the honeybees collected the honey from the nectar of the same flower or various flowers. On the other hand, non-floral honey is normally collected by honeybees from living plant tissues and fruits, as well as from the excretion of insects, commonly aphids, which tap its stylet into the veins of higher plants to harvest sugar deposits (Subrahmanyam, 2007).

In general, there are two main types of honey known as forest honey and apiary honey (Manyi-Loh *et al*., 2011). Forest honey is produced by rock bees (*Apis dorsata*) or wild bees (*Apis cerana indica*) in the forests and is normally harvested using traditional methods such as via bee comb squeezing (Subrahmanyam, 2007). Forest honey products are normally turbid due to the presence of wax, pollen, parts of bees, and bee larvae as well as being prone to microorganism contamination (Hussein *et al*., 2011; Subrahmanyam, 2007). Hence, it is sometimes necessary to process the crude forest honey through filtration system to remove suspended particles or to sterilize using gamma radiation at 25 kGy to remove natural contaminants such as *Clostridium botulinum* prior to marketing (Hussein *et al*., 2011; Postmes *et al*., 1995; Subrahmanyam, 2007). On the other hand, apiary honey is produced by honeybees (*Apis cerana indica* and *Apis mellifera*) in bee yards and is normally harvested using modern extraction methods. Apiary honey products are normally transparent and free of contamination or foreign materials (Subrahmanyam, 2007).

The naming of different honey products is often based on the geographical location where the honey is produced, the trees on which the honey is harvested or the floral source where the honeybees collected the nectar (Lusby *et al.*, 2002). For example, Manuka honey is named based on an indigenous manuka shrub (*Leptospermum scoparium*) in New Zealand where the honeybees (*Apis mellifera*) collected the nectar (Jenkins *et al*., 2011). Another example is Tualang honey which is named based on the Tualang tree (*Koompassia excelsa*) where the Asian rock bees (*Apis dorsata*) build their hives (Ghashm *et al*., 2010).

1.3.2 Nutritional benefits of honey

Honey can be consumed by humans of all ages due to its high nutritional value. When consumed orally, 100 grams of honey can provide 303 kilocalories of energy as its carbohydrates are easily digested and transported rapidly into bloodstream to be utilized into energy to power the body (Blasa *et al*., 2006). Therefore, honey is often recommended for athletes and children (above 1 year old) in order to promote better health and performance (Alvarez-Suarez *et al.*, 2009).

It is reported that toddlers fed with diet containing honey have higher weight increase, higher haemoglobin content in blood, better skin colour, reduce throw up and less susceptible to diseases as compared to toddler fed with diet containing sucrose. Besides, toddlers fed with diet containing honey also show less diarrhoea and produce lighter and thinner faeces due to better calcium uptake into the blood (Bogdanov, 2011a).

In the case of athletes, it is reported that honey can provide a good source of carbohydrate to replenish muscles after training. In the past, honey was considered similar to sugar solution which can cause a rise in blood sugar level, leading to higher insulin production. Higher insulin production often reduces the blood sugar to low level causing hypoglycaemia effect such as dizziness and fatigue. However, recent studies showed that honey intake during training significantly increased the frequency

of heart beat without causing any hypoglycaemia effect as only a mild increase in blood sugar and insulin were detected (Kreider *et al*., 2002, Kreider *et al*., 2000).

1.3.3 Potential hazards of honey

Allergic reaction to honey and honey poisoning are rarely reported in literature but the occurrence of these events cannot be excluded (Bogdanov, 2011a). Similar to other natural food products, honey can be contaminated by heavy metal and pesticides from the environment as well as pollen and toxic substances originated from plants (Bogdanov, 2006). For example, Mad honey originated from Turkey contains plant-based toxic substances such as pyrazolidine alkaloid and diterpenoids. These toxic substances are not lethal upon consumption but can cause negative effects such as dizziness, blurred vision and unconsciousness (Bostan *et al*., 2010) .

There is also health concern in feeding infants (younger than 12 months) with diet containing honey due to the presence of *Clostridium botulinum* which is often linked to infant botulism (Bogdanov, 2011a). The spores of *Clostridium botulinum* can survive but cannot accumulate toxin in honey. However, these spores can germinate within the infant's large intestine and accumulate neurotoxins due to their weak immune system to neutralize these neurotoxins. These neurotoxins can block the release of acetylcholine at the neuromuscular junctions leading to failure in muscle contraction. Generally, infants with botulism will show signs of constipation, difficulty in feeding and lethargy. In severe cases, these conditions can progress to flaccid paralysis of respiratory muscles, posing threat of suffocation (Tanzi & Gabay, 2002). Therefore, it is recommended that only children (above 1 year old) are suitable to take honey diet in order to reduce the risk of *Clostridium botulinum* (Cox & Randy, 2002; Tanzi & Gabay, 2002).

1.3.4 Application of honey in food industry

Since ancient time, honey had been used as sweetener due to its palatable properties and high carbohydrates content. Nowadays, honey is widely used as food additive in poultries, cakes, muffins, ice creams, processed fruit and vegetables as well as in fermented beverages due to its antibacterial and antioxidant properties (Bogdanov, 2011a). The antibacterial and antioxidant properties of honey can help to prevent food spoilage by inhibiting the growth of microorganisms and preventing oxidation spoilage during storage (Chen *et al.*, 2000; McKibben & Engeseth, 2002; Nagai *et al.*, 2006).

In dairy industry, honey is added as prebiotic additive and sweetener into probiotic fermented milk products such as yogurt. This is because honey can inhibit the growth of harmful microorganisms and at the same time enhance the growth of effective bacteria such as *Lactobacillus acidophilus* and *Lactobacillus delbruekii* which are beneficial to human gastrointestinal tract in digesting food and absorbing nutrients (Chick *et al.*, 2001; Sanz *et al*., 2005). In fruit industry, honey is used as coating agent to protect fruits against enzymatic browning and food borne pathogen (Chen *et al*., 2000).

1.4 Origin of Tualang honey

Tualang honey is a multifloral forest honey collected by the world's largest and most ferocious honeybees known as Asian rock bees (*Apis dorsata*) (**Figure 1.2**) which commonly build their hives on Tualang trees (*Koompassia excelsa*) (**Figure 1.3**) (Ghashm *et al*., 2010; Mohamed *et al*., 2010). The hive of Asian rock bee is often disc-like in shape, approximately 2m long and can contain as many as 30,000 bees (Ahmed & Othman, 2013; Mohamed *et al*., 2010).

Figure 1.2 Asian rock bee (*Apis dorsata*) (Gorman, 2010)

Figure 1.3 Tualang tree (*Koompassia excelsa*) in Malaysia (Nodin, 2010)

Tualang tree can be commonly found in Peninsular Malaysia, northeastern Sumatra, Palawan, Borneo and southern Thailand. In the wild, this tree can grow up to approximately 250m tall and Asian rock bees often build their hives on the horizontal branches of Tualang tree. This is because the tree trunk of Tualang tree is often slippery and the branches are formed at least 30m from ground level hence providing some form of protection to the hives from bears (Nurul *et al.*, 2013).

In Thailand, Asian rock bees also often build their hives on the horizontal branches of Keruing tree (*Dipterocarpus*), commonly found in tropical forests. The tree trunk of Keruing tree is smooth and the branches are formed at least 5m from

ground level. These characteristics provide protection to the hives similar to Tualang tree (Mustaffa, 2013).

1.4.1 Origin of MTH

MTH (**Figure 1.4**) is commonly found in the lowland rain forests of Peninsular Malaysia. The chemical composition in MTH collected from different parts of Kedah such as Pedu, Pulai, Sik and Ulu Muda can vary depending on the floral sources available around that particular forest where the Asian rock bees can collect the nectar (Nurul *et al.*, 2013). Traditionally, this local honey has been used by local community as medicinal product to treat various diseases and as food source in Malaysia (Ghashm *et al*., 2010).

Figure 1.4 Agromas® MTH certified by Federal Agricultural and Marketing Authority (FAMA), Malaysia

1.5 Composition of honey

Honey is a solution of concentrated sugar with some complex mixture of other saccharides, proteins, amino acids, organic acids, vitamins, minerals, polyphenols and enzymes (Gheldof *et al.*, 2002). In comparison, different types of honey will have some variations in their composition which show differences in colour, density, aroma and taste, as well as biological properties (Ramirez & Montenegro, 2004). These

variations in composition may be due to the temporal and spatial variation in the source of nectar (Mandal & Mandal, 2011). For example, the different types of flower used by the honeybees, different weather condition when harvesting the honey and different processing procedures may contribute to these variations (Mandal & Mandal, 2011; Ramirez & Montenegro, 2004).

1.5.1 Carbohydrate content in honey

Generally, carbohydrates make up approximately 95 % of the dry weight of honey (Alvarez-Suarez *et al*., 2009). The main sugars in honey are mostly monosaccharides such as glucose and fructose which determine the nutritional and physical properties of honey (Sato & Miyata, 2000). In general, honeys (acacia honey) with high fructose content are sweeter as compared to honeys (rape honey) with high glucose content (Bogdanov, 2011a). Besides monosaccharides, other sugars such as disaccharides (sucrose, trehalose, maltose and turanose), trisaccharides (melezitose) and oligosaccharides (raffinose) are also present in honey but in smaller quantities (Bogdanov, 2011a; Sato & Miyata, 2000). These sugars are formed in honey due to the storage effect of honeybee enzymes and the acidic effect of the honey resulting in acid-catalyzed formation of maltose (Ball, 2007).

In a previous study, the major sugar content in several local honeys such as MTH, Gelam, Acacia and Forest honey were determined using high performance liquid chromatography (HPLC). It was reported that the major sugar content in MTH was found to be 47.13 % glucose, 41.73 % fructose, 1.02 % sucrose and 4.49 % maltose. As for the other local honeys, Gelam, Acacia and Forest honey samples consisted mostly of monosaccharides such as glucose (44.64 % - 50.45 %) and fructose (35.79 % - 44.91 %), as well as disaccharides such as sucrose (0.02 % - 6.09 %) and maltose (0.88 % - 11.69 %) (Chua & Adnan, 2014).

1.5.2 Protein content in honey

Honey contains approximately 0.5 % of proteins which include various amino acids and enzymes such as invertase, diastase and glucose oxidase. Functionally, invertase (glucosidase and sucrase) can convert sucrose into glucose and fructose, diastase (amylase) can convert starch into glycogen and glucose oxidase can convert glucose into H_2O_2 and gluconic acids (Bogdanov *et al.*, 2008; Bogdanov, 2011a). Approximately 18 types of essential and non-essential amino acids can be found in various honeys with proline and lysine being the most prevalent, especially in honeydew honey. Other amino acids such as tryptophan and glutamic acid can also be found but their quantities vary depending on the honey's origin and botanical origin. Typically, honeydew honeys contain higher level of proline as compared to floral honeys (Iglesias *et al.*, 2004).

Based on a previous study, the amino acid composition in MTH, Gelam, and Acacia honey was determined using HPLC and it was found that 70 % (in mass) of the total amino acid content in Acacia honey was contributed by proline. In contrast, only 0.16 % and 0.01 % of proline was found in MTH and Gelam honey respectively. Hence, it was suggested that proline can be potentially used as an amino acid marker to differentiate honeydew honeys from floral honeys (Chua & Adnan, 2014).

1.5.3 Polyphenol content in honey

The polyphenols found in honey are mainly flavonoids (chrysin, luteolin, kaempferol and quercetin), phenolic acids (caffeic acid, benzoic acids and cinnamic acids) and phenolic acid derivatives which contribute to the antioxidant capacity of honey (Martos *et al.*, 2001). In some studies, it was reported that the total polyphenols and flavonoid content found in different honey samples can vary from 56 mg/kg to 500 mg/kg and 2 mg/kg to 46 mg/kg respectively (Al-Mamary *et al.*, 2002; Gheldof & Engeseth, 2002; Kenjeric *et al.*, 2007). These variations depend very much on the botanical origin of honey and the weather condition during harvesting process. In

brief, honey with higher flavonoid content is produced when the honey is harvested during dry season and high temperature (Kenjeric *et al*., 2007).

In a previous study, the polyphenol and flavonoid content in MTH and Borneo tropical honey were determined using Folin-Ciocalteu assay and colorimetric assay developed by Zhishen *et al.* (1999) respectively. It was found that MTH exhibited higher content of polyphenol (383.61 mg gallic acid equivalent (GAE) per kg of honey) and flavonoid (149.77 mg catechin equivalents (CEQ) per kg of honey) when compared to Borneo tropical honey. The polyphenol and flavonoid content in Borneo tropical honey were only 223.20 mg GAE/kg and 31.89 CEQ/kg respectively (Khalil *et al*., 2012).

1.5.4 Organic acid content in honey

The most prevalent acid found in honey is 2,3,4,5,6-pentahydroxyhexanoic acid, which is also known as gluconic acid. Gluconic acid is produced as a by-product when glucose oxidase in honey decompose glucose into H_2O_2 in the presence of water (Iurlina & Fritz, 2005). Besides gluconic acid, other acids such as malic acid, citric acid, succinic acid and acetic acid can also be found in honey but in smaller quantities. Generally, the presence of these acids has given honey an acidic characteristic with pH ranging from 3.4 to 6.1 (Ghazali, 2009).

In a study conducted by Ghazali (2009), it was reported that MTH (Agromas®) had a pH range from 3.39 to 3.90. In comparison, MTH was less acidic when compared to other local honeys such as Gelam honey (pH 3.40 – 3.43), Kelulut Putih honey (pH 2.30 – 2.34) and Kelulut Hitam honey (pH 2.19 – 2.27).

1.5.5 Aromatic volatile compounds in honey

The composition of aromatic volatile compounds between different honey samples can vary depending on their botanical origin (Bogdanov *et al*., 2004). In general, the aromatic volatile compounds found in honey are mainly alcohols, acids, esters,

ketones, aldehydes and terpenes (Zhou *et al.*, 2002). Over the years, many scientific studies have been carried out and more than 500 different types of aromatic compounds have been identified from different honey samples (Bogdanov, 2011a).

In a study conducted by Nurul *et al.* (2013), the chemical compounds in MTH were extracted using 5 solvents (petroleum spirit, hexane, dichloromethane, ethyl acetate and methanol) and analysed using gas chromatography-mass spectrometry (GC-MS). Overall, 35 volatile compounds were detected and classified into hydrocarbons, acids, aldehydes, ketones, alcohols, furans and terpenes (Nurul *et al.*, 2013).

1.5.6 Vitamins, minerals and trace compounds in honey

Various types of vitamins (thiamine, niacin and riboflavin), minerals (calcium and selenium) and trace elements (nickel and chromium) can be found in honey. However, the quantities of these vitamins, minerals and trace elements can vary between different types of honey depending on their botanical and geographical origin (Bogdanov, 2011a). To date, the vitamins, minerals and trace elements in MTH has yet to be quantified.

1.6 Properties of honey

In recent years, many scientific studies were carried out and reported that honey possessed several beneficial properties such as immunomodulatory, antioxidant, antimicrobial, anti-inflammatory, anticancer and wound healing potential. Generally, most of these scientific studies were focused on Manuka honey which was well-known for its antimicrobial activity. Nevertheless, some local researchers believed that MTH probably possessed better properties as compared to imported honeys such as Manuka honey from New Zealand and Jellybush honey from Australia (**Table 1.2**). For example, it was reported previously that MTH was more effective against some gram-negative bacterial strains like *Acinetobacter baumannii* and *Stenotrophomonas*

maltophilia as compared to the well-researched Manuka honey, probably due to its higher content of phenolics and flavonoid (Ahmed & Othman, 2013; Tan *et al*., 2009).

Table 1.2 General properties of MTH and Manuka honey

Properties	Findings	
	MTH	Manuka honey
Immunomodulatory	Improve immune status and reduce the symptoms of sore throat and rhinitis in Malaysian subjects (Sulaiman *et al*., 2011)	Stimulate the production of TNF-α, IL-1β and IL-6 cytokine in human monocytes (Tonks *et al*., 2007)
Anti-inflammatory	Reduce inflammation in the eyes of rabbit after subjected to alkali chemical injury (Bashkaran *et al*., 2011)	Increase the production of IL-6 (anti-inflammatory cytokine) in human peripheral blood monocytes and human monocytes (MM6) cell line (Tonks *et al*., 2003)
Wound healing	Promote wound healing in patients with burn wounds (Rodzaian *et al*., 2011)	Promote wound healing in diabetic patients with neuropathic ulcers (Kamaratos *et al*., 2014)
Antioxidant	High antioxidant activity due to high level of phenolic compounds (Mohamed *et al*., 2010)	High antioxidant activity due to high level of phenolic compounds (Alzahrani *et al*., 2012)
Antimicrobial	Inhibit growth of *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, *Escherichia coli* and *Salmonella typhimurium* (Tan *et al*., 2009)	Inhibit the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* by interrupting their cell division (Jenkins & Cooper, 2012)
Anticancer	Induce apoptosis in human cervical carcinoma (HeLa) and human breast adenocarcinoma (MCF-7 and MDA-MB-231) (Fauzi *et al*., 2011)	Induce apoptosis in murine melanoma (B16.F1), murine colorectal carcinoma (CT26) and human breast cancer (MCF-7) cell lines (Fernandez *et al*., 2013)

1.6.1 Immuno-activating properties of honey

The immuno-activating properties of honey are often related to wound healing as honey is known to clear infection through stimulation of the body's immune system such as increasing the production of lymphocytes and promote the activity of phagocytes (Molan, 2002; Tonks *et al*., 2003). It is believed that this immuno-

activating activity can be found in most honeys but with variation depending on their botanical origin.

It is generally accepted that natural honeys contain substantial amount of immunomodulatory substances such as endotoxin which can trigger and stimulate the immune system (Timm *et al*., 2008). In addition, the acidic pH of honey (normally ranging from pH 3 to pH 6) may also help macrophages in the process to kill bacteria by providing suitable acidic condition to ingest the targeted bacteria into phagocytic vacuole. Furthermore, honey also contains high level of glucose which can be used as substrate for glycolysis to provide energy for macrophages to perform their functions (Molan, 1992).

In a previous study, Abuharfeil *et al.* (1999) investigated the effect of Multifloral honey on the proliferative activity of human peripheral blood lymphocytes. In this study, 0.1 %, 0.2 %, 0.4 % and 0.8 % of Multifloral honey were used to stimulate cell proliferation in B lymphocytes and T lymphocytes. It was reported that 0.1 % and 0.2 % of Multifloral honey were optimal concentrations in stimulating the proliferation of B lymphocytes and T lymphocytes (Abuharfeil *et al.*, 1999). Al-Waili and Haq (2004) further evaluated the effects of Multifloral honey on antibody production in mice against thymus-dependent antigen (sheep red blood cells) and thymus-independent antigens (*Escherichia coli*). Based on their results, a higher level of antibody production was observed in mice fed with regular diet plus Multifloral honey as compared to mice fed with just regular diet. This suggests that antibody production can be stimulated by honey in the process to fight thymus-dependent and thymus-independent antigens (Al-Waili & Hag, 2004). These studies show that honey possesses stimulatory effect on immune response and hence can be potentially used in therapeutic applications in helping immuno-compromised patient

Another study was conducted by Chepulis (2007) to investigate the effect of long term (52 weeks) 10 % Honeydew honey feeding on lymphocytes numbers and neutrophils phagocytosis in rats (Sprague Dawley). In this study, the percentage of

phagocytic neutrophils and lymphocytes in rats fed with 10 % Honeydew honey and regular diet (95 g/kg skim milk powder, 120 g/kg casein, 160 g/kg oil, 50 g/kg cellulose, 50 g/kg mineral mix, 5 g/kg sugar-free vitamin mix and 365 g/kg starch) was significantly ($P < 0.0001$) higher when compared to rats fed with sugar-free regular diet. This shows that honey can enhance immune responses by elevating the production of phagocytic neutrophils and lymphocytes (Chepulis, 2007). In addition, by using Jungle honey collected from blossom and timber by wild bees in the tropical forest of Nigeria, Fukuda *et al.* (2011) found that Jungle honey possessed chemotactic activity for neutrophils. It was reported that the number of peritoneal cells extracted from honey-treated mice ($5.13 \pm 0.28 \times 10^6$ cells/mouse) was 4-fold higher when compared to control mice ($0.17 \pm 0.11 \times 10^6$ cells/mouse). Under microscopic observation, these peritoneal cells were determined as neutrophils. Besides, by using mice implanted with Lewis Lung Carcinoma/2 (LL/2) cells model pretreated with Jungle honey, they found that tumor incidence was 20 % in Jungle honey-injected mice as compared to 100 % in control mice. The production of ROS such as O_2^- and H_2O_2 was significantly ($P < 0.001$) higher in peritoneal cells extracted from Jungle honey-treated mice as compared to control mice. Collectively, these results suggest that Jungle honey can stimulate the activation of neutrophils to produce ROS which can contribute to the antitumor activity of honey (Fukuda *et al.*, 2011).

Besides stimulating immune cell proliferation and antibody production, honey was also found to enhance the production of various cytokines in immune cells. In a study conducted by Tonks *et al.* (2007), it was found Manuka honey enhanced the production of TNF-α, IL-1β and IL-6 cytokines in MM6 cells. It was also reported that the active compound in Manuka honey that induced cytokine production in MM6 cells was a 5.8-kDa heat-sensitive compound. This compound stimulated the MM6 cells through interaction with toll-like receptor 4 (TLR4), which was responsible in recognizing antigens such as the lipopolysaccharides isolated from Gram-negative bacteria. Collectively, these results suggest that Manuka honey can enhance the

production of cytokines in monocytes through interaction with TLR4 (Tonks *et al*., 2007).

Besides the 5.8-kDa compound found in Manuka honey, there were also several findings that managed to identify other components responsible for the immuno-activating activity in honey. Some studies reported that this immuno-activating activity may be due to the presence of natural stimulant such as lectin or bacterial endotoxin (lipopolysaccharide) which can stimulate immunomodulatory responses in immune cells (Abuharfeil *et al*., 1999; Timm *et al.*, 2008). Other studies suggested that this immuno-activating activity may be due to the presence of active compounds such as caffeic acid, chrysin or apalbumin1 (a royal jelly protein) which can also be found in honey (Lin *et al*., 2012; Majtan *et al.*, 2006).

To date, the understanding of immunomodulatory property of MTH is limited. In 2011, a study was conducted by Sulaiman *et al.* (2011) to investigate the effectiveness of MTH in reducing acute respiratory symptoms among Malaysians. In this study, the volunteers were influenza-vaccinated and given 20 g MTH (oral consumption) for 42 days. For control, the volunteers were also influenza-vaccinated but were not given MTH. All volunteers were asked to score each acute respiratory symptoms such as sore throat and rhinitis that they had throughout the study. Based on their results, it was found that daily consumption of MTH had significantly reduced the symptoms of sore throat and rhinitis in Malaysian subjects by improving their immune status as compared to control group (Sulaiman *et al*., 2011). In this study, the exact mechanisms by which MTH can boost the immune system and the compounds responsible for this immuno-activating effect are not known and hence this represents an area for further study.

1.6.2 Immuno-suppressive properties of honey

Besides immuno-activating properties, some studies also showed that honey possesses immuno-suppressive properties (Duddukuri *et al.*, 2001; Mesaik *et al.*, 2008) These

findings were consistent with the traditional belief that honey can be used to treat diseases related to pollen hypersensitivity and reduce allergic reactions such as hay fever. In some cases, it is also believed that the immuno-suppressive properties of honey are often related to its anti-inflammatory properties (Bogdanov, 2011a).

A study was conducted by Mesaik *et al.* (2008) to investigate the effects of *Plectranthus* honey, *Ziziphus* honey, *Citrus* honey, *Acacia modesta* honey, Swiss honey, Chestnut honey and Clover honey on phagocytic respiratory burst in human whole blood, human polymorphonuclear neutrophils (PMNs), mice peritoneal macrophages and mononuclear cells (MNCs). It was reported that higher concentration of honey (32 µg/ml) resulted in lower ROS production (hypochlorous acid, H_2O_2 and hydroxyl free radicals) as compared to lower concentration of honey (2 µg/ml). Both hypochlorous acid and hydroxyl free radicals were primarily produced by phagocytic myeloperoxidase undergoing oxidative burst. Hence, it was suggested that honey possibly altered the level of ROS production by inhibiting phagocytic myeloperoxidase. However, the level of phagocytic myeloperoxidase was not measured in this study and therefore the direct inhibition of honey in phagocytic myeloperoxidase cannot be determined. The identification of specific compounds responsible for this inhibitory activity are still underway (Mesaik *et al*., 2008). This result shows that honey possesses immuno-suppressive effect by inhibiting ROS production possibly through the inhibition of phagocytic myeloperoxidase.

Besides inhibiting ROS production, some honeys were also found to inhibit the production of antibody in immune cells. For example, in another study conducted by Duddukuri *et al.* (2001), it was found that Rock bee honey significantly ($p < 0.01$) inhibited the production of ovalbumin-specific lgA, lgM, lgG_1 and lgG_{2b} in mice intraperitoneally injected with ovalbumin and Rock bee honey when compared to controls. The exact mechanism of how Rock bee honey inhibited the production of these antibodies in mice is still unclear and the purification of key compounds responsible for this immuno-suppressive effect is still underway (Duddukuri *et al*.,

2001). Nevertheless, this study shows that honey may possess immuno-suppressive effect in regulating antibody production.

To date, the immuno-suppressive activity of MTH has not been studied extensively. In a local study conducted by Ashaari *et al.* (2013), it was reported that ingestion of MTH improved the symptoms of allergic rhinitis (hay fever) in Malaysian patients. Allergic rhinitis is an inflammatory disease commonly characterized as inflammation of the mucosa surface leading to airway blockage and sinuses. In this study, allergic rhinitis patients were given loratidine (antihistamine) followed by 1 g/kg body weight of honey daily for 8 weeks. For control, allergic rhinitis patients were also given similar amount of loratidine and honey-flavored corn syrup as placebo. The symptoms of allergic rhinitis such as nasal blockage, sneezing, nasal itchiness, eye itchiness, palatal itchiness, hyposmia and rhinorrhea were observed throughout the study. Overall, the MTH-treated group showed significant progressive improvement, whereas the improvement in control group were found to decline following the termination of loratidine. The exact mechanisms of how MTH improve the symptoms of allergic rhinitis were not determined in this study. Nevertheless, Ashaari *et al.* (2013) postulated several mechanisms to explain the improvement in the symptoms of allergic rhinitis after MTH ingestion. They postulated that MTH could have suppressed lgE-mediated hypersensitivity reaction in these patients. The ingestion of MTH may also induce oral tolerance to the aeroallergens present in MTH. This can possibly decrease the chance of an overwhelming immune responses such as anaphylactic reaction when exposed the similar aeroallergen subsequently. Thirdly, MTH can possibly reduce nasal inflammation due to its anti-inflammatory activity and hence open up the nasal airway (Ashaari *et al.*, 2013).

1.6.3 Wound healing properties of honey

Traditionally, honey has been used to enhance the healing of ulcers, burns and infected wounds (Rodzaian *et al*., 2011). In a normal wound healing process, series of

events such as inflammation, cell division and tissue remodeling occur to remove the damaged tissue and replace with new tissue. During the inflammatory process, neutrophils and macrophages are attracted to the injured region. The function of neutrophils is to remove foreign materials, bacteria and cell debris through phagocytosis or proteolytic mechanisms. On the other hand, the function of macrophages is to perform phagocytosis and produce specific mediators such as ROS and cytokines to initiate the wound healing process (Tonks *et al*., 2003).

A study was conducted by Kamaratos *et al.* (2014) to investigate the effect of Manuka honey-impregnated dressing on the healing of neuropathic diabetic foot ulcers. In this study, type II diabetic patients with lower limb neuropathic ulcers were recruited and treated with Manuka honey-impregnated dressing for 16 weeks. For control, the patients were treated with conventional saline-soaked gauze dressing. It was reported that the mean duration of ulcers healing for Manuka honey-treated patients (31 ± 4 days) was significantly ($p < 0.05$) shorter when compared to control (43 ± 3 days). Overall, it was suggested that the wound healing activity of Manuka honey represented a potential effective treatment for neuropathic diabetic foot ulcers with shorter wound healing duration (Kamaratos *et al.*, 2014).

It is believed that the wound healing activity of honey is related to its antimicrobial and anti-inflammatory activities (Rodzaian *et al*., 2011; Tonks *et al*., 2003). In brief, the antimicrobial activity of honey is mainly due to the presence of organic acids (acidic pH), H_2O_2 and high osmolarity with low water activity which can effectively remove infections from the wounds and eventually accelerate the wound healing process (Rodzaian *et al*., 2011; Sukur *et al.*, 2011). During wound healing process, various pro-inflammatory cytokines (IL-1β and TNF-α) and anti-inflammatory cytokines (IL-6 and IL-10) are being secreted by macrophages in order to initiate and control the wound healing process. The pro-inflammatory cytokine like TNF-α can affect almost all types of tissues and is chemotactic for macrophages. Functionally, TNF-α can promote the activation of macrophages, promote

angiogenesis, stimulate fibroblast proliferation and initiate fibroblast to synthesize prostaglandin and collagenase during wound healing process. Besides, TNF-α can also increase the level of IL-6, an anti-inflammatory cytokine which can promote the epithelialization of the wound. (Tonks *et al*., 2003). In addition, honey also contains significant amount of nutrients which can be used to promote cell division and cell growth during the epithelialization process (Rodzaian *et al*., 2011).

1.6.3.1 Wound healing properties of MTH

A local study was conducted by Rodzaian *et al.* (2011) to investigate the healing properties of MTH and silver-based dressing in partial thickness burn wounds. It was reported that MTH-based dressing exhibited shorter average epithelization time (18 days), lower mean number of dressing changes (5 dressing changes) and shorter length of hospital stay (5 days) as compared to silver-based dressing which required 21 days for epithelization, 6 dressing changes and 10 days of hospital stay. Rodzaian *et al.* (2011) also speculated that the wound healing activity of MTH may be due to the ability of MTH to promote cell reparative and inhibit the growth of major wound infecting bacteria. Besides, the high nutrient content in MTH may also contribute to its wound healing activity by enhancing angiogenesis and epithelization. This finding shows that MTH-based dressing can be potentially used as an alternative in burn wound treatment because it is probably effective as compared to conventional dressing in promoting wound healing process (Rodzaian *et al*., 2011).

Similar finding was obtained by Khoo *et al.* (2010) who reported that MTH showed better antibacterial activity and wound healing effect when applied on full-thickness burn wounds in rats when compared to hydrofibre. In their study, burn wounds were created on the dorsum of rats and inoculated with *Pseudomonas aeruginosa, Klebsiella pneumonia* and *Acinetobacter baumannii*. The wounds were treated topically with undiluted MTH (0.1 ml/cm^2), hydrofibre and hydrofibre silver respectively for 21 days. It was found that MTH treatment reduced the wound size by

12.86 % from the original 100 mm^2 after 3 days ($p = 0.010$) and was further reduced by 33.94 % after 9 days ($p = 0.001$). In comparison, hydrofibre silver treatment reduced the wound size by 2.20 % after 3 days ($p = 0.010$) and was further reduced by 13.74 % after 9 days ($p = 0.003$). Additionally, on day 21, only MTH-treated burn wounds inoculated with *Pseudomonas aeruginosa* healed completely. This finding shows that MTH possesses wound healing activity and it is an effective topical dressing for full-thickness burn wounds (Khoo *et al*., 2010).

In another animal study conducted by Bashkaran *et al.* (2011), it was found that MTH treatment was as good as the conventional treatment in treating alkali injury on the eyes of rabbits. In this study, the rabbits were treated with 1 drop of MTH (30 %) diluted in normal saline for 4 times per day for a week and oral administration of 1.0 g MTH per kg of weight per day for a week. For control, the rabbits were treated with conventional treatment using 1 drop of 1 % Prednisolone acetate, 1 drop of 0.3 % Ciprofloxacin for 4 times per day for a week and oral administration of 0.6 g ascorbic acid per kg of weight per day for a week. Based on their result, it was reported that MTH treatment was as good as the conventional treatment in treating corneal edema, epithelial healing and conjunctival hyperemia. There was no significant difference ($p > 0.05$) in the clinical inflammatory features (**Appendix 1**) observed between both treatments. This result shows that MTH possesses anti-inflammatory properties as well as wound healing potential and hence can be used as alternative to conventional treatment in treating alkali injury (Bashkaran *et al*., 2011).

1.6.4 Antioxidant properties of honey

The development of many chronic diseases is often associated with oxidative stress due to the imbalance between free radical production and level of antioxidants. This oxidative stress can lead to various cellular damages such as damaging the structural and functional properties of lipids, proteins and nucleic acids as well as causing

biological complications such as aging, mutagenesis, carcinogenesis and atherosclerosis (Kishore *et al.*, 2011).

In recent years, the antioxidant activity of various honey samples had been tested and a significant relationship was observed between the antioxidant activity and the amount of phenolic compounds present in honey (Bogdanov, 2011a). Among all the antioxidant compounds found in honeys, the most important classes are phenolic acids and flavonoids which can inhibit metal ions such as iron and copper, which behave as catalyst in the formation of free radicals (Makawi *et al.*, 2009). Besides phenolic acids and flavonoids, some honeys also contain significant amount of other antioxidant compounds such as protein, amino acids, ascorbic acids, organic acids, hydroxymethylfurfural and Maillard reaction products, as well as enzymes including glucose oxidase and catalase (Bogdanov, 2011a; Makawi *et al*., 2009).

A study was conducted by Alzahrani *et al.* (2012) to evaluate the antioxidant activity of Manuka honey, Acacia honey and Wild Carrot honey using Folin assay and ferric reducing ability of plasma (FRAP) assay. It was found that Manuka honey had the highest total phenolic content (899.09 ± 11.75 mg gallic acid/kg honey) when compared to Acacia honey (627.56 ± 44.03 mg gallic acid/kg honey) and Wild carrot honey (503.09 ± 8.29 mg gallic acid/kg honey). In terms of antioxidant potential, the antioxidant activity in Manuka honey (1.2106 ± 0.005 AU) and Acacia honey (1.366 ± 0.06 AU) were found to be higher when compared to Wild Carrot honey (0.6386 ± 0.05 AU). In this study, it was also speculated that Manuka honey may contain methylglyoxal (MGO) and trimethoxybenzoic acid that potentially contributed to its antioxidant activity (Alzahrani *et al*., 2012).

1.6.4.1 Antioxidant properties of MTH

Similar to other honeys, the total phenolic content and antioxidant potential in MTH were also successfully determined in several previous studies. For example, a study was conducted by Mohamed *et al*. (2010) to evaluate the antioxidant properties of

MTH based on the colour intensity (spectrometric detection at 450 nm), total phenolic content (Folin assay) and total antioxidant activity (FRAP assay). It was reported that the colour intensity, total phenolic content and total antioxidant activity of MTH were comparable and within the range of other honeys such as Slovenian honeys (Forest honey, Fir honey and Chestnut honey) and Multifloral honey (**Table 1.3**).

Table 1.3 The colour intensity, total phenolic content and total antioxidant activity of MTH, Slovenian honeys and Multifloral honey

Honey	Colour intensity at 450nm (mAU)	Total phenolic content (mg gallic acid/kg honey)	Total antioxidant activity (µM Fe(II)/kg honey)
MTH	489.5 ± 1.7	251.7 ± 7.9	322.1 ± 9.7
Forest	467.0 ± 74	233.9 ± 21.7	426.4 ± 41.5
Fir	405.0 ± 60	241.4 ± 39.5	478.5 ± 95.5
Chestnut	495.0 ± 73	199.9 ± 34.1	360.1 ± 66.5
Multifloral	415.0 ± 10	170.4 ± 1.7	361.9 ± 10.8

The results were expressed as mean ± standard deviation (Mohamed *et al.*, 2010)

It was also highlighted that the colour intensity of honey samples correlated with its total phenolic content as well as antioxidant activity (Mohamed *et al*., 2010). As reported by Bogdanov (2011a), honey samples with dark colour intensity contained higher phenolic content and potentially better antioxidative power. Similar findings were also reported by Kishore *et al.* (2011), Khalil *et al.* (2011) as well as Khalil *et al.* (2012). These researchers suggested that the high level of phenolic compounds found in MTH may contribute to its high antioxidant capacity and free radical scavenging activity (Kishore *et al*., 2011; Khalil *et al*., 2012; Khalil *et al*., 2011). In fact, Kishore *et al.* (2011) highlighted that MTH may possess even better antioxidant activity as compared to other honeys such as local Gelam honey, Pineapple honey and Indian Forest honey (**Table 1.4**).

Table 1.4 The total phenolic content, total flavonoid content and total antioxidant activity of MTH, Gelam honey, Indian Forest honey and Pineapple honey

Honey	Total phenolic content (mg gallic acid/100g honey)	Total flavonoid content (mg quercetin/100g honey)	Total antioxidant activity (μM Fe(II)/100g honey)
MTH	83.96 ± 4.53^{a}	50.45 ± 1.83^{a}	121.89 ± 3.87^{a}
Gelam	74.12 ± 2.52^{b}	45.11 ± 0.71^{a}	115.61 ± 3.86^{a}
Indian Forest	45.63 ± 0.66^{c}	36.69 ± 0.74^{b}	73.35 ± 4.04^{b}
Pineapple	27.75 ± 0.80^{d}	24.74 ± 0.35^{c}	47.92 ± 1.76^{c}

The results were expressed as mean ± standard deviation. Significantly different values ($p < 0.05$) were represented by different letters. (Kishore *et al.*, 2011)

Additionally, Khalil *et al.* (2011) highlighted that MTH exhibited the highest content of flavonoids, phenolic compounds, total antioxidant activity and radical scavenging activity (IC_{50} of DPPH inhibition) as compared to other local honeys such as Gelam honey and Borneo Tropical honey (**Table 1.5**). They also managed to identify 6 phenolic acids (gallic acid, caffeic acid, syringic acid, trans-cinnamic, benzoic acid and p-coumaric acid) and 5 flavonoids (catechin, luteolin, apigenin, naringenin and kaempferol) in their MTH (AgroMas®) samples using HPLC. However, the content of flavonoids, phenolic compounds and total antioxidant activity of MTH were significantly lower when compared to the Manuka honey (**Table 1.5**).

Table 1.5 The total phenolic content, total flavonoid content, total antioxidant activity and radical scavenging activity of MTH, Gelam honey, Borneo Tropical honey and Manuka honey

Honey	Total phenolic content (mg gallic acid/kg honey)	Total flavonoid content (mg quercetin/kg honey)	Total antioxidant activity (µM Fe(II)/kg honey)	Radical scavenging activity (mg/ml)
MTH	42.23 ± 0.64^{b}	25.31 ± 0.37^{b}	892.15 ± 4.97^{c}	5.24 ± 0.40^{e}
Gelam	26.20 ± 0.56^{d}	19.47 ± 0.23^{d}	644.28 ± 9.53^{d}	14.36 ± 0.83^{b}
Borneo Tropical	15.21 ± 0.51^{f}	11.52 ± 0.27^{f}	492.04 ± 11.25^{e}	17.51 ± 0.51^{a}
Manuka	52.63 ± 1.21^{a}	34.55 ± 0.45^{a}	1295.34 ± 10.35^{a}	4.71 ± 0.36^{e}

The results were expressed as mean ± standard deviation. Significantly different values ($p < 0.05$) were represented by different letters. (Khalil *et al.*, 2011)

In another study conducted by Khalil *et al.* (2012), it was reported that the polyphenol and flavonoid contents of MTH samples (AgroMas®) collected from 11 different regions in Kedah Rain Forest, Malaysia ranged from 305.47 to 419.86 mg gallic acid/kg honey (Folin assay) and 135.29 to 165.34 mg catechin/kg honey (total flavonoid assay) respectively. It was also reported that the total antioxidant activity ranged from 273.46 to 292.34 µM Fe(II)/kg MTH (FRAP assay) (Khalil *et al*., 2012).

Collectively, these results show that MTH has better antioxidant properties when compared to other local honeys but may vary depending on the source and origin of the MTH samples. In contrast, MTH still has comparatively lower antioxidant properties as compared to Manuka honey.

1.6.5 Antimicrobial properties of honey

Traditionally, honey has been used to treat infected wounds with no negative effects on wound healing process (Molan, 1992). To date, various honey samples have been tested for standardized level of antimicrobial activity prior to marketing (Mandal &

Mandal, 2011). Among all the tested honey samples, Tualang honey and Manuka honey are most well known for their significant antimicrobial activity and are proven to be effective against a broad range of bacteria, fungi, viruses and parasites (Mandal & Mandal, 2011; Molan, 1992).

The Manuka honey has been widely researched and its antimicrobial potential is renowned worldwide. It has been reported that Manuka honey has inhibitory effect against 60 species of bacteria including some pathogenic strains such as *Helicobacter pylori* and MRSA (Jenkins *et al*., 2011; Mandal & Mandal, 2011; Molan, 1992). In a study conducted by Jenkins *et al.* (2011), it was reported that 5 % to 20 % weight/volume (w/v) Manuka honey was sufficient to inhibit the growth of MRSA by interrupting its cell division (Jenkins *et al*., 2011). In another study conducted by Jenkins and Cooper (2012), the combinations of 5 antibiotics (Rifampicin, Tetracycline, Imipenem, Mupirocin and Colistin) and 5 % (w/v) Manuka honey (Manukacare 18+) were found to have better inhibition against MRSA and *Pseudomonas aeruginosa* as compared to antibiotics alone (**Table 1.6**) (Jenkins & Cooper, 2012). In this study, the minimum inhibitory concentration (MIC) for each combination of antibiotics and 5 % Manuka honey was determined using broth dilution method.

Table 1.6 MIC for each combination of Rifampicin, Tetracycline, Imipenem, Mupirocin and Colistin with 5 % (w/v) Manuka honey against MRSA and *Pseudomonas aeruginosa*

Antibiotic	MIC (µg/ml) antibiotic			
	MRSA		*Pseudomonas aeruginosa*	
	Antibiotic alone	Antibiotic + 5 % Manuka	Antibiotic alone	Antibiotic + 5 % Manuka
Rifampicin	0.0156	0.0156	8	4
Tetracycline	0.5	0.0312	32	16
Colistin	-	-	2	0.5
Imipenem	16	0.05	-	-
Mupirocin	0.06	0.0078	-	-

(Jenkin & Cooper, 2012)

Overall, these findings have provided a new avenue of future treatments for wound infections caused by MRSA and possibly other antibiotics-resistant bacteria strains using honey. To date, microbial resistance to honey has not been reported and hence honey remains a preferred choice to be used as antimicrobial agent especially against antibiotic-resistant bacteria (Mandal & Mandal, 2011).

The antibacterial activity in honey can be either bacteriostatic or bactericidal depending on the concentration of honey being used against a particular bacteria (Taormina *et al.*, 2001). Generally, higher concentrations of honey (5 % to 50 %) were found to be bactericidal (Molan, 1992). In fact, it has been suggested that the antibacterial activity of honey may be due to several factors such as the presence of antibacterial compounds like H_2O_2, the presence of various sugar components which contributes to high osmolarity with low water activity, the presence of organic acids which contributes to acidic pH as well as the presence of different phenolic compounds and phytochemical antibacterial substances like royal jelly acid and defensing-1 protein added by honeybees into the honey (Molan, 1992; Taormina *et al.*, 2001).

One of the main antimicrobial component contributing to the antimicrobial activity in honey is H_2O_2 (Temaru *et al.*, 2007). This product is produced when glucose oxidase decompose glucose into gluconic acid and H_2O_2 in the presence of water during the dilution of honey (Iurlina & Fritz, 2005). When H_2O_2 is being broken down by catalase, free radicals are produced which can directly kill bacteria. Subsequently, these free radicals are removed by the antioxidant compounds such as phenolic and flavonoid present in honey through electron donation mechanism in order to reduce further damage to the host cells (Manyi-Loh *et al*., 2011). However, some honey products such as Jellybush honey from Australia and Manuka honey from New Zealand also show antimicrobial activity even in the absence of H_2O_2 (Alvarez-Suarez *et al*., 2009). Some researchers suggested that the antimicrobial activity in these honey products may be due to the presence of non-peroxide biological active components such as methyl syringate and MGO. Hence, these honey products are often known as 'non-peroxide' honeys (Manyi-Loh *et al*., 2011).

Besides H_2O_2, the sugar components present in honey also contribute to the antimicrobial properties of the honey. These sugar components have high affinity for water molecules and hence leaving minimal water to support the growth of microorganisms. As a result, the microorganisms become dehydrated and eventually die due to water deficiency (Malika *et al.*, 2004).

The presence of organic acids and gluconic acids in honey has also given honey an acidic characteristic with pH ranging from 3.4 to 6.1. This acidic condition is sufficient to inhibit the growth of several pathogens such as *Escherichia coli* and *Salmonella typhimurium*, which require optimum pH from 6.0 to 7.0 and 6.5 to 7.5 respectively for optimal growth (Mandal & Mandal, 2011).

In addition, the presence of phenolic compounds such as benzoic acid, cinnamic acid and flavonoids also contributes to the antimicrobial activity of honey even though this contribution is relatively small as compared to H_2O_2. Nonetheless, the chemical reaction between H_2O_2 and benzoic acid can yield peroxyacids which is a

more powerful antimicrobial component and oxidizing agent. Similar to free radicals, peroxyacid can also be removed by antioxidant compounds present in honey through electron donation mechanism to prevent further damage to the host cells (Weston, 2000).

1.6.5.1 Antimicrobial properties of MTH

Similar to Manuka honey, MTH had also been reported to be effective against several microorganisms as well as human pathogens such as *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, *Escherichia coli* and *Salmonella typhimurium* (Mandal & Mandal, 2011; Tan *et al*., 2009). A study was conducted by Tan *et al.* (2009) to compare the antibacterial activity of MTH and Manuka honey against wound and enteric microorganisms. In this study, the MIC of MTH and Manuka honey against 8 Gram-negative bacteria (*Stenotrophomonas maltophilia*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Salmonella enterica*, *Proteus mirabilis*, *Shigella flexneri* and *Enterobacter cloacae*) as well as 5 Gram-positive bacteria (MRSA, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus pyogenes* and coagulase-negative *Staphylococcus aureus*) were determined using broth dilution method. Based on their results, it was reported that the MIC for MTH and Manuka honey ranged from 8.75 % to 25.00 % and 8.75 % to 20.00 % respectively (**Table 1.7**).

Table 1.7 MIC values for MTH and Manuka honey against 13 microorganisms

Microorganisms	MIC (%)	
	MTH	Manuka
Stenotrophomonas maltophilia	8.75	8.75
Escherichia coli	22.50	17.50
Pseudomonas aeruginosa	17.50	17.50
Acinetobacter baumannii	11.25	12.50
Salmonella enterica	15.00	15.00
Proteus mirabilis	20.00	17.50
Shigella flexneri	20.00	17.50
Enterobacter cloacae	25.00	20.00
MRSA	12.50	8.75
Staphylococcus aureus	20.00	11.25
Streptococcus agalactiae	20.00	15.00
Streptococcus pyogenes	12.50	11.25
Coagulase-negative *Staphylococcus aureus*	12.50	11.25

(Tan *et al*., 2009)

Overall, MTH showed variations in its antimicrobial activity but these variations were comparable to Manuka honey. These findings suggest that MTH can be potentially used as an alternative antimicrobial agent against certain microorganisms such as *Acinetobacter baumannii*, *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* (Tan *et al*., 2009).

1.6.6 Anticancer properties of honey

To date, several chemotherapeutic drugs used in cancer treatments are losing their effectiveness due to the development of drug resistance in some cancer cells. The development of drug resistance in cancer cells may be due to several factors such as individual metabolic variations, genetic heterogeneity in the tumor cells or the expression of drug transporters which limit the accumulation of drug within the cells. For example, the growth of breast cancer cells can initially be attenuated using tamoxifen (TAM). However, prolonged treatment of TAM can cause resistance in the

breast cancer cells and eventually the tumor cells will continue to grow even in the presence of the drug (Yaacob *et al.*, 2013).

In order to overcome this issue, researchers are now focusing on the potential use of natural products such as honey and cordyceps as anticancer or anti-proliferative agent (Fauzi *et al*., 2011; Ghashm *et al*., 2010). Over the years, various honey samples had been tested against different cancer cell lines such as colon cancer (HCT-15 and HT-29), bladder cancer (253J, T24, RT4 and MBT-2), fibrosarcoma and mammary carcinoma in order to evaluate their anticancer and anti-proliferation abilities (Ghashm *et al.*, 2010; Jaganathan & Mandal, 2010; Swellam *et al*., 2003).

A study was conducted by Fernandez *et al.* (2013) to investigate the anticancer activity of Manuka honey on murine melanoma (B16.F1), murine colorectal carcinoma (CT26) and human breast cancer (MCF-7) cell lines. In this study, all cancer cell lines were treated with Manuka honey (0.3 % to 2.5 %) for 24 hours, 48 hours and 72 hours. For positive control, the cancer cells were treated with 10 ng/ml taxol. Based on their result, the percentages of cell viability observed in B16.F1 cell line treated with 0.3 %, 0.6 %, 1.25 % and 2.5 % Manuka honey for 24 hours were 85 %, 75 %, 60 % and 40 % as compared to control (90 %). When the incubation time increased to 72 hours, the reduction in cell viability became more apparent. Similar trends were also observed in CT26 and MCF-7 cell lines, suggesting that the anticancer effect of Manuka honey on these cancer cell lines was in a time and concentration dependent manner. Additionally, Fernandez *et al.* (2013) also reported that the anticancer activity of Manuka honey was mediated through the activation of caspase 9-dependent apoptosis pathway, leading to the induction of caspase 3, reduction in Bcl-2 expression (cell-survival regulatory protein consisting pro-apoptotic and anti-apoptotic regulators), DNA fragmentation and eventually cell death (Fernandez *et al*., 2013).

The anticancer and anti-proliferation activities in honey are mostly due to the presence of several antioxidant compounds such as phenolic acids, flavonoid and

hydroxymethylfurfural as well as polyphenols such as caffeic acid, chrysin, kaempferol and pinocembrin (Jaganathan & Mandal, 2009; Tsiapara *et al*., 2009). These phenolic compounds are able to inhibit cancer cell proliferation and transformation by down-regulating various cellular enzymatic pathways such as cyclooxygenase, protein tyrosine kinase and ornithine decarboxylase pathways (Ghashm *et al*., 2010). Besides, honey can also inhibit the growth and proliferation of cancer cells through the regulation of cell cycle, induction of mitochondrial outer membrane permeabilization, activation of mitochondrial pathways, induction of apoptosis pathways, regulation of oxidative stress, regulation of inflammation process and inhibition of angiogenesis (Fauzi *et al*., 2011; Pichichero *et al*., 2010). In addition, some honey products can also activate and increase the population of neutrophils in bloodstream to elevate the production of ROS in order to combat the growth of cancer cells (Fukuda *et al*., 2011).

1.6.6.1 Anticancer properties of MTH

A study was conducted by Fauzi *et al.* (2011) to investigate the anticancer potential of MTH in human cervical carcinoma (HeLa), human breast adenocarcinoma (MCF-7 and MDA-MB-231) and normal human breast epithelial (MCF-10A). In this study, the cancer cells were treated with MTH (1 % to 10 %) for up to 72 hours and the cytotoxic effect of MTH was determined. It was reported that MTH was cytotoxic to all cancer cell lines in a time-dependent and concentration-dependent manner but not cytotoxic to the normal breast epithelial cells. Besides, the maximum cytotoxic effect and effective concentration of 50 % inhibition (EC_{50}) were also determined in this study (**Table 1.8**).

Table 1.8 Cytotoxic effect of MTH on HeLa, MCF-7, MDA-MB-231 and MCF-10A cell lines

Cell lines	Maximum cytotoxic effect of MTH			EC_{50} (%)
	Concentration of MTH (%)	Incubation duration (hour)	Cell death (%)	
HeLa	5	72	100	2.4
MCF-7	5	24	91	2.4
MDA-MB-231	10	24	93	2.8
MCF-10A	5	72	28	-

(Fauzi *et al.*, 2011)

Using flow cytometry approach, it was also reported that the percentages of MDA-MB-231 (81.1 %), MCF-7 (73.8 %) and HeLa (73.4 %) cell lines with reduced mitochondrial membrane potential after MTH treatment (based on EC_{50} values) for 24 hours were significantly ($p < 0.05$) higher when compared to each untreated cell lines (between 16.1 % and 21.0 %). All cancer cell lines treated with MTH (based on EC_{50} values) for 6 hours also showed apoptosis and activation of caspase-3/7 and caspase 9 when analyzed using fluorescence microscopy approach, indicating the involvement of mitochondrial apoptotic pathway during apoptosis. These findings show that MTH has significant anticancer activity by inducing apoptosis in cancer cells through depolarization of the mitochondrial membrane. In addition, it was also suggested that the anticancer activity of MTH can be potentially due to the presence of polyphenols such as kaempferol, chrysin, caffeic acid and caffeic acid phenyl ester by down-regulating various enzymatic pathways (Fauzi *et al.*, 2011).

Similar findings were also obtained by Ghashm *et al.* (2010) who reported that MTH had anti-proliferative and apoptotic effect on oral squamous cell carcinoma (OSCC) and human osteosarcoma (HOS) cell lines. In this study, the cell viability of OSCC and HOS cell lines treated with 1 % to 20 % MTH for various incubation time (3 hours, 6 hours, 12 hours, 24 hours and 48 hours) was determined using MTT assay. It was found that the cell viability in OSCC and HOS cell lines were reduced by MTH

in a concentration and time dependent manner, with EC_{50} of 4.0 % and 3.5 % respectively. Using annexin V apoptosis assay kit and flow cytometry approach, it was also reported that MTH can induce apoptosis in OSCC and HOS cell lines in concentration and time dependent manner. In contrast, most of the untreated cells were still alive after incubation. Similarly, Ghashm *et al.* (2010) also speculated that the anticancer activity of MTH in their study may be due to the presence of phenolic compounds such as flavonoids (Ghashm *et al*., 2010).

1.7 Research question and rationale of study

Based on literature review, MTH was found to possess immunoregulatory, wound healing and antimicrobial activities. It was also reported that the immunomodulatory effect of MTH in PBMCs could be either immuno-activating or immuno-suppressive. This triggered our interest to investigate the immunomodulatory effect of MTH in regulating the gene expression profile, cell activation and cytokines production in PBMCs. In this study, we hoped to provide scientific evidence to support and justify the application of MTH particularly in wound healing management from the aspect of immunomodulation.

1.8 Aims and objectives

This study was aimed at providing a holistic picture of the immunomodulatory effect of MTH in PBMCs. The general aims of this research project include investigating the potential immunomodulatory effect of MTH on gene expression profile, cell activation and cytokines production and in PBMCs. This study was not aimed at studying the composition of MTH. In fact, the immunomodulatory effect of MTH in PBMCs was studied based on the whole MTH and not its specific composition.

Specific objectives:

1. To determine the optimal concentration of MTH and incubation time which can yield at least 90 % cell viability in THP-1 and U-937 monocytic cell lines before testing with PBMCs. This was achieved by determining the cytotoxic effect of MTH in these cells using MTT assay.

2. To screen the gene expression profile in MTH-treated PBMCs using Agilent microarray platform. The gene expression profile was analysed using GeneSpring GX software (version 11.5). Gene ontology analysis was also carried out using Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources online software (version 6.7).

3. To investigate the immunomodulatory effect of MTH on PBMCs activation. This was achieved by identifying different PBMCs subpopulations using lineage specific marker antibodies conjugated with fluorochromes and analysed using flow cytometry approach. Cell activation marker (CD69) was also used to identify activated PBMCs subpopulations after MTH treatment.

4. To examine the effect of MTH on type 1 and type 2 cytokines production in PBMCs. This was achieved by quantifying the level of these cytokines in MTH-treated PBMCs using ELISA approach.

CHAPTER 2: GENERAL METHODOLOGY

The cytotoxic effect of MTH was initially determined in human monocytic cell lines (THP-1 and U-937) before testing with PBMCs isolated from healthy donors. This was carried out to determine the optimal concentrations of MTH and incubation time that can yield at least 90 % cell viability in PBMCs before proceeding to downstream assays and experiments such as microarray, flow cytometry and ELISA. In this chapter, the general methods used to culture and maintain human monocytic cell lines, isolation of PBMCs from blood samples, cell culture media preparation and chemical reagent preparation were listed accordingly. In subsequent chapters, a more detailed methodology for each assay was listed separately.

2.1 Source of human monocytic cell lines

In biomedical research, several monocytic cell lines such as THP-1, U-937 and MM6 are commonly used to study immune responses such as cytokines production, expression of inflammatory genes and activation of transcription factors *in vitro* (Chanput *et al.*, 2015). In present study, both THP-1 and U-937 cell lines were selected and tested against MTH.

The THP-1 and U-937 cell lines were obtained from the American Type Culture Collection (ATCC). The THP-1 cell line is a human monocytic leukaemia cell line derived from the blood of a 1-year old male patient with acute monocytic leukaemia. The U-937 cell line is a human pro-monocytic leukaemia cell line derived from the histiocytic lymphoma of a 37-years old male patient. Morphologically, both THP-1 and U-937 cells are large and round monocytes with a single nucleus. The main advantage of using these monocytic cell lines is their homogeneous genetic background which minimizes the degree of variability in cell phenotypes. Besides, these cell lines can also be cultured *in vitro* up to 25 passages (approximately 3 months) without affecting its sensitivity and cell activity (Chanput *et al*., 2015).

Both THP-1 and U-937 cell lines used in this study were suspension cells. These cell lines were propagated in Roswell Park Memorial Institute media (RPMI) supplemented with 10 % fetal bovine serum (FBS) and incubated at 37 °C in a humidified atmosphere of 5 % carbon dioxide for optimal growth condition.

2.1.1 Thawing frozen cells

Initially, frozen THP-1 and U-937 cell lines were stored in cryovials (Thermo Fisher Scientific, Massachusetts, USA) and kept in liquid nitrogen freezer (Statebourne, Washington, USA) at -196 °C. During the thawing process, the frozen cells were removed from liquid nitrogen freezer and gently swirled in water bath (Julabo, Pennsylvania, USA) at 37 °C until a small piece of ice was left in the cryovial. The cryovial was wiped with 70 % ethanol (Labcon, California, USA) and transferred into Biosafety Cabinet Class II (Esco, Pennsylvania, USA). Inside the Biosafety Cabinet Class II, a 50 ml falcon tube (BD, New Jersey, USA) was labelled accordingly and filled with 5 ml of pre-warmed R10 medium. In order to recover the frozen cells, 1 ml of pre-warmed R10 medium was pipetted (Brand, Wertheim, Germany) in dropwise manner into the cryovial and mixed gently. The thawed cell suspension was transferred into the labelled 50 ml falcon tube and centrifuged (Eppendorf, Stevenage, England) at 1800 rpm for 5 minutes at 23 °C using swing-bucket rotor. The supernatant was discarded into Virkon solution and the cell pellet was resuspended gently with appropriate volume (normally 3 ml to 5 ml) of pre-warmed R10 medium to break the cell clump. The cell suspension was transferred into either T25 or T75 tissue culture flask (Orange Scientific, Braine-I'Alleud, Belgium) depending on the initial number of cells in the cryovial. Next, additional R10 medium was added into the tissue culture flask if necessary. Lastly, the cell suspension was incubated at 37 °C in a humidified atmosphere of 5 % carbon dioxide.

2.1.2 Growing and maintaining human monocytic cell lines

In order to maintain the THP-1 and U-937 cell lines, appropriate volume of pre-warmed R10 medium was added into the flask every 2 to 3 days to reset the pH of medium and to achieve a suitable cell density for each cell line. This procedure was conducted inside the Biosafety Cabinet Class II. On a daily basis, the colour of R10 medium was examined for pH change and the cultures were examined under inverted microscope (Nikon, Tokyo, Japan) to determine their confluency. These cultures were passaged when the colour of R10 medium became orange and when they were approximately 80 % confluent.

2.1.3 Passaging human monocytic cell lines

For the passage process, the human monocytic cell cultures were transferred into 50 ml falcon tube inside Biosafety Cabinet Class II. These cell suspensions were centrifuged at 1800 rpm for 5 minutes at 23 °C using swing-bucket rotor to remove any metabolic wastes, cell debris and dead cells in the suspension. The supernatant was discarded into Virkon solution and the cell pellet was resuspended gently with 3 ml of pre-warmed R10 medium to break the cell clump. The cell suspension was subjected to cell counting (**Section 2.1.5**) to determine the total number of viable cells. Based on the total number of viable cells, suitable volume of cell suspension was transferred into a new culture flask and appropriate volume of pre-warmed R10 medium was added to achieve the desired seeding density. The seeding density as recommended by ATCC for THP-1 and U-937 cell lines were 2 x 10^5 cells/ml and 1 x10^5 cells/ml respectively. The passaged cell cultures were incubated at 37 °C in a humidified atmosphere of 5 % carbon dioxide.

2.1.4 Freezing cells (cryopreservation)

Practically, the cell density suitable for freezing should be between 5 x 10^6 cells to 10 x 10^6 cells in 1 ml of freezing reagent. The human monocytic cell cultures ready for

cryopreservation were transferred into 50 ml falcon tube inside Biosafety Cabinet Class II. These cell suspensions were centrifuged at 1800 rpm for 5 minutes at 23 °C using swing-bucket rotor to remove any metabolic wastes, cell debris and dead cells in the suspension. The supernatant was discarded into Virkon solution and the cell pellet was resuspended gently with 3 ml pre-warmed R10 medium to break the cell clump. The cell suspension was subjected to cell counting (**Section 2.1.5**) to determine the total number of viable cells. After knowing the total number of viable cells, the cell suspension was centrifuged again at 1800 rpm for 5 minutes at 23 °C using swing-bucket rotor to remove the R10 medium. The supernatant was discarded into Virkon solution and the cell pellet was resuspended with appropriate volume of pre-chilled freezing reagent to achieve the desired cell density per 1 ml of freezing reagent. Next, 1 ml of the homogeneous cell suspension was aliquoted into labelled cryovials accordingly. The labelled cryovials were placed in pre-chilled Mr Frosty freezing container (Thermo Fisher Scientific, Massachusetts, USA) containing isopropanol and stored in -80 °C freezer (Angelantoni Industrie, Massa Martana, Italy) overnight to allow a controlled rate of freezing process (approximately 1 °C decrease in temperature per minute). The cryovials were then transferred into liquid nitrogen freezer for long term storage.

2.1.5 Cell counting

After harvesting the suspension cells from tissue culture flask, 50 µl of cell suspension and 50 µl of Trypan blue exclusion solution (Sigma-Aldrich, Missouri, USA) (ratio 1:1) were mixed evenly in a 1.5 ml microcentrifuge tube. Approximately 10 µl of cell suspension was pipetted into the loading chambers of a hemacytometer (Hirschmann Laborgerate, Eberstadt, Germany) as shown in **Figure 2.1** below.

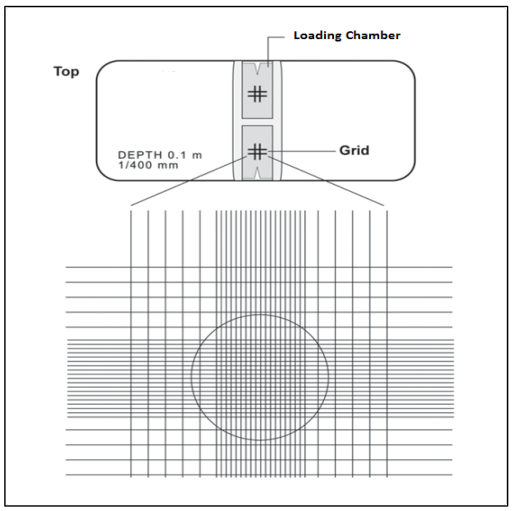


Figure 2.1 Layout of the gridlines in the loading chamber of a hemacytometer

The hemacytometer was placed under an inverted microscope and observation was made using 10x objective lens. Under microscopic observation, the viable cells appeared as bright cells while dead cells were stained blue. Based on the layout in **Figure 2.1** above, all the viable cells located within the large central gridded square (1 mm^2) was counted and the total number of viable cells per ml was calculated using the formula below.

Number of viable cells per ml: Average number of viable cells x Dilution factor x 10^4

2.2 Source of PBMCs

The PBMCs are blood cells with round nucleus such as monocytes, macrophages, B cells, T cells, NK cells and natural killer T cells, which play a crucial role in the immune system. Over the years, PBMCs have been used widely in many immunological and clinical studies because PBMCs can mimic the *in vivo* system of immune responses very closely (Mihajlovic *et al.*, 2014). In fact, the application of PBMCs in immunological studies has enabled researchers to study and understand the mechanisms involved in human immune system in the process of fighting infection as well as cancer diseases (Martini *et al.*, 2005).

In this study, the PBMCs were isolated from blood samples collected from 6 healthy Malaysian donors (mean age 26 years old) using density gradient centrifugation (ethics reference number: BT27042011, University of Nottingham Malaysia Campus) (**Appendix 2**). Once isolated from blood samples, these fresh PBMCs were maintained in R10 medium and incubated at 37 °C in a humidified atmosphere of 5 % carbon dioxide.

2.2.1 Isolation of PBMCs from blood samples

The collection of blood samples from healthy donors was conducted by trained medical personnel in the University of Nottingham Malaysia Campus. During blood collection, a sterile 25 ml syringe attached to a sterile hypodermic needle (Terumo, Somerset, New Jersey) was used to draw 25 ml of blood from the forearm of the donors. The blood collected was transferred into 50 ml falcon tube containing 200 µl of heparin sodium salt solution (20 mg/ml) (Nacalai tesque, Kyoto, Japan) and mixed evenly by gently inverting the tube few times. All blood samples were labelled accordingly and the isolation of PBMCs was conducted within the same day.

Inside the Biosafety Cabinet Class II, 20 ml of blood was diluted with 20 ml of phosphate buffer saline (PBS 1x) (ratio 1:1). The diluted blood was mixed evenly by gently inverting the tube few times. Next, 10 ml of Ficoll-Paque Plus solution (GE Healthcare, Buckinghamshire, England) was transferred into two 50 ml falcon tubes. Using a serological pipette (Nunc, Massachusetts, USA), 20 ml of diluted blood was pipetted slowly to overlay onto the Ficoll-Paque Plus solution in each of the 50 ml falcon tubes (**Figure 2.2**).

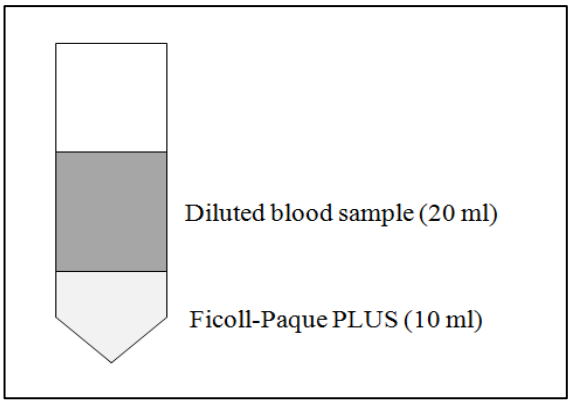


Figure 2.2 Layers of blood and Ficoll-Paque Plus solution before density gradient centrifugation

The layers of diluted blood and Ficoll-Paque Plus solution were centrifuged at 2000 rpm for 20 minutes at 23 °C using swing-bucket rotor without brake. After centrifugation, 4 resultant layers (**Figure 2.3**) were obtained which included the plasma (top layer), PBMCs (second layer), Ficoll-Paque Plus solution (third layer) and erythrocytes as well as granulocytes (bottom layer).

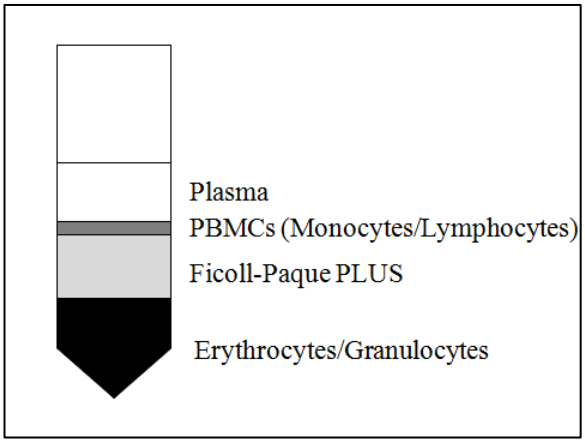


Figure 2.3 Layers of blood and Ficoll-Paque Plus solution after density gradient centrifugation

The PBMCs layered between the interface of plasma and Ficoll-Paque solution was harvested carefully using pasteur pipette and transferred into a new 50 ml falcon tube. The PBMCs were washed twice with approximately 30 ml to 50 ml of

phosphate buffer saline (PBS 1x) and centrifuged at 1600 rpm for 10 minutes at 23 °C using swing-bucket rotor. The supernatant was discarded and the cell pellet was resuspended gently with 5 ml of pre-warmed R10 medium to break the cell clump. The cell suspension was subjected to cell counting (**Section 2.1.5**) to determine the total number of viable cells. After knowing the number of viable cells, the fresh PBMCs were seeded directly into 96-wells U bottom plates (Orange Scientific, Braine-I'Alleud, Belgium) for experimental setup.

2.3 Media preparation

In this study, R10 medium was used to support the growth of THP-1, U-937 as well as to maintain the survival of PBMCs. All the components for each of the following supplemented RPMI were measured by either using a weighing scale (Sartorius, Goettingen, Germany) or graduated 50 ml falcon tubes which were then transferred into 500 ml Schott's bottle (Schott Duran, Mainz, Germany) and subsequently filtered using Nalgene Filtration System inside Biosafety Cabinet Class II. All filtered RPMI media supplemented with FBS were kept in 4 °C fridge (Panasonic, Osaka, Japan) and pre-warmed in water bath at 37 °C before use.

RPMI media with 10 % FBS (R10): 5.2 g RPMI powder (Sigma-Aldrich, Missouri, USA), 1 g sodium bicarbonate (Sigma-Aldrich, Missouri, USA), 50 ml FBS (JR Scientific, California, USA) and 5 ml penicillin streptomycin (Cellgro, Manassas, Virginia) were dissolved in 445 ml distilled water.

RPMI media with 5 % FBS (R5): 5.2 g RPMI powder and 25 ml FBS were dissolved in 475 ml distilled water.

RPMI media with no FBS (R0): 5.2 g RPMI powder was dissolved in 500 ml distilled water.

2.4 Chemical solutions preparation

All the components for each of the following solutions were measured using pipette or weighing scale and transferred into either 500 ml Schott's, 50 ml falcon tube, 1.5 ml microcentrifuge tube, 500 ml volumetric flask, 250 ml volumetric flask, 100 ml volumetric flask or 25 ml volumetric flask (all from Favorit, Puchong, Malaysia) depending on the total final volume.

2.4.1 Cell culture reagents

Freezing reagent: 1 ml dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Missouri, USA) and 9 ml FBS were mixed evenly and filtered into 50 ml falcon tube using 0.45 µm minisart filter (Sartorius, Goettingen, Germany). The filtered freezing reagent was kept in -20 °C freezer and thawed in water bath at 37 °C before use. Once thawed, the filtered freezing reagent was stored on ice until it was ready to use.

Phosphate buffer saline (PBS 20x): 1.44 g sodium dihydrogen phosphate (Merck, Darmstadt, Germany), 80 g sodium chloride, 2 g potassium dihydrogen phosphate and 2 g potassium chloride (all from R & M Chemicals, Alberta, Canada) were dissolved in 500 ml distilled water. The pH was adjusted to pH 7.2 using 2 M sodium hydroxide solution (Merck, Darmstadt, Germany) and autoclaved (Tomy, Tokyo, Japan) at 121 °C for 15 minutes.

Phosphate buffer saline (PBS 1x): 2.5 ml PBS 20x was diluted with 47.5 ml distilled water in 50 ml falcon tube and mixed evenly.

Heparin sodium salt solution (20 mg/ml): 50 mg heparin sodium salt powder was dissolved in 2.5 ml phosphate buffer saline (PBS 1x) and filtered into 1.5 ml microcentrifuge tube using 0.45 µm minisart filter. The filtered heparin sodium salt solution was kept in 4 °C fridge. The heparin sodium salt solution is an anticoagulant that can prevent the formation of blood clots in the blood samples collected from healthy donors before the isolation of PBMCs.

Phorbol 12-myristate 13-acetate (PMA): 5 mg PMA powder (Nacalai tesque, Kyoto, Japan) was dissolved in 1 ml DMSO (Sigma-Aldrich, Missouri, USA). This stock reagent (5 mg/ml) was kept in -20 °C freezer. PMA is a mitogen commonly used to stimulate gene expression, biosynthesis of protein, cell differentiation and enzymatic activity in different immune cells (Goel *et al.*, 2007).

Ionomycin calcium salt from *Streptomyces conglobatus* (I): 1 mg of ionomycin powder (Nacalai tesque, Kyoto, Japan) was dissolved in 1 ml DMSO. This stock reagent (1 mg/ml) was kept in -20 °C freezer. In addition to PMA, ionomycin is also used in immune cells stimulation to trigger calcium release for the signalling of nuclear factor of activated T cells (NFAT) which is expressed in most immune cells (Goel *et al*., 2007).

2.4.2 Cytotoxic assay reagents

The cytotoxic effect of MTH on THP-1 cell line, U-937 cell line and PBMCs was initially determined using MTT assay. The MTT assay is a colorimetric method that involves the conversion of water soluble MTT reagent by dehydrogenase in the mitochondria of viable cells into an insoluble formazan that directly correlates to the number of viable cells. The formazan is then solubilized in DMSO and quantified by measuring its absorbance at 570 nm using a standard microplate absorbance reader (Berridge & Tan, 1993). Besides MTT, another formazan-based cell viability detection assay which can also be used is 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay. In the presence of viable cells, the tetrazolium salt in XXT assay is cleaved by succinate reductase in the mitochondria of viable cells to form formazan that directly correlates to the number of viable cells (Silva *et al.*, 2008). In addition to formazan-based detection method, there are also other cell viability detection methods such as trypan blue exclusion and propidium iodide staining method. However, the trypan blue exclusion method may be less accurate as compared to formazan-based method because the viable cells are counted

manually by the operator under microscopic observation. As for propidium iodide staining, this method requires expensive detection equipment such as flow cytometer and fluorescence microscope.

MTT reagent (5 mg/ml): 25 mg MTT powder (Invitrogen, Massachusetts, USA) was dissolved in 5 ml PBS 1x. Occasionally, the MTT solution was vortexed using vortex machine (LMS, Turnov, Czech) to dissolve all the MTT powder. Due to light sensitivity, the MTT reagent was kept in 50 ml falcon tube wrapped with aluminium foil. The MTT reagent was stored in 4 °C fridge.

2.4.3 RT-qPCR reagents

The RT-qPCR is a method commonly used to study gene expression. Theoretically, this method reverse transcribes messenger ribonucleic acid (mRNA) into complementary deoxyribonucleic acid (cDNA) using reverse transcriptase and further amplifies these cDNA using DNA polymerase while incorporating SYBR Green fluorescence dye into the newly synthesized double stranded DNA for subsequent detection and measurement. Another mode of detection is by using the TaqMan probe (consist of a fluorophore and quencher in close proximity) that specifically hybridizes to the complementary target sites of the targeted gene. During DNA amplification, the Taq polymerase will cleave the TaqMan probe to break the close proximity between the fluorophore and quencher. Thus, this will relieve the quenching effect and allow the fluorophore to emit fluorescence which can be subsequently detected using qPCR thermal cycler. In both detection methods, the fluorescence emitted is directly proportional to the amount of DNA template present in the PCR process (Holland *et al.*, 1991). In comparison, the TaqMan probe platform is more specific as compared to the SYBR Green platform. Nevertheless, the SYBR Green platform was used in present study because it was more cost effective when working with multiple genes without compromising its specificity.

Generally, there are two types of qPCR quantification, namely relative quantification and absolute quantification. The relative quantification is based on the relative expression of the target gene compared to another reference gene (housekeeping gene). Housekeeping genes such as glyceraldehyde-3-phosphate (GapDH), albumin, actins, 18S ribosomal ribonucleic (rRNA) and 28S rRNA are commonly used as reference because these genes are present in all nucleated cells and their expressions are considered stable in various tissues even under experimental treatment conditions. On the other hand, absolute quantification is determined based on a standard curve of known transcript concentration. Practically, RT-qPCR can produce rapid quantification results and more accurate as compared to conventional agarose gel based PCR (Pfaffl, 2001). In this study, RT-qPCR was used to validate the microarray results by looking at the gene expression levels in PBMCs treated with MTH.

1 M Tris chloride solution (pH 8): 3.94 g Tris chloride (Fisher, Pennsylvania, USA) was dissolved in 25 ml distilled water. The pH was adjusted to pH 8 using 2 M sodium hydroxide solution. The solution was autoclaved at 121 °C for 15 minutes.

0.5 M ethylenediaminetetraacetic acid (EDTA) (pH 8): 4.65 g EDTA (Promega, Wisconsin, USA) was dissolved in 25 ml distilled water. The pH was adjusted to pH 8 using 2 M sodium hydroxide solution. The solution was autoclaved at 121 °C for 15 minutes.

TE buffer (pH 8): 1 ml of 1 M Tris chloride solution and 0.2 ml of 0.5 M EDTA solution were mixed evenly.

2.4.4 Flow cytometry reagents

Flow cytometry is a powerful research tool which can be used to analyse various microscopic particles such as cells. Basically, flow cytometry utilizes antibodies conjugated with fluorochromes to detect the presence of specific cell markers and can simultaneously determine multiple characteristics of cells within a mixed population

based on size, phenotype and structural complexity (Rahman, 2006). In this study, flow cytometry was used to identify the PBMCs subpopulation (helper T cell, cytotoxic T cell, B cell and NK cell) after MTH treatment and determine the immunomodulatory effect of MTH on PBMCs activation.

FACS buffer: 0.5 g bovine serum albumin (Sigma-Aldrich, Missouri, USA), 5 ml FBS and 2.5 ml PBS 20x solution were added into 42.5 ml distilled water and filtered using 0.45 µm minisart filter. The filtered FACS buffer was kept in 4 °C fridge.

2.4.5 ELISA reagents

ELISA is a technique commonly used to quantify the concentration of specific proteins or antigens in cell lysate, serum or supernatant solutions using enzyme-linked antibodies. Upon binding of proteins or antigens, the enzymes (horseradish peroxidase, alkaline phosphatase and carbonic anhydrase) can convert the colourless substrate solutions such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 3,3',5,5'-tetramethylbenzidine substrate solution (TMB) into coloured product which can be measured directly using spectrophotometer. Next, the colour change intensity is compared with a standard curve to calculate the concentration of proteins in the initial samples.

There are 2 types of ELISA, namely the direct ELISA and sandwich ELISA. The direct ELISA uses a solid-phase binding support to capture the tested protein. On the other hand, the sandwich ELISA uses a matched pair of protein-specific capturing antibodies and detection antibodies which can significantly improve its specificity and sensitivity to detect picogram quantities of proteins. This makes the sandwich ELISA a preferred choice for measuring cytokines in biological samples (Lequin, 2005). Hence, the sandwich ELISA approach was used in this study to quantify the production of cytokines in PBMCs after MTH treatment. Besides ELISA, western blot coupled with gel electrophoresis can also be used as alternative to detect the presence

of specific proteins in supernatant solutions. However, this method is less quantitative as compared to ELISA because the quantification of protein is based on the thickness of band compared to a standard ladder marker with known size (Kurien & Scofield, 2006).

PBS: 1.16 g disodium hydrogen phosphate (Merck, Darmstadt, Germany), 8.0 g sodium chloride, 0.2 g potassium dihydrogen phosphate and 0.2 g potassium chloride were dissolved in 1000 ml distilled water. The pH was adjusted to pH 7.4 using 2 M sodium hydroxide and autoclaved at 121 °C for 15 minutes.

Assay diluent: 5 ml FBS was added into 45 ml PBS solution and filtered using 0.45 µm minisart filter. The filtered assay diluent was kept in 4 °C fridge.

Coating buffer: 8.40 g sodium hydrogen carbonate (Systerm, Shah Alam, Malaysia) and 3.56 g sodium carbonate (R & M Chemicals, Alberta, Canada) were dissolved in 1000 ml distilled water. The pH was adjusted to pH 9.5 using sodium hydroxide and autoclaved at 121 °C for 15 minutes.

Wash buffer: 0.5 ml Tween-20 (Acros Organics, Geel, Belgium) was added into 199.5 ml PBS solution and mixed evenly.

Stop solution for ELISA (2 N sulphuric acid): 9.8 ml sulphuric acid (R & M Chemicals, Alberta, Canada) was added into 90.2 ml distilled water in the fume hood (Esco, Pennsylvania, USA).

Capturing antibody solutions: The pre-titrated capturing antibodies for IFN-γ, IL-2, IL-4 and IL-10 were diluted (1:200) in coating buffer and kept in 4 °C fridge.

Detection antibody solutions: The pre-titrated biotinylated detection antibodies for IFN-γ, IL-2, IL-4 and IL-10 were diluted (1:200) in assay diluent and kept in 4 °C fridge.

Avidin-horseradish peroxidase solutions (Avidin-HRP): The Avidin-HRP for IFN-γ, IL-2, IL-4 and IL-10 were diluted (1:1000) in assay diluent and kept in 4 °C fridge.

TMB solution: The TMB Substrate A solution was mixed immediately (1:1) with TMB Substrate B solution before use.

Standard solutions: The lyophilized standards for IFN-γ, IL-2, IL-4 and IL-10 were reconstituted with 200 µl assay diluent and mixed well for 15 minutes. The stock standard solutions for IFN-γ and IL-2 were serially diluted with assay diluent to produce 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml, 15.6 pg/ml and 7.8 pg/ml standard solutions. Similarly, the stock standard solutions for IL-4 and IL-10 were also serially diluted with assay diluent to produce 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml, 15.6 pg/ml 7.8 pg/ml and 3.9 pg/ml standard solutions.

CHAPTER 3: METHOD DEVELOPMENT AND OPTIMIZATION

3.1 Introduction

Traditionally, the MTH has been used by local community as medicinal product and as food source (Ghashm *et al*., 2010). In recent years, this local honey has gained more attention among the science community and some researchers believe that this local honey may have better properties as compared to other local honey like Gelam honey as well as the more well-known Manuka honey originated from New Zealand. As different honey products are produced from different floral sources, there are often variations in their composition and biochemical activities (Fauzi *et al*., 2011). To date, many publications have reported that MTH contains various bioactive components such as phenolic acids, flavonoids and bio-chemical compounds (**Table 3.1**).

Table 3.1 List of active chemical compounds found in MTH and Manuka honey

Phenolic acids/Flavonoids		Bio-chemical compounds	
MTH	Manuka honey	MTH	Manuka honey
Benzoic acid	Benzoic acid	5-(hydroxymethyl)-furfural	5-(hydroxymethyl)-furfural
Gallic acid	Gallic acid	2-furylmethylketone	Methylglyoxal
Syringic acid	Syringic acid	5-methyl furfural	4-methylacetophenone
Caffeic acid	Apigenin	Acetic acid	2,3-dimethoxynaphthalene
Catechin	Salicylic acid	Furfural alcohol	1-methoxy-4-propyl-benzene
Luteolin	Methyl syringate	Palmitic acid	4-hydroxy-3-methoxybenzoic acid methyl ester
Kaempferol	Pinocembrin	Ethyl linoleate	2,6-dimethoxybenzoic acid benzyl ester
P-coumaric acid	Chrysin	Oleic acid	4,4-dimethoxystilbene
Naringenin	Abscisic acid	Linoleic acid	1-(2-hydroxy-6-methoxyphenyl)-ethanone
Cinnamic acid	Phenyllatic acid	Octadecanoic acid	1,4-bis(methoxyphenyl)-1-pentanone

(Ahmed & Othman, 2013; Khalil *et al*., 2012; Kishore *et al*., 2011)

Based on literature review, different honey samples such as Manuka honey, Pasture honey, Jellybush honey and Kanuka honey had been tested against MM6, THP-1, U-937 cell lines as well as human peripheral blood lymphocytes in order to determine the cytotoxic effect of these honeys on different immune cells (Abuharfeil *et al.*, 1999; Gannabathula *et al.*, 2011; Tonks *et al.*, 2003). In a previous study, Abuharfeil *et al.* (1999) reported that 0.1 % (v/v) and 0.2 % (v/v) of Multifloral honey stimulated cell proliferation in B lymphocytes (60.8 %) and T lymphocytes (54.4 %) respectively after 72 hours of incubation. This immuno-stimulatory activity may be due to the presence of natural immunomodulatory substances such as endotoxins or lectins in the Multifloral honey which can trigger cell activation and proliferation. However, increasing the concentration of Multifloral honey (0.1 % to 0.8 %) subsequently reduced the proliferation of B lymphocytes and T lymphocytes, most probably due to the osmotic effect of the honey where water molecules were drawn out from cells (Abuharfeil *et al*., 1999). In another study, Tonks *et al.* (2003) reported that the cellular viability of MM6 cell line and human monocytes was greater than 90 % after treated with 1 % (w/v) Manuka honey, Pasture honey and Jellybush honey for 24 hours. Collectively, these previous studies suggested that different types of honey will have different cytotoxic effect on immune cells.

The cytotoxic effect of MTH was also reported in several previous studies. Kannan *et al.* (2009) reported that 0.0195 % (v/v) MTH enhanced cell proliferation (105.3 % increment than control) in human osteoblast cell line (CRL 1543) cultured in Dulbecco's modified eagle medium supplemented with 10 % FBS and 1 % penicillin. As the MTH concentration increased to 5 %, the percentage of cell viability in CRL 1543 cell line decreased to below 20 % (Kannan *et al.*, 2009). In another study, Fauzi *et al.* (2011) reported that MTH showed significant cytotoxic effect in HeLa, MCF-7 and MDA-MB-231 cancer cell lines in a concentration and time dependent manner. In this study, the HeLa cancer cell line treated with 5 % (v/v) MTH resulted in 100 % cell death after 72 hours of incubation. In addition, MCF-7 and MDA-MB-231 cancer

cell lines treated with 5 % (v/v) and 10 % (v/v) MTH resulted in 91 % and 93 % cell death, respectively after 24 hours of incubation (Fauzi *et al*., 2011). Collectively, these previous studies suggested that lower concentration of MTH (less than 1 %) can potentially stimulate cell proliferation. In contrast, higher concentration of MTH (greater than 1 %) can potentially induce cell death.

Therefore, it was crucial to determine the optimal concentration of honey and incubation time that can yield a high percentage of viable cells (preferably 90 %) after honey treatment so that the subsequent downstream assays can be carried out more accurately. Based on previous studies as mentioned above, it was speculated that the optimal concentration of MTH could be between 0.0195 % and 10 % while the optimal incubation duration could be between 24 hours and 72 hours. Hence, the cytotoxic effect of MTH was initially determined in THP-1 and U-937 cell lines using MTT assay before proceeding to PBMCs isolated from blood samples.

The MTT assay was selected because this photometric assay was quantitative, commonly used and more accurate as compared to trypan blue exclusion method. The THP-1 and U-937 cell lines were selected because these cell lines were commonly used to study immune responses such as cytokine production and gene expression *in vitro*. For example, it was reported previously that THP-1 and U-937 cell lines treated with arabinogalactan protein isolated from New Zealand Kanuka honey produced higher level of TNF-α (25 pg/ml and 8 pg/ml, respectively) when compared to untreated cell lines (< 1 pg/ml) (Gannabathula *et al*., 2011). Besides, both these cell lines were human monocytes and therefore can be used to mimic the immunomodulatory responses in PBMCs after MTH treatment.

Objectives:

1. To determine the optimal concentration of MTH (Agromas®, Selangor, Malaysia) and incubation time that can yield at least 90 % cellular viability in THP-1 and U-937 cell lines as well as PBMCs.

2. To compare the cytotoxic effect of MTH with another imported Manuka honey (Berringa, Currumbin Waters, Australia). The Manuka honey was selected for comparison because this honey was well-researched and often considered as the gold standard for all honeys.

3.2 Materials and methods

3.2.1 Cell seeding

During the experimental setup for cytotoxic assay, each honey treatment for the human monocytic cell lines (THP-1 and U-937) and PBMCs was performed in triplicates and duplicates respectively. Each experiment was independently repeated for 3 times. The cytotoxicity assay for PBMCs was carried out in duplicates due to the limited amount of fresh PBMCs isolated from blood samples (25 ml) and the high seeding number of PBMCs (2 x 10^5 cells per well) during the cell seeding step.

The human monocytic cell lines and PBMCs were subjected to cell counting (**Section 2.1.5**) to determine the total number of viable cells. Based on the number of viable cells, the human monocytic cell lines and PBMCs were seeded into 96-well U bottom plate containing 1 x 10^4 cells and 2 x 10^5 cells respectively in 100 µl of R10 medium per well. The seeded cells were incubated overnight at 37 °C in a humidified atmosphere of 5 % carbon dioxide. For both THP-1 and U-937 cell lines, honey treatment was carried out when the cells reached about 70 % to 80 % confluence. As for PBMCs, honey treatment was carried out after the PBMCs were rested overnight (approximately 16 hours).

3.2.2 Honey preparation

Based on previous studies, it was speculated that the optimal concentration of MTH could be between 0.0195 % and 10 % (Fauzi *et al.*, 2011; Kannan *et al.*, 2009). Therefore, two ranges of honey concentration were tested in this study. The first range was 2 %, 4 %, 6 %, 8 % and 10 % which included higher honey concentrations. The

second range was 0.125 %, 0.25 %, 0.5 %, 1 % and 2 % which focused mainly on the lower honey concentrations. The same batch of MTH (source: Kuala Nerang, Kedah, batch no: 9556461110013, production date: May 2010) and Manuka honey (source: Currumbin Waters, Australia, batch no: 19/04/14/4/6936/3, production date: April 2011) were used throughout the whole study. The different concentrations of honey solutions were freshly prepared inside the Biosafety Cabinet Class II by diluting the stock honey solution with R5 or R0 media and filtered using 0.45 µm minisart filter.

The choice of using either R5 or R0 as dilution media depended on the types of cells involved during the experimental setup. In brief, R0 medium was used to treat human monocytic cell lines and R5 medium was used to treat PBMCs. The 5 % FBS was selected based on preliminary findings during protocol optimization. Previously, the PBMCs were resuspended in R0 medium prior to honey treatment but it was found that these PBMCs cannot survive for more than 24 hours in the absence of FBS. Hence, FBS (5 % and 10 %) was added into RPMI medium and it was found that both 5 % and 10 % FBS successfully sustained the survival of PBMCs. Considering that FBS also had growth promoting properties, it was therefore crucial to keep the percentage of FBS as low as possible while not compromising the survival rate of PBMCs. Therefore, the minimal percentage of FBS (5 %) was used during honey dilution for PBMCs. On the other hand, R0 medium was used for human monocytic cell lines because these cell lines can survive even in the absence of FBS.

Using serial dilution method, the stock MTH was directly diluted with pre-warmed R5 or R0 media to produce 10 %, 8 %, 6 %, 4 % and 2 % MTH solutions (v/v) (**Figure 3.1**) as well as 2 %, 1 %, 0.5 %, 0.25 % and 0.125 % MTH solutions (v/v) (**Figure 3.2**).

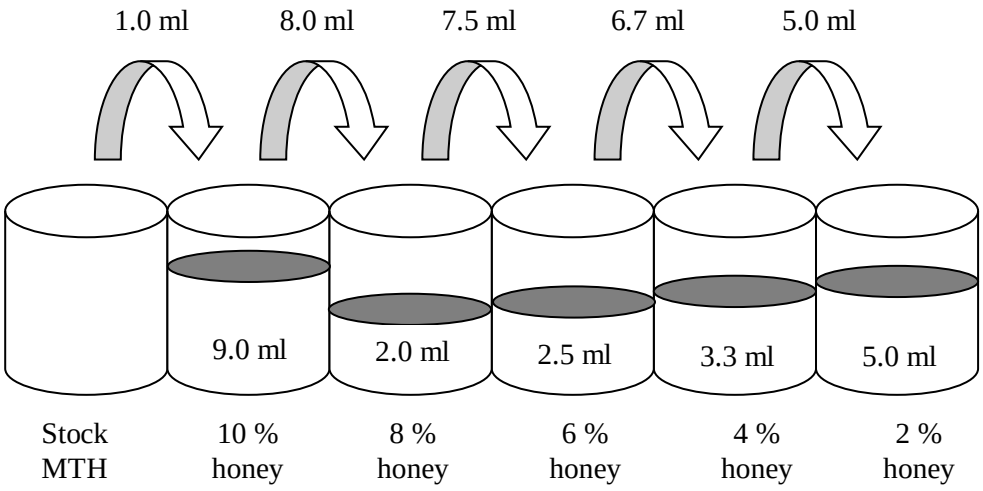


Figure 3.1 Dilution of stock MTH to produce 10 %, 8 %, 6 %, 4 % and 2 % MTH solutions in a total volume of 10 ml.

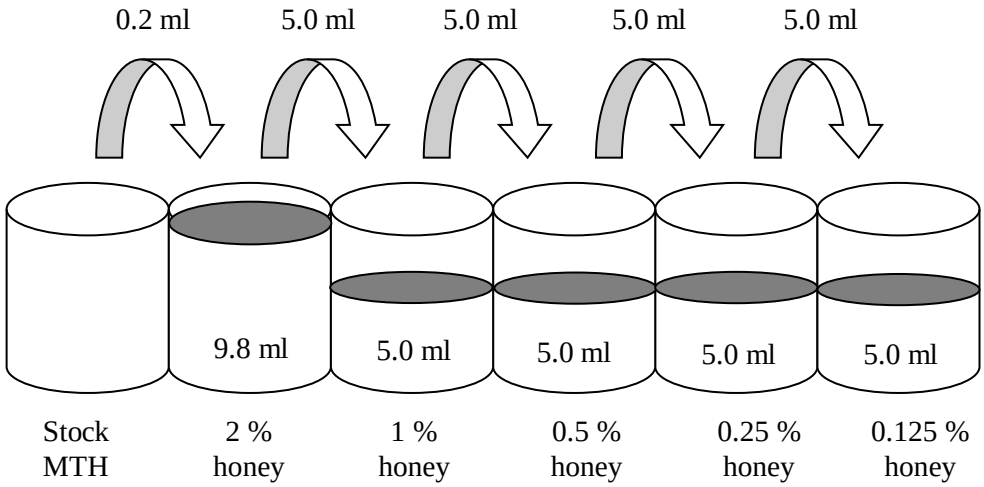


Figure 3.2 Dilution of stock MTH to produce 2 %, 1 %, 0.5 %, 0.25 % and 0.125 % MTH solutions in a total volume of 10 ml.

Due to its high viscosity, the stock Manuka honey solution was initially diluted with pre-warmed R5 or R0 media (ratio 1:1) and vortexed thoroughly to create a homogeneous 50 % Manuka honey solution. Using similar serial dilution method, the 50 % Manuka honey solution was further diluted with pre-warmed R5 or R0 media to produce 10 %, 8 % , 6 %, 4 %, 2 %, 1 % Manuka honey solutions (v/v) as well as 2 %, 1 %, 0.5 %, 0.25 % and 0.125 % Manuka honey solutions (v/v).

3.2.3 Honey treatment

After overnight incubation, the seeded cells for cytotoxic assay were centrifuged at 1800 rpm for 5 minutes at 23 °C. The supernatant was discarded and the cell pellet was treated and resuspended with 100 µl of different concentrations of MTH and Manuka honey solutions. Negative controls were resuspended with 100 µl of R0 medium or R5 medium. Based on previous studies, it was speculated that the optimal incubation duration could be between 24 hours and 72 hours (Fauzi *et al*., 2011; Kannan *et al*., 2009). Therefore, the honey-treated cells were incubated at 37 °C in a humidified atmosphere of 5 % carbon dioxide for 16 hours, 24 hours, 48 hours and 72 hours.

3.2.4 Cytotoxicity assay

The cytotoxic effect of MTH and Manuka honey on THP-1, U-937 and PBMCs was determined and quantified using MTT viability assay. After honey treatment, the cells were centrifuged at 1800 rpm for 5 minutes at 23 °C and the supernatant was discarded. The cell pellet was resuspended with 100 µl of pre-warmed R0 medium followed by 10 µl of MTT reagent (5 mg/ml). Next, the treated cells were covered with aluminium foil and incubated at 37 °C in a humidified atmosphere of 5 % carbon dioxide for 4 hours. After 4 hours of incubation, the treated cells were centrifuged at 1800 rpm for 5 minutes at 23 °C and 75 µl of medium was removed from each well and discarded. Next, 50 µl of DMSO solution was added and mixed thoroughly into each well. The treated cell lines were covered with aluminium foil and incubated at 37 °C in a humidified atmosphere of 5 % carbon dioxide for another 10 minutes. After 10 minutes of incubation, the solutions in each well were mixed thoroughly again and colorimetric absorbance measurement was read at 540 nm using Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Massachusetts, USA). The absorbance results were corrected with the absorbance readings of blank (containing only R0

medium, MTT reagent and DMSO) and the percentage of cell viability was calculated using the formula below.

$$\textit{Percentage of cell viability (\%)} = \frac{A_{540\ nm}\ \textit{Treated Cell Lines}}{A_{540\ nm}\ \textit{Control Cell Lines}}\ X\ 100\ \%$$

$A_{540\ nm}$ Treated Cell Lines: Absorbance of honey-treated cell lines – Absorbance of blank at 540 nm

$A_{540\ nm}$ Control Cell Lines: Absorbance of control cell lines – Absorbance of blank at 540 nm

3.2.5 Statistical analysis

All data entry and analyses were conducted using GraphPad Prism version 5.02. The values were expressed as mean and standard error of mean (SEM). Two-way analysis of variance (ANOVA) followed by Bonferroni posttest were used to compare overall difference between group means. The level of statistical significance was set at $p < 0.05$.

The Bonferroni posttest is a statistical posttest which can prevent any data from incorrectly regarded as statistically significant simply by lowering the p value (divide the p value by the number of comparisons being made) when several comparisons are being performed simultaneously on a single data set. This posttest ensures that the 5 % probability is applied to the entire comparisons and not separately to each individual comparison (Abdi, 2007).

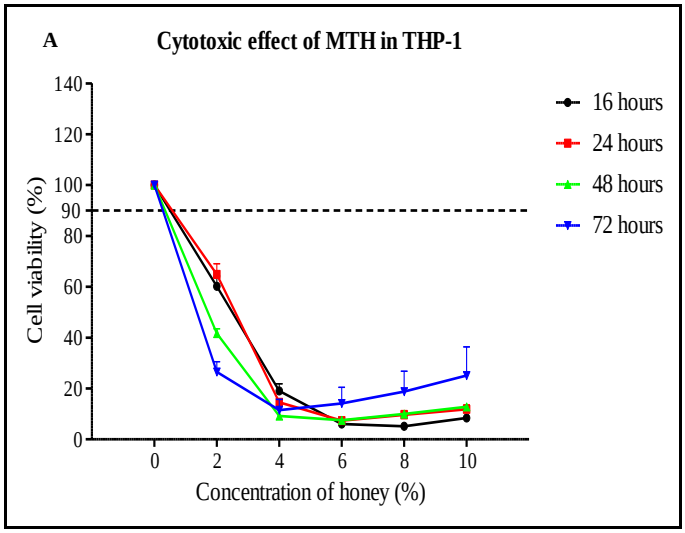
3.3 Results

3.3.1 Cytotoxic effect of MTH and Manuka honey (2 % to 10 %) in THP-1 and U-937 cell lines

Initially, both THP-1 and U-937 cell lines were cultured and treated with MTH and Manuka honey (2 % to 10 %) at different incubation durations (16 hours, 24 hours, 48 hours and 72 hours). This was carried out to determine the optimal honey

concentration and incubation duration that can yield at least 90 % cell viability before proceeding to PBMCs isolated from blood samples. The cytotoxic effect of both MTH and Manuka honey on these human monocytic cell lines was shown in **Figure 3.3** and **Figure 3.4**.

At higher concentrations of MTH (**Figure 3.3**), the percentage of cell viability in THP-1 and U-937 cell lines decreased in a concentration dependent manner from 100 % to approximately 10 % - 30 % as the concentrations of MTH increased from 2 % to 10 % respectively. This trend was observed in all tested incubation durations. It was also found that the percentage of cell viability in both THP-1 and U-937 cell lines treated with 2 % and 4 % MTH decreased drastically in a time dependent manner. However, when the THP-1 and U-937 cell lines were treated with 6 %, 8 % and 10 % MTH, the percentage of cell viability increased slightly in an inversely time dependent manner.



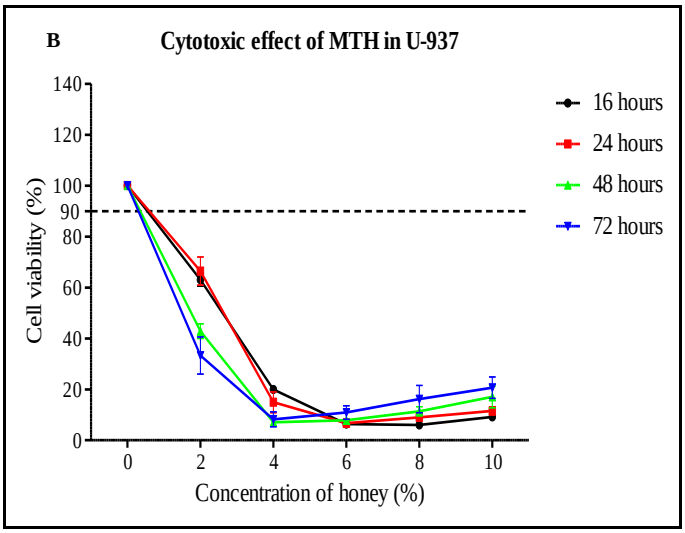


Figure 3.3 Cytotoxic effect of MTH in THP-1 (**A**) and U-937 (**B**) cell lines treated with MTH (2 %, 4 %, 6 %, 8 % and 10 %) and different incubation durations (16 hours, 24 hours, 48 hours and 72 hours). All values were represented as mean ± SEM for triplicates from 3 independent experiments.

Similar to MTH, the percentage of cell viability in THP-1 and U-937 cell lines also decreased in a concentration dependent manner from 100 % to approximately 10 % - 20 % as the concentrations of Manuka honey increased from 2 % to 10 % for all tested incubation durations (**Figure 3.4**). It was also found that the percentage of cell viability in THP-1 cell line treated with 2 % and 4 % Manuka honey decreased drastically in a time dependent manner but similar trend was not observed in U-937 cell line (**Figure 3.4**).

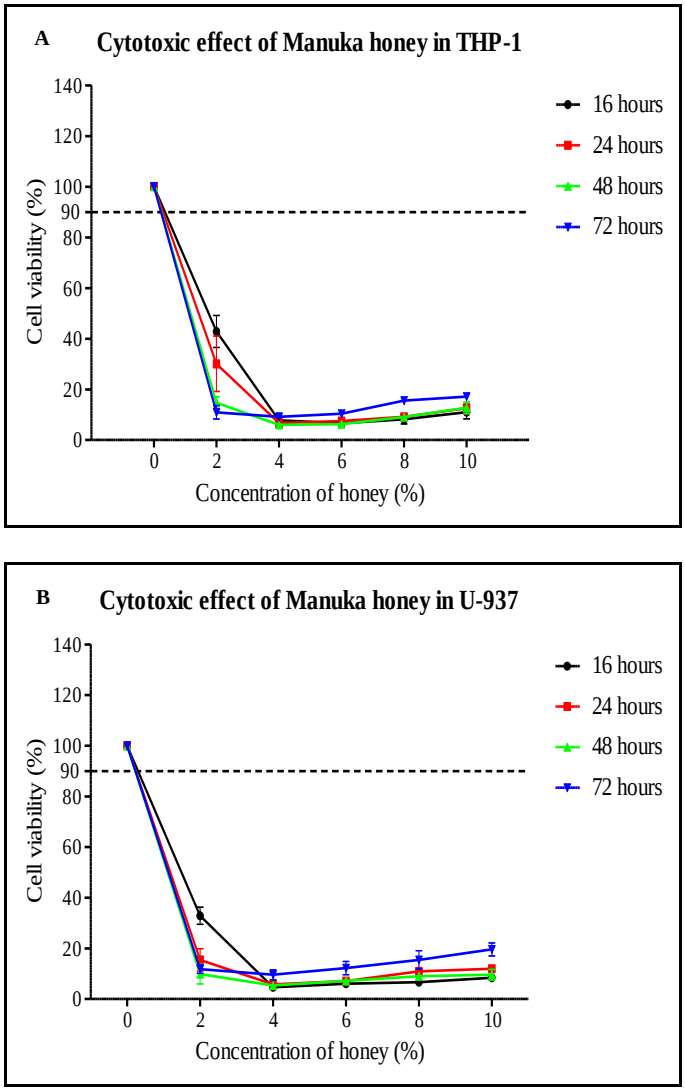


Figure 3.4 Cytotoxic effect of Manuka honey in THP-1 (**A**) and U-937 (**B**) cell lines treated with Manuka honey (2 %, 4 %, 6 %, 8 % and 10 %) and different incubation durations (16 hours, 24 hours, 48 hours and 72 hours). All values were represented as mean ± SEM for triplicates from 3 independent experiments.

Collectively, both THP-1 and U-937 cell lines showed relatively low percentage of cell viability when treated with MTH and Manuka honey at concentrations between 2 % to 10 %. The targeted 90 % cell viability honey treatment was not achievable. Therefore, this range of honey concentration (2 % to 10 %) was not suitable to be used in subsequent immunoassays experimental setup because low percentage of cell viability will give less accurate results and hence making the

downstream experiments difficult to be analysed. In order to overcome this, another range of honey concentration (0.125 % to 2 %) was tested against THP-1 and U-937 cell lines before proceeding to PBMCs.

In terms of incubation duration, it was found that 72 hours of incubation resulted in lowest percentage of cell viability in both THP-1 and U-937 cell lines treated with 2 % MTH and Manuka honey. Therefore, only 16 hours, 24 hours and 48 hours were selected for subsequent optimization experiments.

3.3.2 Cytotoxic effect of MTH and Manuka honey (0.125 % to 2 %) in THP-1 and U-937 cell lines

The cytotoxic effect of MTH and Manuka honey on THP-1 and U-937 cell lines treated with lower concentrations of MTH and Manuka honey (0.125 % to 2 %) and different incubation durations (16 hours, 24 hours and 48 hours) was shown in **Figure 3.5** and **Figure 3.6**.

Based on **Figure 3.5,** both THP-1 and U-937 cell lines responded to MTH (0.125 % to 2 %) in a concentration dependent manner. As the concentration of MTH increased from 0.125 % to 2 %, the percentage of cell viability in THP-1 cell line decreased gradually in a concentration dependent manner from 100 % to approximately 30 % - 50 % respectively. On the other hand, the percentage of cell viability in U-937 cell line increased gradually first to approximately 120 % (except 24 hours and 48 hours of incubation) at 0.125 % MTH before decreasing to approximately 20 % - 70 % as the MTH concentration increased to 2 %.

No clear trend was observed in the percentage of cell viability in THP-1 cell line treated with MTH for different incubation durations. On the other hand, it was found that the percentage of cell viability in U-937 cell line treated with MTH decreased in a time dependent manner as the concentration of MTH increased from 0.125 % to 2 %. Briefly, it was found that the longer incubation durations (24 hours

and 48 hours resulted in lower percentage of cell viability when compared to 16 hours (**Figure 3.5**).

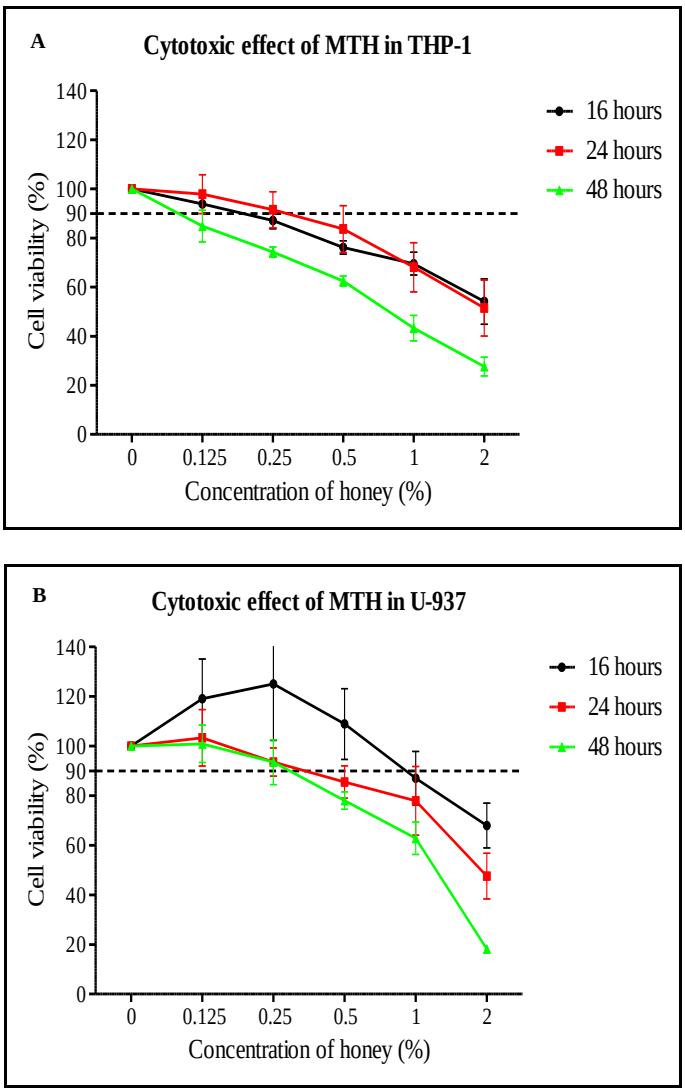


Figure 3.5 Cytotoxic effect of MTH in THP-1 (**A**) and U-937 (**B**) cell lines treated with MTH (0.125 %, 0.25 %, 0.5 %, 1 % and 2 %) and different incubation durations (16 hours, 24 hours and 48 hours). All values were represented as mean ± SEM for triplicates from 3 independent experiments.

At low concentrations of Manuka honey (**Figure 3.6**), both THP-1 and U-937 cell lines responded to Manuka honey in concentration dependent manner. Generally, the percentage of cell viability in both cell lines decreased from 100 % to approximately 20 % - 50 % as the concentrations of Manuka honey increased from

0.125 % to 2 % respectively. Nevertheless, slight increase of approximately 110 % in cell viability was also observed in THP-1 cell line (24 hours of incubation) as well as U-937 cell line (16 hours) upon 0.125 % Manuka honey treatment. This trend was similar when compared to U-937 cell line upon 0.125 % MTH treatment.

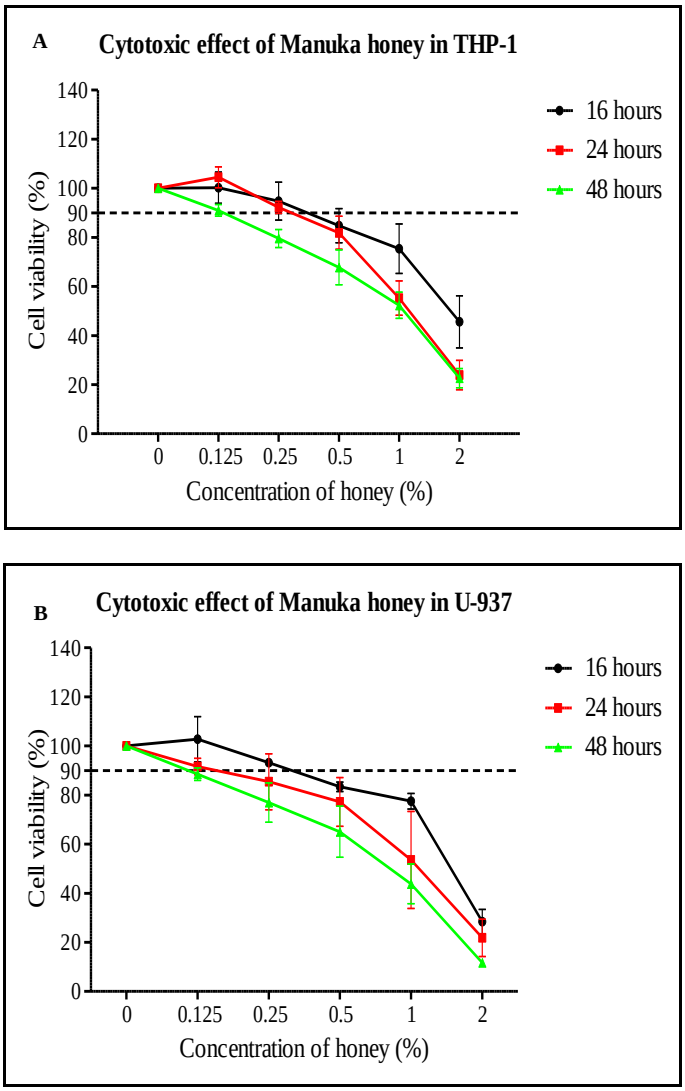


Figure 3.6 Cytotoxic effect of Manuka honey in THP-1 (**A**) and U-937 (**B**) cell lines treated with Manuka honey (0.125 %, 0.25 %, 0.5 %, 1 % and 2 %) and different incubation durations (16 hours, 24 hours and 48 hours). All values were represented as mean ± SEM for triplicates from 3 independent experiments.

Collectively, the targeted 90 % cell viability in both THP-1 and U-937 cell lines was achieved at these lower honey concentrations. This suggested that similar 90 % cell viability may also be achievable in PBMCs using these honey concentrations (0.125 % to 2 %). In terms of incubation duration, it was observed that all the tested incubation duration (16 hours, 24 hours and 48 hours) were able to yield the targeted 90 % cell viability in both THP-1 and U-937 cell lines when treated with 0.125 % MTH and Manuka honey. Hence, all 3 incubation durations were selected for cell viability assessment using PBMCs.

3.3.3 Cytotoxic effect of MTH and Manuka honey (0.125 % to 2 %) in PBMCs

The cytotoxic effect of MTH and Manuka honey in PBMCs treated with 0.125 % to 2 % of honey and different incubation durations (16 hours, 24 hours and 48 hours) was shown in **Figure 3.7** and **Figure 3.8**.

Based on **Figure 3.7**, the percentage of cell viability in PBMCs treated with MTH increased gradually to approximately 120 % as the concentrations of MTH increased from 0.125 % to 2 % for all tested incubation durations (except 2 % MTH for 48 hours of incubation). In contrast, PBMCs treated with 2 % MTH for 48 hours of incubation showed a decrease in the percentage of cell viability from 120 % (at 1 % MTH) to 78 %. Statistical analysis showed that PBMCs treated with 2 % MTH for 16 hours and 48 hours ($p < 0.01$) as well as 24 hours and 48 hours ($p < 0.05$) were statistically significant. In terms of incubation durations, it was found that the percentage of cell viability in PBMCs decreased gradually as the incubation duration increased from 16 hours to 48 hours. This suggested that longer incubation duration (48 hours) resulted in greater cytotoxic effect as compared to shorter incubation duration (16 hours and 24 hours) in MTH-treated PBMCs.

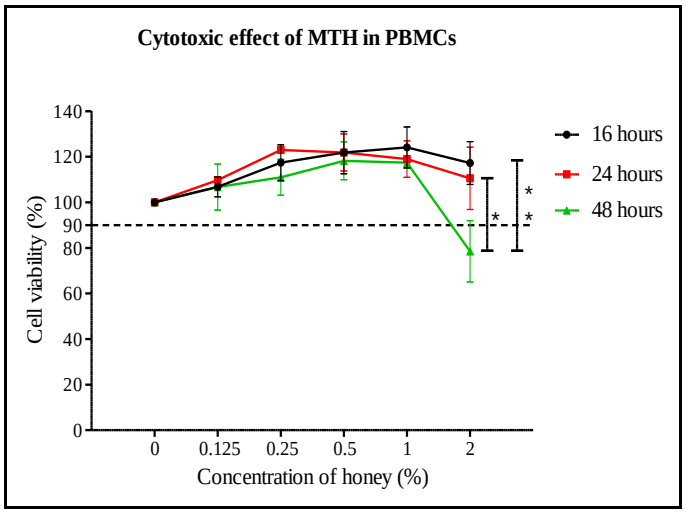


Figure 3.7 Cytotoxic effect of MTH in PBMCs treated with different concentrations of honey (0.125 %, 0.25 %, 0.5 %, 1 % and 2 %) and incubation durations (16 hours, 24 hours and 48 hours). All values were represented as mean ± SEM for duplicates from 3 independent experiments. (*) represented $p < 0.05$ and (**) represented $p < 0.01$ analyzed using two-way ANOVA and Bonferroni posttest.

As for Manuka honey (**Figure 3.8**), the percentage of cell viability in PBMCs increased gradually from 100 % to approximately 110 % - 120 % as the concentrations of Manuka honey increased from 0.125 % to 1 % for 16 hours and 48 hours of incubation. However, the percentage of cell viability decreased drastically to approximately 40 % - 70 % when the concentration of Manuka honey was further increased to 2 %. As for 24 hours of incubation, the percentage of cell viability decreased gradually from 100 % to approximately 60 % when the concentrations of Manuka honey increased from 0.125 % to 2 %. Statistical analysis showed that PBMCs treated with 0.25 % Manuka honey for 16 hours and 24 hours ($p < 0.05$) as well as PBMCs treated with 2 % Manuka honey for 16 hours and 48 hours ($p < 0.05$) were statistically significant. In terms of incubation duration, it was found that the percentage of cell viability in PBMCs decreased gradually as the incubation durations increased from 16 hours to 24 hours. This suggested that 24 hours of incubation resulted in greater cytotoxic effect as compared to shorter incubation duration (16

hours). However, the percentage of cell viability was greatly improved when the incubation duration was further increased from 24 hours to 48 hours.

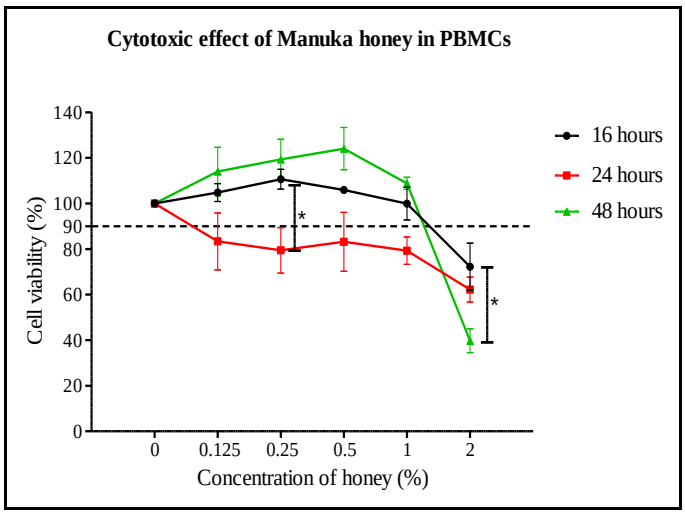


Figure 3.8 Cytotoxic effect of Manuka honey in PBMCs treated with different concentrations of honey (0.125 %, 0.25 %, 0.5 %, 1 % and 2 %) and incubation durations (16 hours, 24 hours and 48 hours). All values were represented as mean ± SEM for duplicates from 3 independent experiments. (*) represented $p < 0.05$ analyzed using two-way ANOVA and Bonferroni posttest.

Overall, the targeted 90 % cell viability in PBMCs after MTH and Manuka honey treatment was achieved with optimal combination of honey concentrations and incubation durations as shown in **Table 3.2** below.

Table 3.2 Optimal combination of honey concentrations and incubation durations that yielded at least 90 % cell viability in PBMCs after honey treatment

MTH		Manuka honey	
Concentration (%)	Incubation duration (hours)	Concentration (%)	Incubation duration (hours)
0.125	16, 24, 48	0.125	16, 48
0.25	16, 24, 48	0.25	16, 48
0.5	16, 24, 48	0.5	16, 48
1	16, 24, 48	1	16, 48
2	16, 24	-	-

3.4 Discussion

Based on these preliminary cytotoxicity results, it was found that THP-1, U-937 cell line and PBMCs responded to MTH and Manuka honey in certain degree of concentration dependent and time dependent manner. Generally, both MTH and Manuka honey showed greater cytotoxic effect (low percentage of cell viability) at honey concentrations ranging from 0.5 % to 10 % at all tested incubation durations. Under these treatment conditions, the targeted 90 % cell viability was not achievable.

It was speculated that the cytotoxic effect of MTH and Manuka honey in THP-1, U-937 cell line and PBMCs may be due to the presence of strong antioxidant compounds, possibly polyphenol, phenolic acids and flavonoid in both honeys. This was because high levels of phenolic compounds were found in these honeys. In this study, the total phenolic content in the MTH was found to be slightly higher (435.23 mg GAE per kg honey) (**Appendix 3**) as compared to 419.86 mg GAE per kg honey reported by Khalil *et al.* (2012) in previous study using the same MTH. As for the Manuka honey used in this study, the total phenolic content was found to be 850.62 mg GAE per kg honey which was slightly lower as compared to 899.09 mg GAE per kg honey reported previously by Alzahrani *et al.* (2012). It was also reported previously that these phenolic compounds can inhibit cell proliferation by down regulating cellular enzymatic pathway such as protein tyrosine kinase (Ghashm *et al.*, 2010). The protein tyrosine kinase is an enzyme responsible to phosphorylate protein molecules in the cells by transferring a phosphate group from adenosine triphosphate (ATP) molecules. In living cells, phosphorylation of protein molecules is crucial and often related to cellular activities such as cell division and cell signalling (Rao *et al.*, 1993). Therefore, any suppression on protein tyrosine kinase can potentially lead to reduction of phosphorylation of protein molecules that can affect cell growth and cell survival. Besides, the cytotoxic effect of MTH and Manuka honey may also be due to the osmotic effect of honey solutions (0.5 % to 10 %). In a previous study, Abuharfeil *et al.* (1999) reported that increasing concentration of Multifloral honey (0.1 % to 0.8

%) reduced the proliferation of lymphocytes (B cells and T cells), most probably due to the osmotic effect of the honey where water molecules were drawn out from cells.

At honey concentrations below 0.5 %, the percentage of cell viability in THP-1, U-937 cell lines and PBMCs were higher (approximately 70 % to 120 %) when treated with MTH and Manuka honey. Under these treatment conditions, the targeted 90 % cell viability was achievable. In fact, the enhancement in cell viability was more apparent in PBMCs treated with MTH when compared to Manuka honey. It was also found that the enhancement in cell viability in PBMCs was higher when compared to THP-1 and U-937 cell lines treated with the same concentrations of MTH (0.125 % to 0.5 %). This could be possibly due to the different cell populations present in PBMCs as compared to THP-1 and U-937 cell lines. As mentioned in **Section 2.1**, both THP-1 and U-937 cell lines were human monocytes with uniform genetic background. In contrast, the PBMCs were isolated from healthy donors and consisted of B cells, T cells, monocytes, macrophages and NK cells (**Section 2.2**). Hence, it was speculated that the higher cell viability observed in MTH-treated PBMCs may be possibly due to the accumulative effect of different responses in various PBMCs subpopulations as compared to the genetically uniform THP-1 and U-937 cell lines. These preliminary results were also slightly different when compared to the findings reported by Tonks *et al.* (2003). In their study, the cellular viability of human monocytes was greater than 90 % when treated with 1 % Manuka honey. In present study, it was found that the concentration of MTH and Manuka honey can be further reduced to 0.125 % without compromising the targeted 90 % cell viability (except for PBMCs treated with Manuka honey for 24 hours).

3.5 Concluding remarks

There was a clear trend that higher concentration of MTH and Manuka honey (4 % - 10 %) resulted in greater cytotoxic effect (approximately 50 % cell viability). In contrast, lower range of honey concentrations (0.125 % - 2 %) showed certain degree

of cell proliferative potential with lesser cytotoxic effect. Overall, the combinations of honey concentrations and incubation durations that yielded at least 90 % cell viability in PBMCs were successfully determined in **Table 3.2**. These optimal parameters were used in subsequent PBMC treatments prior to analysis using different immunoassays in the following chapters.

CHAPTER 4: GENE EXPRESSION PROFILE IN PBMCs UPON MTH TREATMENT

4.1 Introduction

Gene expression is a process where information from a gene is used to synthesize a functional gene product, commonly proteins. This process is crucial for the development and biological functions of all living organisms. The gene expression process can be categorized into several stages such as transcription, ribonucleic acid (RNA) processing, translation and protein folding. In eukaryotic system, the transcription process is initiated when RNA polymerase bind to a strand of DNA which encodes the genetic information and transcribe a copy of pre-mRNA. This copy of pre-mRNA will undergo RNA processing such as 5' capping (addition of 7-methylguanosine to the 5' end of the RNA) and polyadenylation (addition of multiple adenines to the 3' end of the RNA) to protect the pre-mRNA from degradation by exonucleases. Besides, the pre-mRNA will also undergo RNA splicing to remove the non-coding segments (intron) while retaining the coding segments (exon) and become functional mRNA. Next, this mRNA will be transported from the nucleus to the cytoplasm to be translated into proteins by ribosome. During translation, each mRNA is translated into a liner chain of amino acids which will then undergo protein folding to develop into a 3-dimentional functional protein (Kohler & Hurt, 2007).

In this study, the gene expression profile in MTH-treated PBMCs was determined in order to study the immunomodulatory effect of MTH in regulating gene expression related to immune responses. This was achieved by screening the gene expression profile in MTH-treated PBMCs using Agilent microarray approach. Microarray is a technique commonly used to detect and quantify large number of nucleic acids or proteins in biological samples such as cells, tissues or bodily fluids (Eady *et al*., 2015). This technique is often used in probing blood serum for disease diagnosis and profiling expression in cells treated with drugs. Practically, microarray uses a miniaturized solid support or a chip (glass slide or nylon membrane) spotted

with capture elements such as cDNA, oligonucleotide, protein or antibody, which can bind to targeted nucleic acids or proteins tagged with fluorescent dyes (commonly cyanine-3 or cyanine-5) in the biological samples. After binding, the microarray chip is washed and scanned using laser scanner for data analysis. The relative amount of targeted nucleic acids or proteins can be determined by comparing the signal intensities between biological samples such as healthy and diseased or treated and control (Wiltgen & Tilz, 2007). The main advantage of using microarray in gene expression study is the flexibility to simultaneously detect many targets with low sample volume (10 ng of starting material) (Arezi *et al.*, 2012). As compared to other gene expression analysis techniques such as RNA sequencing and RT-qPCR, microarray can provide a comprehensive whole-genome coverage in a single experiment, rapid results and lower cost per sample. The choice of using microarray also gave the advantage of screening the expression of genes other than immune related genes, which can be potentially studied in future studies (Arezi *et al*., 2012).

In addition, gene expression study can be carried out either using one-colour microarray (intensity-based) or two-colour microarray (ratio-based) platforms. In present study, the gene expression profile in PBMCs after MTH treatment was determined using one-colour microarray platform because it gave the advantage to compare another microarrays on the same glass slide directly. Besides, the one-colour Agilent microarray platform uses cyanine-3 dye which is more stable and less susceptible to degradation due to environmental factors such as ozone and pH change as compared to cyanine-5 dye (Xiao *et al*., 2006).

Based on literature review, it was reported previously that 1 % Manuka honey, Jellybush honey and Pasture honey significantly increased the production of IL-1β, IL-6 and TNF-α in human monocytes and MM6 cell line (Tonks *et al*., 2007; Tonks *et al*., 2003). In another study, qPCR analyses showed that 0.25 % Manuka honey significantly ($p < 0.001$) upregulated the gene expression of *IL1R* (+0.50 fold) and *TGF-B* (+7.00 fold) in unstimulated THP-1 cell line (Bean, 2012). These findings

suggested that honey can generally regulate the expression of various genes in the immune system. Similarly, it was believed that MTH may also possess similar immunomodulatory effect in regulating the expression of various genes in PBMCs. To date, the gene expression profile in MTH-treated PBMCs has not been reported. Therefore, this was the first study ever conducted to investigate this aspect and hence it was crucial to have an overview of the gene expression profile in MTH-treated PBMCs.

Objectives:

1. To determine the overall gene expression profile in MTH-treated PBMCs using Agilent microarray platform and validate the selected genes of interest using RT-qPCR approach.
2. To select immune-related genes for subsequent cytokine study
3. To determine potential immune-related pathways being regulated by MTH in PBMCs using DAVID software

4.2 Materials and methods

4.2.1 Cell seeding

Due to the limited number of arrays on a single microarray chip, PBMCs were isolated from 4 healthy donors and seeded into 6-well flat bottom plates containing 5 x 10^6 cells in 2 ml of R5 medium per well. These PBMCs were rested at 37 °C in a humidified atmosphere of 5 % carbon dioxide for 6 hours prior to MTH treatment.

4.2.2 MTH preparation and treatment

Based on the cell viability results obtained in Chapter 3, it was found that the minimum 0.125 % MTH was sufficient to treat PBMCs while maintaining the cell viability by at least 90 %. In terms of incubation duration, it was found that the 90 % cell viability in PBMCs was still achievable after MTH treatment for up to 48 hours.

Hence, the minimum MTH concentration (0.125 %) and an average incubation duration (24 hours) were selected to treat the PBMCs prior to microarray analysis.

The 0.125 % MTH was serially diluted from stock MTH solution as described in **Section 3.2.2** using R5 medium. After 6 hours resting, the PBMCs were centrifuged at 1800 rpm for 5 minutes at 23 °C and the supernatant was discarded. The PBMCs pellets were resuspended and treated with 2 ml of 0.125 % MTH. The MTH-treated PBMCs were incubated at 37 °C in a humidified atmosphere of 5 % carbon dioxide for 24 hours. In this experimental setup, untreated PBMCs with R5 medium only was used as control.

4.2.3 RNA extraction

After 24 hours of incubation, the PBMCs were scraped gently using cell scraper (SPL Life Sciences, Seoul, Korea) and transferred into 2 ml centrifuge tube. The PBMCs were centrifuged at 1800 rpm for 5 minutes at 23 °C to discard supernatant and subsequently washed twice using PBS 1x solution. Next, RNA was extracted from the PBMCs using Qiagen RNeasy Mini kit according to manufacturer's instruction. Briefly, 600 µl RLT cell lysis buffer was added to the PBMCs cell pellet and vortexed thoroughly. The lysate was homogenized by passing the lysate 10 times through a 20-gauge needle fitted to a syringe. After homogenization, 600 µl 70 % ethanol was added to the lysate and mixed properly. Next, 700 µl of the mixture was transferred to RNeasy spin column placed in a 2 ml collection tube and centrifuged at 10,000 rpm for 15 seconds to discard the flow-through. This step was repeated for the remaining lysate solution using the same RNeasy spin column. Next, the RNeasy spin column was washed with 350 µl RW1 wash buffer. In order to remove DNA contamination, 10 µl DNase solution and 70 µl RDD buffer were added to the RNeasy spin column and incubated on the benchtop for 15 minutes. After 15 minutes, 350 µl RW1 wash buffer was added to the RNeasy spin column and centrifuged to wash away the DNase solution, followed by 500 µl RPE buffer to wash the spin column membrane. In order

to dry the spin column membrane and prevent ethanol carried over during RNA elution, another 500 µl RPE buffer was added to the RNeasy spin column and centrifuged at 10,000 rpm for 2 minutes. Finally, 30 µl RNase-free water was added and centrifuged at 10,000 rpm for 1 minute to elute the RNA into sterile RNase-free and DNase-free microcentrifuge tube. This step was repeated using the same eluate to obtain higher RNA concentration. All RNA samples were stored in -80 °C freezer until further analysis.

4.2.4 RNA concentration, purity and integrity assessment

All RNA samples were measured at 260/280 nm (value ≥ 2 indicating absence of protein) using microplate spectrophotometer (Biotek, Beijing, China) to determine the purity and amount of RNA extracted from the PBMCs.

The RNA purity and integrity assessment were also determined in Genomax Technologies Lab (Singapore) using Nanodrop and Agilent RNA 6000 nano chip with Agilent 2100 Bioanalyzer. The RNA integrity number (RIN) for all RNA samples were preferably ≥ 7, indicating acceptable RNA purity and integrity for downstream microarray and RT-qPCR experiments.

4.2.5 Microarray and data analysis

The microarray analysis was carried out at Genomax Technologies Lab (Singapore). Briefly, 100 ng of total RNA was labelled with Low Input Quick Amp Labelling kit (Agilent, California, USA) according to manufacturer's instruction. During this labelling step, the total RNA was reverse transcribed into double stranded cDNA by priming with oligo-dT primers containing the recognition site for T7 RNA polymerase. The *in vitro* transcription of cDNA with T7 RNA polymerase produced cyanine-3 labelled complementary RNA (cRNA). Next, 600 ng of cyanine-3 labelled cRNA was hybridized onto Agilent SurePrint G3 Human GE 8x60K microarray, which covered the entire human genome (50,599 features) at 10 rpm and 65 °C for 17

hours in Agilent hybridization oven. After hybridization, the microarray slide was washed in wash buffer 1 for 1 minute at room temperature and another minute in wash buffer 2 at 37 °C. After washing, the microarray slide was scanned using Agilent High Resolution Microarray Scanner (C-model). The raw signal data was extracted from the TIFF image using Agilent Feature Extraction Software (version 10.7.1.1) and analysed using GeneSpring GX software (version 11.5). Probeset flag filtering (detected/not detected/compromised) and probeset expression filtering (expression larger than 20 signal intensity) were applied as probeset quality controls. Gene ontology and pathway analysis was carried out using the DAVID Bioinformatics Resources online software (version 6.7).

4.2.6 RT-qPCR analysis

In order to validate the microarray data, the same RNA samples used for microarray analysis were used to measure the amount of targeted genes using RT-qPCR approach. The reverse transcription of RNA to cDNA was carried out using Qiagen QuantiTect Reverse Transcription kit according to manufacturer's instruction. Inside the DNA/RNA UV-Cleaner biosafety cabinet, 1.5 µg template RNA, 3 µl genomic DNA Wipeout buffer and appropriate volume of RNase-free water were added into a sterile microcentrifuge tube in a total reaction volume of 21 µl. The mixture was mixed gently and incubated at 45 °C for 2 minutes then placed immediately on ice. Next, 1.5 µl Quantiscript reverse transcriptase, 6 µl Quantiscript RT buffer (5x) and 1.5 µl RT primer mix were added into the mixture and incubated at 42 °C for 15 minutes to initiate the reverse transcription reaction. In order to terminate the reverse transcription reaction, the mixture was incubated at 95 °C for 3 minutes to inactivate the Quantiscript reverse transcriptase. The cDNA template was immediately stored on ice prior to relative qPCR analysis.

The relative qPCR was carried out on BioRad CFX Connect Real-Time System using Qiagen QuantiFast SYBR Green PCR kit according to manufacturer's

instruction. Briefly, 1 µl cDNA template, 12.5 µl QuantiFast SYBR Green PCR master mix (2x), 2.5 µl primers and appropriate volume of RNase-free water were added into PCR strips (Eppendorf, Stevenage, England) in a total reaction volume of 25 µl. During the 2-step qPCR amplification, the PCR initial heat activation was set at 95 °C for 5 minutes, followed by 40 cycles of denaturation at 95 °C for 10 seconds and combined annealing and primer extension at 60 °C for 30 seconds. A melting curve analysis was performed at the end of the reaction at 60 °C to 95 °C for 20 minutes. The relative qPCR was carried out in triplicates. The expression patterns of the genes of interest were normalized relative to *GapDH* expression (housekeeping gene) using comparative C_T Livak method ($2^{-\Delta\Delta CT}$). The following primers were used in the qPCR reactions: *GapDH*: Hs_GAPDH_1_SG QuantiTect Primer Assay (NM_002046 / 95 bp), *IFNG*: Hs_IFNG_1_SG QuantiTect Primer Assay (NM_000619 / 130 bp), *IL2*: Hs_IL2_1_SG QuantiTect Primer Assay (NM_000586 / 121 bp), *IL4*: Hs_IL4_1_SG QuantiTect Primer Assay (NM_000589 / 89 bp) and *IL10*: Hs_IL10_ 1_SG QuantiTect Primer Assay (NM_000572 / 113 bp).

Similarly, another 5 cytokine and chemokine related genes (*IL20*, *IL24*, *CXCL1*, *CXCL3* and *CXCL9*) were also selected and validated using Eppendorf Mastercycler Realplex (Model ep*gradient S*) and Qiagen QuantiFast SYBR Green PCR kit according to manufacturer's instruction as stated above. The following primers were used in the qPCR reactions: *IL20*: Hs_IL20_1_SG QuantiTect Primer Assay (NM_018724 / 134 bp), *IL24*: Hs_IL24_SG QuantiTect Primer Assay (NM_001185156 / 128 bp), *CXCL1*: Hs_CXCL1_SG QuantiTect Primer Assay (NM_001511 / 120 bp), *CXCL3*: Hs_CXCL3_SG QuantiTect Primer Assay (NM_002090 / 110 bp) and *CXCL9*: Hs_CXCL9_1_SG QuantiTect Primer Assay (NM_0024116 / 69 bp).

4.2.7 Statistical analysis

For microarray analysis, all data entry and analysis were conducted using GeneSpring GX software (version 11.5). The values were expressed as mean of fold change (fold change cut-off set at 2.00 fold changes) and significant analysis was performed using paired T-test with statistical significance level set at $p < 0.05$. Paired T-test was used to compare the mean of untreated and MTH-treated groups obtained from the same donors.

For RT-qPCR analysis, all data entry and analysis were conducted using GraphPad Prism version 5.02. The values were expressed as mean ± SEM. Paired T-test was also used to compare the mean of untreated and MTH-treated groups obtained from the same donors. The level of statistical significance was set at $p < 0.05$.

4.3 Results

4.3.1 RNA concentration, purity and integrity assessment

The RNA concentration (ng/µl), purity (absorbance 260/280 nm) and RIN for all RNA samples extracted from untreated PBMCs and PBMCs treated with MTH for 24 hours were shown in **Table 4.1** below. Based on the RNA purity assessment, the absorbance readings for 260/280 nm were around 2.0, indicating all RNA samples were not contaminated with proteins. As for RNA integrity assessment, the RIN for all RNA samples were above 7, indicating the RNA samples were intact and hence acceptable for microarray analysis.

Table 4.1 RNA concentration, purity and RIN assessment

Sample	Concentration (ng/µl)	Absorbance 260/280 nm	RIN
Donor 1 (Control)	339.6	2.09	7.9
Donor 1 (Treated)	332.2	2.15	9.2
Donor 2 (Control)	459.1	2.10	9.7
Donor 2 (Treated)	530.0	2.08	8.6
Donor 3 (Control)	210.2	2.10	8.8
Donor 3 (Treated)	414.2	2.07	8.3
Donor 4 (Control)	225.1	2.03	9.7
Donor 4 (Treated)	345.0	2.04	7.6

4.3.2 Immunomodulatory effect of MTH on gene expression profile in PBMCs

The microarray raw data were subjected to probeset flag filtering, probeset expression filtering, normalization, statistical analysis and fold change analysis prior to gene ontology analysis (**Figure 4.1**). From a total of 50,599 targeted genes on the microarray chip, 24,648 genes passed through the probeset quality control (**Figure 4.2A**). Among these 24,648 genes, 361 genes with at least 2.00 fold changes and p values < 0.05 were filtered and subjected to gene ontology analysis (**Figure 4.2B**).

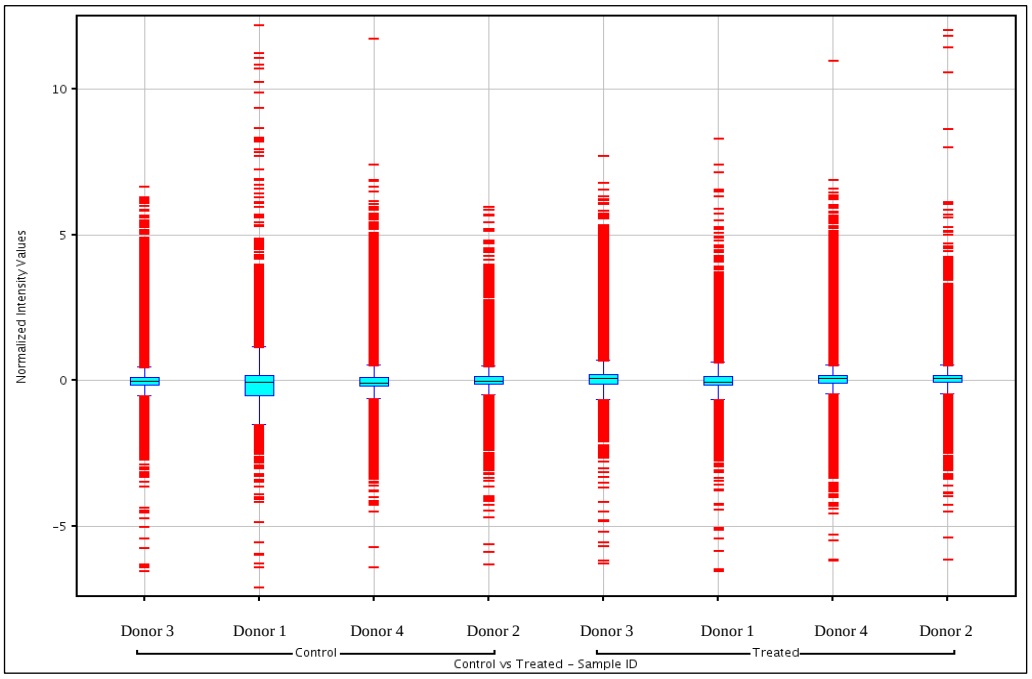


Figure 4.1 Box-whisker plot on normalized microarray data which enable direct comparison across all RNA samples.

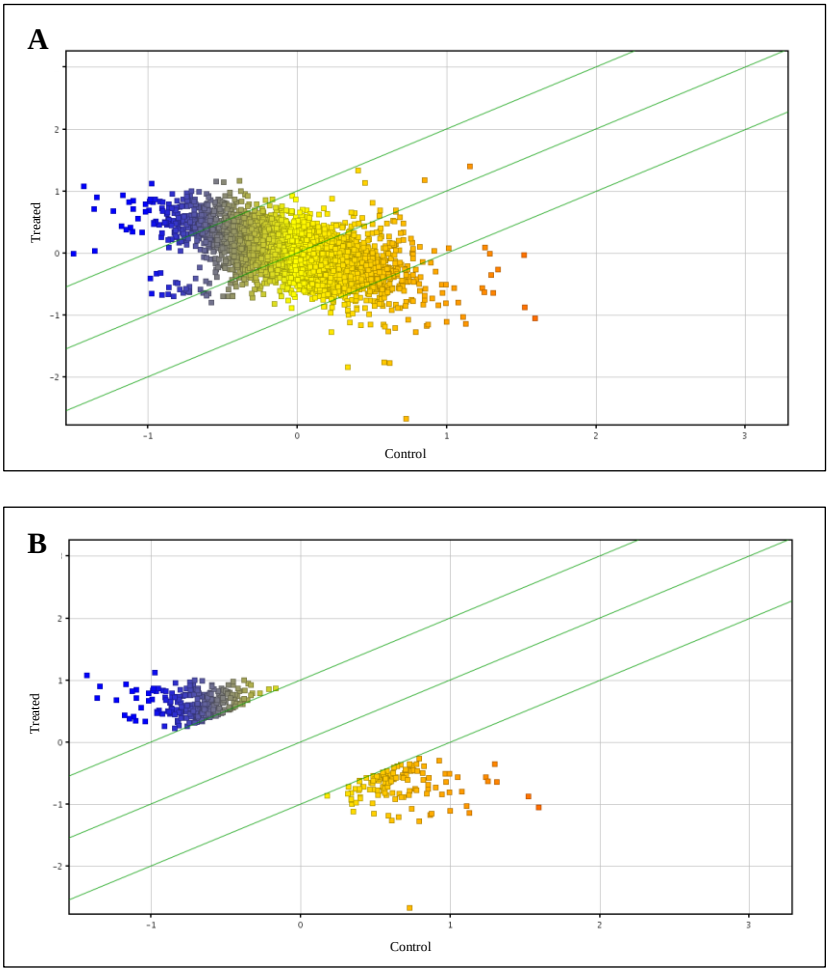


Figure 4.2 Total number of genes that passed through the probeset quality control (**A**) and total number of genes with at least 2.00 fold changes and p values < 0.05 (**B**). The upper and lower green lines represented the upregulation and downregulation of 2.00 fold changes.

Among these 361 genes with at least 2.00 fold changes and p values < 0.05, it was found that 44 genes were associated with the regulation of immune responses based on gene ontology analysis using online DAVID software (**Table 4.2**). Among the 44 genes associated with the regulation of immune responses, 7 genes of interest were selected and validated using RT-qPCR analysis. These genes were interferon gamma (*IFNG*), interleukin-10 (*IL10*), interleukin-20 (*IL20*), interleukin-24 (*IL24*), chemokine (C-X-C motif) ligand 1 (*CXCL1*), chemokine (C-X-C motif) ligand 3 (*CXCL3*) and chemokine (C-X-C motif) ligand 9 (*CXCL9*). The selection of these

genes of interest was based on their specific functions in the immune system, which can potentially contribute to the beneficial properties of MTH such as antimicrobial, wound healing and immunoregulatory function. In addition, interleukin-2 (*IL2*) and interleukin-4 (*IL4*) were also selected to serve as negative controls in subsequent immunoassays.

Based on the microarray data, the expression of *IFNG* was significantly downregulated by 6.20 fold and the expression of *IL10* was significantly upregulated by 2.36 fold (**Table 4.2**). Functionally, the *IFNG* gene encodes IFN-γ (type 1 cytokine) which is crucial in promoting pro-inflammatory process in response to microbial infection (Young & Hardy, 1995). On the other hand, the *IL10* gene encodes IL-10 (type 2 cytokine) which is crucial in regulating prolong inflammatory responses and initiate wound healing process (Saraiva & O'Garra, 2010). This finding suggested that MTH potentially possess wound healing activity by regulating the production of type 1 and type 2 cytokines in PBMCs.

The expression of several others genes associated with cytokines and chemokine production were also significantly regulated in PBMCs treated with 0.125 % MTH for 24 hours. These genes were *IL20* (+4.76), *IL24* (+3.88), *CXCL*1 (+3.29), *CXCL3* (+3.17) and *CXCL9* (-10.46) (**Table 4.2**). The expression of these cytokines and chemokines genes play an important role in the recruitment of neutrophils and formation of blood vessels (Addison *et al.*, 2000; Scapini *et al.*, 2004). This also supported the immunomodulatory potential of MTH in regulating immune responses particularly during wound healing process.

Table 4.2 Microarray gene expression data associated with the regulation of immune responses

Sequence name	GenBank accession	Fold change
Chemokine (CXC family):		
Chemokine ligand 9 (*CXCL9*) #	NM_002416	-10.46
Chemokine ligand 1 (*CXCL1*) #	NM_001511	+3.29
Chemokine ligand 3 (*CXCL3*) #	NM_002090	+3.17
Chemokine ligand 2 (*CXCL2*)	NM_002089	+2.91
Chemokine ligand 5 (*CXCL5*)	NM_002994	+2.67
Chemokine (CC family):		
Chemokine ligand 20 (*CCL20*)	NM_004591	+2.51
Chemokine ligand 3-like 3 (*CCL3L3*)	NM_001001437	+2.09
Hematopoietins cytokine:		
Colony stimulating factor 3 (*CSF3*)	NM_000759	+2.61
Interleukin-23 alpha (*IL23A*)	NM_016584	+2.55
Interleukin-12 (*IL12*)	NM_002187	+2.43
Interleukin-6 (*IL6*)	NM_000600	+2.41
Interleukin-10 family:		
Interleukin-20 (*IL20*) #	NM_018724	+4.76
Interleukin-24 (*IL24*) #	NM_001185156	+3.88
Interleukin-10 (*IL10*) #	NM_000572	+2.36
Tumor necrosis factor (TNF) family:		
Tumor necrosis factor superfamily member 15 (*TNFSF15*)	NM_005118	+2.43
Tumor necrosis factor superfamily member 10D (*TNFRSF10D*)	NM_003840	+2.32
Tumor necrosis factor superfamily member 13B (*TNFSF13B*)	NM_006573	+2.30
Interferon:		
Interferon gamma (*IFNG*) #	NM_000619	-6.20

Sequence name	GenBank accession	Fold change
Interleukin-1 family:		
Interleukin 1 alpha (*IL1A*)	NM_000575	+2.98
Transforming growth factor beta (TGF-β) family:		
Inhibin beta A (*INHBA*)	NM_002192	+2.77
Platelet-derived growth factor (PDGF) family:		
Vascular endothelial growth factor A (*VEGFA*)	NM_001025370	+2.14
Others:		
Guanylate binding protein 1 (*GBP1*)	NR_003133	-4.38
Interferon alpha inducible protein (*IFI6*)	NM_022873	-3.10
Interferon-induced protein 44-like (*IFI44L*)	NM_006820	-2.95
2'-5'-oligoadenylate synthetase 3 (*OAS3*)	NM_006187	-2.94
Pentraxin-related protein (*PTX3*)	NM_002852	+2.88
2'-5'-oligoadenylate synthetase 1 (*OAS1*)	NM_002534	-2.75
Solute carrier family 11 (*SLC11A1*)	NM_000578	+2.58
Fms-related tyrosine kinase 1 (*FLT1*)	NM_001160031	+2.45
UL16 binding protein (*ULBP1*)	NM_025218	-2.40
Interleukin 1 receptor antagonist (*IL1RN*)	NM_173843	+2.40
2'-5'-oligoadenylate synthetase 2 (*OAS2*)	NM_001032731	-2.39
DEAD box polypeptide 58 (*DDX58*)	NM_014314	-2.33
Interferon induced protein 35 (*IFI35*)	NM_005533	-2.30
Fc fragment of IgA receptor (*FCAR*)	NM_002000	+2.30
Triggering receptor expressed on myeloid cells 1 (*TREM1*)	NM_018643	+2.28
Mitogen activated protein kinase kinase kinase 13 (*MAP3K13*)	NM_004721	+2.24
Guanylate binding protein 5 (*GBP5*)	NM_052942	-2.19
Toll-like receptor 2 (*TLR2*)	NM_003264	+2.16
Guanylate binding protein 3 (*GBP3*)	NM_018284	-2.10

Sequence name	GenBank accession	Fold change
Leukocyte immunoglobulin-like receptor subfamily A (*LILRA6*)	NM_104098	+2.06
NLR domain containing protein 3 (*NLRP3*)	NM_001079821	+2.06
Complement component 3 (*C3*)	NM_000064	+2.03
Thrombospondin 1 (*THBS1*)	NM_003246	+2.00

The fold change represented mean values obtained from 4 biological replicates. The + and - signs indicated upregulated and downregulated genes respectively. The genes with " # " sign were validated using RT-qPCR.

Based on RT-qPCR analysis (**Figure 4.3**), it was found that the expression pattern for *IFNG* and *IL10* were consistent with their respective microarray data. Compared to their respective controls, the expression of *IFNG* was significantly ($p = 0.0379$) downregulated by 14.40 fold in PBMCs upon 0.125 % MTH treatment for 24 hours. In contrast, the expression of *IL10* was significantly ($p = 0.0134$) upregulated by 4.07 fold in MTH-treated PBMCs. On the other hand, the expression of *IL2* was downregulated by 1.38 fold and the expression of *IL4* was upregulated by 0.22 fold in MTH-treated PBMCs when compared to their respective controls. These gene expressions (*IL2* and *IL4*) were not statistically significant.

It was also found that the gene expression of *IL20*, *IL24*, *CXCL1* and *CXCL3* were consistent with their respective microarray data. Based on RT-qPCR analysis (**Figure 4.3**), the expression of *IL20* ($p = 0.0203$), *IL24* ($p = 0.0493$), *CXCL1* ($p = 0.0500$) and *CXCL3* ($p = 0.0436$) were significantly upregulated by 7.87 fold, 4.41 fold, 6.34 fold and 4.03 fold respectively in MTH-treated PBMCs when compared to their respective controls.

In contrast, the expression of *CXCL9* was not significantly regulated in PBMCs after MTH treatment even though the gene expression was downregulated by 41.32 fold (**Figure 4.3**). This could be possibly due to the large SEM for the expression of *CXCL9* as compared to other genes. Based on raw data, it was found

that the Donor 4 in particular showed a higher *CXCL9* gene expression level (average fold change of 148.32) when compared to other 3 donors (average fold changes of 5.66). This data was not omitted to avoid biasness towards Donor 4.

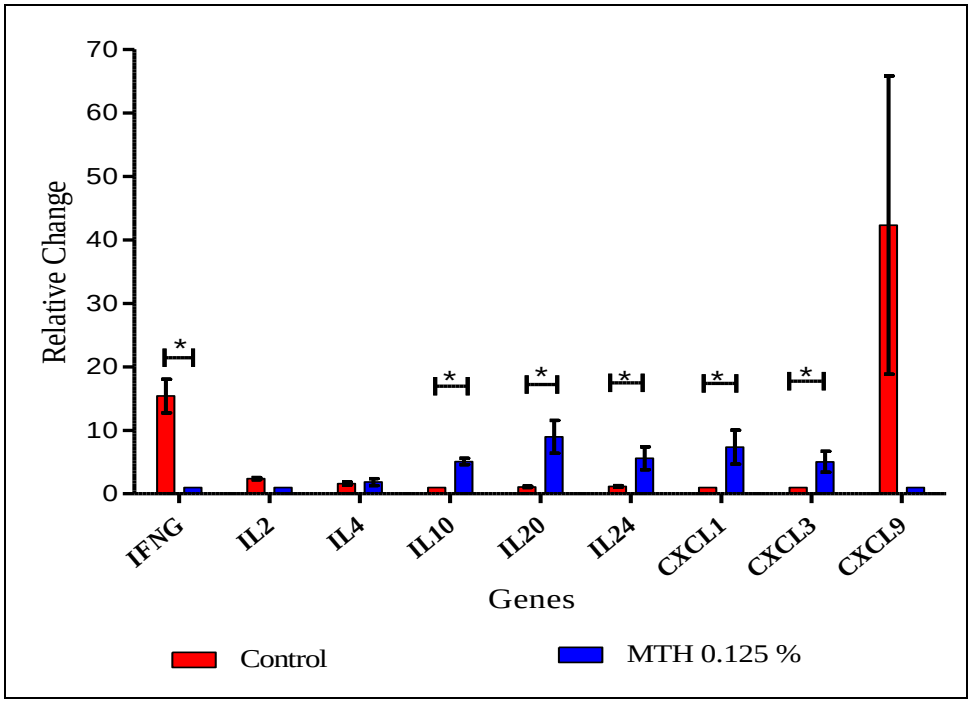


Figure 4.3 Transcriptional alteration of *IFNG*, *IL2*, *IL4*, *IL10*, *IL20*, *IL24*, *CXCL1*, *CXCL3* and *CXCL9* upon 0.125 % MTH treatment for 24 hours. All values were represented as mean ± SEM from 4 biological replicates. (*) represented $p < 0.05$ analyzed using paired T-test between control and MTH-treated samples.

Among the genes validated using RT-qPCR, *IFNG* and *IL10* were further studied at protein level using ELISA approach. Both these genes were selected for further study because it was known that the production of IFN-γ can be regulated by IL-10 and vice versa. This can provide a better understanding in explaining the immunomodulatory effect of MTH in regulating pro-inflammatory and immunoregulatory responses in the human immune system. In addition, *IL2* (type 1 cytokine) and *IL4* (type 2 cytokine) were also selected to serve as negative controls (**Table 4.3**)

Table 4.3 Genes selected for ELISA study

Genes	Expression level (fold change)	
	Microarray	RT-qPCR
IFNG	-6.20	-14.40
IL10	+ 2.36	+ 4.07
IL2	< 2.00	-1.38
IL4	< 2.00	+ 0.22

The fold change represented mean values obtained from 4 biological replicates. The + and - signs indicated upregulated and downregulated genes respectively.

4.3.3 Potential pathway altered by MTH in PBMCs

The 44 genes were associated with the regulation of immune responses were analyzed using online DAVID software and 1 potential pathway was determined based on Kyoto Encyclopedia of Genes and Genomes (KEGG) database. This pathway was identified as the cytokine network pathway (**Figure 4.4**).

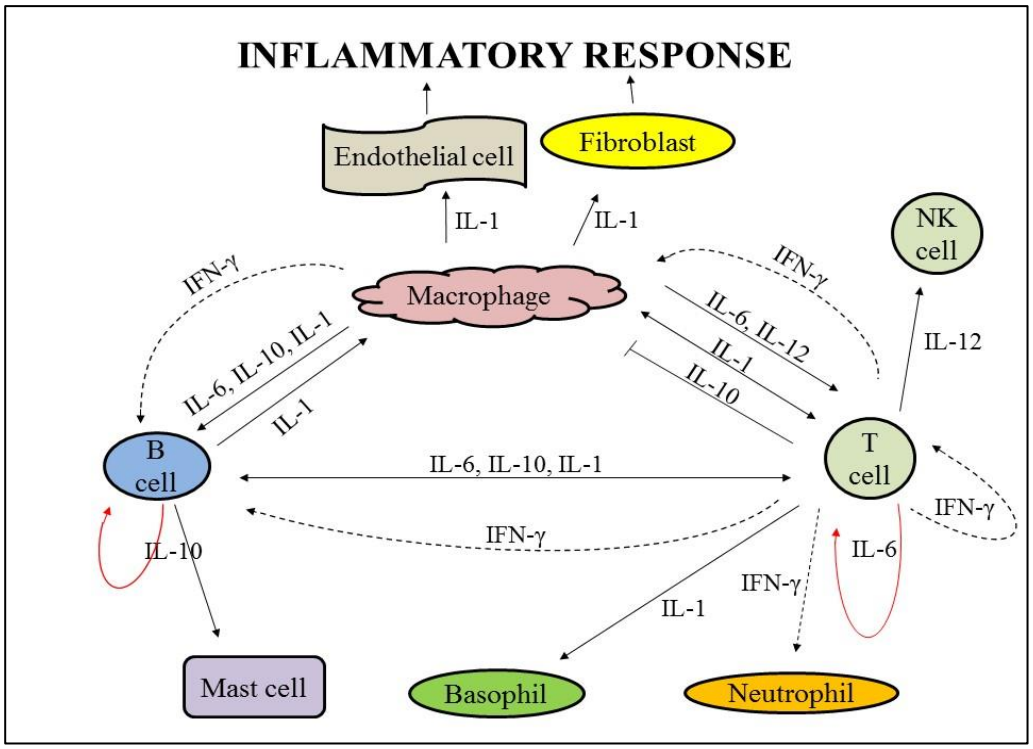


Figure 4.4 Cytokine network. The 5 genes which were significantly regulated in PBMCs after 0.125 % MTH treatment for 24 hours. These genes were *IFNG*, *IL1*, *IL6*, *IL10* and *IL12*. (⇢) represented stimulatory effect, (⊣) represented inhibitory effect and (→) represented self-regulatory effect. 'Solid arrow' represented gene expression upregulated and 'dotted arrow' represented gene expression downregulated. (Modified from KEGG, 2012)

In the cytokine network pathway, it was proposed that MTH may possess potential immunomodulatory effect to regulate gene expression in different immune cells such as macrophages, T cells and B cells to secrete various type-1 cytokines (IFN-γ and IL-12), type-2 cytokines (IL-6 and IL-10) as well as IL-1 (**Figure 4.4**). This suggested that MTH may potentially activate different PBMCs subpopulations.

The immunomodulatory effect of MTH in regulating the expression of *IFNG* and *IL10* triggered our interest to investigate the translation of these genes at protein level because both these proteins were known to regulate pro-inflammatory and immunoregulatory responses in the human immune system. Theoretically, IFN-γ can

promote differentiation of B cells into plasma cells, enhance T cells proliferation (positive feedback effect) and stimulate phagocytosis activity in macrophages by elevating the expression of MHC class II molecules (Schroder *et al.*, 2004). The production of IFN-γ can also stimulate neutrophils to secrete various pro-inflammatory cytokines such as TNF-α and IL-1β, which are involved in immune cells proliferation and differentiation (Ellis & Beaman, 2004). However, microarray analysis showed that the expression of *IFNG* in present study was downregulated by 6.20 fold in MTH-treated PBMCs. This suggested that MTH may possibly possess immuno-suppressive activity in regulating pro-inflammatory responses in PBMCs.

As for *IL10*, the expression of this gene was upregulated by 2.36 fold in MTH-treated PBMCs. The production of this cytokine can regulate the inflammatory effect in mast cells in response to allergic reaction, inhibit the production of type 1 cytokines in T cells as well as inhibit the phagocytic activity in macrophages by downregulating the expression of co-stimulatory molecules (CD40), which is essential in the activation of macrophage. Besides, the production of IL-10 in B cells also has a positive feedback effect which can enhance B cell survival, proliferation and antibody production (Duque & Descoteaux, 2014). This suggested that MTH may possibly possess both immuno-suppressive and immuno-activating potential in different immune cells.

Besides *IFNG* and *IL10*, the expression of *IL1* was also upregulated by 2.98 fold in MTH-treated PBMCs. The production of IL-1 can enhance antibody production in B cells, stimulate cell proliferation in helper T cells by functioning as a co-stimulator and promote phagocytosis activity in macrophages (Duque & Descoteaux, 2014). The production of IL-1 can also stimulate basophils to release chemical mediators such as histamine and proteolytic enzymes in response to allergic inflammation (Suzukawa *et al.*, 2008). Additionally, IL-1 can activate mitogen-activated protein kinase kinase kinases (MAPKKKs) in endothelial cells leading to the activation of transcription factor nuclear factor-kB (NF-kB) to initiate expression of

immune genes such as chemokines (chemotactic cytokine) and E-selectin (adhesion molecules that bind leukocytes) during wound healing (Pober & Sessa, 2007). Similarly, IL-1 can also stimulate fibroblast activation to maintain the homeostasis of adjacent cells and orchestra the movement of inflammatory infiltrates leading to inflammation process (Linthout *et al.*, 2014). These regulations are crucial especially during the initiation of wound healing process. This suggested that MTH may possess wound healing potential.

Additionally, the expression of *IL6* was upregulated by 2.41 fold in MTH-treated PBMCs. Theoretically, the production of IL-6 can regulate cell proliferation of T cells as well as to control the expression of chemokine receptors on T cells (positive feedback effect) (Hunter & Jones, 2015). This suggested that MTH can possibly possess immuno-activating effect on T cells.

As for *IL-12*, the expression of this gene was upregulated by 2.43 fold in MTH-treated PBMCs. Theoretically, the production of IL-12 can regulate the differentiation of naïve T cells into Th1 cells as well as stimulate IFN-γ and TNF-α production in T and NK cells (Teng *et al*., 2015).

4.4 Discussion

The gene expression profile in PBMCs treated with 0.125 % MTH for 24 hours was determined using Agilent's microarray platform. Collectively, the microarray data showed that MTH possessed immunomodulatory effect in PBMCs by regulating the expression of various chemokines, hematopoietin, PDGF, TGF-β, interferon, IL-10, IL-1 and TNF (**Table 4.2**). For example, MTH was found to upregulate the expression of various chemokine genes such as *CXCL1* (+3.29 fold), *CXCL2* (+2.91 fold), *CXCL3* (+3.17 fold) and *CXCL5* (+2.67 fold), which can stimulate the recruitment of leukocytes to the site of infection as well as to trigger the initiation of wound healing process (Graves & Jiang, 1995). This supported the wound healing activity in MTH. The immunomodulatory effect of MTH in upregulating the expression of

hematopoietin cytokine such as *IL12* (+2.43 fold) also suggested that MTH may potentially possess antitumor and antimicrobial activities. Functionally, the expression of *IL12* can stimulate cell proliferation in cytotoxic T cells as well as to promote the cytotoxic activity in NK cells (Teng *et al*., 2015).

Besides, MTH was also found to upregulate the expression of PDGF such as *VEGFA* (+2.14 fold), which can stimulate the formation of new blood vessels (Martini *et al*., 2005). This suggested that MTH may possess wound healing potential. As for TGF-β family, the immunomodulatory effect of MTH in upregulating the expression of *INHBA* (+2.77 fold) suggested that MTH may possess antitumor activity. Previously, it was reported that the expression of *INHBA* can suppress cell proliferation in gonadal stromal tumor cells (Mather *et al.*, 1992). In addition, the immunomodulatory effect of MTH in upregulating the expression of *IL10* related family such as *IL20* (+4.76 fold) and *IL24* (+3.88 fold) suggested that MTH may potentially possess anti-inflammatory and immuno-suppressive activities. Functionally, the expression of these genes are often related to the termination of inflammation process and inhibition of pro-inflammatory cytokines such as IFN-γ and TNF-α (Martini *et al*., 2005).

Furthermore, it was also found that the expression for *IL1* family such as *IL1A* (+2.98 fold) was upregulated by MTH. Functionally, the expression of *IL1A* can induce B cell proliferation to promote immunoglobulin secretion (Doan *et al*., 2008b). Besides *IL1A*, MTH also upregulate the expression of TNF family such as *TNFSF13B* (+2.30 fold) which can potentially induce cell proliferation and differentiation in B cells (Martini *et al*., 2005). This supported the immuno-stimulating activity in MTH specifically in enhancing the activities of B cells.

In contrast, MTH was found to downregulate the expression of interferon family such as *IFNG* (-6.20 fold). As mentioned previously, *IFNG* can promote cell proliferation and differentiation in B cells and T cells as well as elevate phagocytosis activity in macrophages (Schroder *et al*., 2004). Hence, the downregulation in the

expression of *IFNG* in MTH-treated PBMCs suggested that MTH may possess immuno-suppressive activity in regulating pro-inflammatory responses in PBMCs.

Among all the immune related genes regulated by MTH in PBMCs, 9 genes (*IFNG*, *IL2*, *IL4*, *IL10*, *IL20*, *IL24*, *CXCL1*, *CXCL3* and *CXCL9*) were validated using RT-qPCR. Based on the result, it was found that the expression pattern for *IL10*, *IFNG*, *CXCL1*, *CXCL3*, *IL20* and *IL24* were consistent with their respective microarray data and were statistically significant when compared to their respective controls. This suggested that MTH may potentially possess immunoregulatory activity in regulating the expression of *IL10*, *IFNG*, *CXCL1*, *CXCL3*, *IL20* and *IL24* in PBMCs.

In present study, it was found that the expression of *IL10* were significantly upregulated by 2.36 fold (microarray) and 4.07 fold (RT-qPCR) in MTH-treated PBMCs when compared to control. The *IL10* gene encodes for IL-10 cytokine which plays a crucial role in modulating the expression of various cytokines, soluble mediators and cell surface molecules in immune cells (Moore *et al*., 2001). This cytokine is also known to exert immunoregulatory activities in preventing inflammatory and autoimmune diseases. This cytokine is commonly produced by immune cells in both innate and adaptive immune system such as CD4+ helper T cells, CD8+ cytotoxic T cells, B cells, dendritic cells, macrophages, NK cells, mast cells, eosinophils and neutrophils (Saraiva & O'Garra, 2010). Functionally, IL-10 can inhibit the production of IFN-γ and IL-2 in Th1 cells as well as the production of IL-4 and IL-5 in Th2 cells. Besides its effect on Th1 and Th2 cytokines, IL-10 can also inhibit the production of pro-inflammatory cytokines (IL-6, IL-8, IL-12 and TNF-α), growth factors (granulocytes-macrophages colony-stimulating factor [GM-CSF]), chemokines (MIP-1α and CXCL-10), expression of MHC class II molecules and costimulatory molecules (CD80, CD86 and CD88) in activated monocytes and macrophages (Borish, 1998; Moore *et al*., 2001). In contrast, the immunoregulatory effect of IL-10 on NK cells and B cells is mainly stimulatory. IL-10 can enhance the

cytotoxic activity and cell proliferation in activated NK cells. As for B cells, IL-10 can promote differentiation of B cells towards plasma cells as well as enhance IgM synthesis by preventing B cells undergoing apoptosis (Asadullah *et al.*, 2003). Therefore, the upregulation in the expression of *IL10* in MTH-treated PBMCs suggested that MTH may possibly possess immuno-regulatory and anti-inflammatory activities.

Similar microarray and RT-qPCR analysis also showed that the expression of *IFNG* were significantly downregulated by 6.20 fold and 14.40 fold respectively in MTH-treated PBMCs when compared to control. During immune responses, the production of IFN-γ is commonly suppressed by IL-10, IL-4 and TGF-β. Similar suppressive effect was also observed in present study where the gene expression of *IFNG* was significantly downregulated in MTH-treated PBMCs, possibly due to the upregulation in the expression of *IL10*. Functionally, the *IFNG* gene encodes for IFN-γ cytokine which is known to exert immunoregulatory and pro-inflammatory activities. This cytokine is commonly produced by CD4+ helper T cells type 1, CD8+ cytotoxic T cells, B cells, NK cells, NKT cells and APCs such as monocytes, macrophages and dendritic cells (Schroder *et al*., 2004). The IFN-γ can induce direct antitumor and antimicrobial activities in macrophages by up-regulating antigen processing and presentation (Young & Hardy, 1995). IFN-γ can also regulate the proliferation, maturation and differentiation of other immune cells as well as leukocyte attraction (Boehm *et al.*, 1997). In addition, IFN-γ can enhance NK cell activity which is crucial in early host defense against infection as well as regulating B cell functions such as immunoglobulin production and class switching (Carnaud *et al*., 1999). Hence, the downregulation in the expression of *IFNG* in MTH-treated PBMCs suggested that MTH may possibly possess immunoregulatory activity in suppressing pro-inflammatory responses in PBMCs.

As for *IL20*, it was found that the gene expression of *IL20* in MTH-treated PBMCs were significantly upregulated by 4.76 fold and 7.87 fold respectively in

microarray and RT-qPCR analysis. Similarly, the gene expression of *IL24* in MTH-treated PBMCs were also significantly upregulated by 3.88 fold in microarray analysis and 4.41 fold in RT-qPCR analysis. Both the *IL20* and *IL24* genes encode for IL-20 and IL-24 cytokines respectively, which belong to the IL-10 cytokine family (Ouyang *et al.*, 2011). IL-20 is commonly produced by monocytes, granulocytes, dendritic cells, fibroblasts and keratinocytes. On the other hand, IL-24 is commonly produced by monocytes, macrophages, endothelial cells and type 2 helper T cells (Rutz *et al.*, 2014). Both IL-20 and IL-24 bind to IL-20 receptor β-subunit (IL-20RB) and are involved in the induction of antimicrobial responses in epithelial cells by promoting the production of various antimicrobial peptides such as β-defensins and psoriasin (Pestka *et al*., 2004; Sa *et al.*, 2007). Besides, both cytokines can also stimulate the proliferation and differentiation of epithelial cells to strengthen the physical barrier against invading microbes as well as to stimulate the production of various chemokines in epithelial cells to facilitate the recruitment of leukocytes to the site of inflammation (Sa *et al*., 2007). Furthermore, both IL-20 and IL-24 can induce the expression of VEGF-A to promote angiogenesis during wound healing (Bao *et al*., 2009). This suggested that MTH may potentially possess antimicrobial and wound healing activity.

In present study, the expression of chemokine related genes such as *CXCL1, CXCL3* and *CXCL9* were also found to be regulated by MTH. The gene expression of *CXCL1* in MTH-treated PBMCs were significantly upregulated by 3.29 fold in microarray analysis and 6.34 fold in RT-qPCR analysis. The *CXCL1* gene encodes for CXCL1 chemokine which is a chemotactic cytokine belonging to the CXC chemokine family. The CXCL1 is also known as growth-related oncogene alpha (GRO-α) and is commonly produced by neutrophils, macrophages and epithelial cells. This chemokine binds to chemokine receptor CXCR2 and is involved in the chemotactic activity for neutrophils to facilitate inflammation, wound healing and angiogenesis responses (Addison *et al*., 2000). In addition, the gene expression of *CXCL3* in PBMCs were

significantly upregulated by 3.17 fold and 4.03 fold in microarray and RT-qPCR analysis respectively. The *CXCL3* gene encodes for CXCL3 chemokine which is also known as growth-related oncogene gamma (GRO-γ). This chemokine is commonly produced by macrophages and epithelial cells. Similar to *CXCL1*, this chemokine also binds to chemokine receptor CXCR2 and is involved in the chemotactic activity for neutrophils and activate leukocytes to facilitate inflammation (Addison *et al*., 2000). The recruitment of neutrophils plays a crucial role in angiogenesis response because neutrophils can synthesize angiogenic molecules such as VEGF-A to promote the formation of new blood vessels during wound healing (Scapini *et al*., 2004). Similarly, this also suggested that MTH may possibly promote wound healing activity.

As for *CXCL9*, both microarray and RT-qPCR analysis showed that the gene expression of *CXCL9* were downregulated by 10.46 fold and 41.32 fold respectively in MTH-treated PBMCs. However, these findings were not statistically significant possibly due to the huge variation in the data collected from each donor. Hypothetically, these huge differences may be contributed by the genetic variation between different donors as well as environmental factor such as diet. Theoretically, the *CXCL9* gene encodes for CXCL9 chemokine which is a small chemotactic cytokine belonging to the CXC chemokine family. This chemokine is commonly produced by mononuclear cells, endothelial cells and fibroblast (Loos *et al*., 2006). This chemokine binds to chemokine receptor CXCR3 and is involved in the chemotactic activity for activated T cells and NK cells to facilitate anti-tumor immunity resulting in angiostatic response and tumor regression (Wenzel *et al*., 2005). In the future, statistical significance can be improved with a larger sampling population and genetic variation between donors can be reduced by using randomized block experimental design. In randomized block experimental design, the donors can be divided into blocks where the variation within each block can be reduced as compared to the variation between different blocks (Howard, 2002).

4.5 Concluding remarks

Overall, present findings showed that MTH possessed immunomodulatory effect in PBMCs treated with 0.125 % MTH for 24 hours by significantly regulating 44 genes associated with immune responses. Among these genes, it was found that MTH significantly regulated the expression of genes related to the production of type 1 and type 2 cytokines such as *IFNG* and *IL10*. Based on these findings, it was speculated that MTH may potentially trigger cell activation in different PBMCs subpopulations leading to the expression of immune-related genes. Hence, the cell population and cell activation in PBMCs subpopulations (helper T cells, cytotoxic T cells, B cells and NK cells) were subsequently quantified using flow cytometry approach. Additionally, the immunomodulatory effect of MTH in regulating the expression of *IFNG* and *IL10* was also studied at protein level using ELISA approach.

CHAPTER 5: IMMUNOMODULATORY EFFECT OF MTH ON PBMCs ACTIVATION

5.1 Introduction

The human immune system comprised various immune cells which derived from pluripotent hematopoietic stem cells. Generally, these cells can be classified into myeloid lineage and lymphoid lineage. Upon stimulation, cells of the myeloid lineage can differentiate into granulocytic cells (neutrophils, basophils and eosinophils) as well as agranular phagocytic cells (monocytes, macrophages and dendritic cells) which are involved in innate defences. Besides that, cells of the myeloid lineage can also differentiate into erythrocytes and platelet which are involved in gas transport and blood clotting respectively. On the other hand, cells of the lymphoid lineage can differentiate into B lymphocytes and T lymphocytes which are involved in acquired immune responses as well as differentiate into NK cells which can bridge both innate and acquired immune systems (Doan *et al*., 2008a).

The PBMCs are commonly isolated from peripheral whole blood through density gradient centrifugation. Generally, these cells are blood cells with round nucleus such as monocytes, macrophages, B cells, T cells, NK cells and natural killer T cells (Mihajlovic *et al*., 2014). In human, the percentages of PBMCs can vary between different individuals depending on their genetic background and environmental factor such as diet. Typically, the percentages of cells types within the PBMCs fall between 70 – 90 % of lymphocytes, 10 – 30 % of monocytes and 1 – 2 % of dendritic cells. Within the 70 – 90 % lymphocytes, 70 – 85 % are CD3+ T cells (45 – 70 % of PBMCs), 5 – 20 % B cells (approximately 15 % of PBMCs) and 5 – 20 % NK cells (approximately 15 % of PBMCs). Within the CD3+ T cells, approximately 25 – 60 % are CD4+ T cells and 5 – 30 % are CD8+ T cells (Miyahira, 2012). In many immunological studies, PBMCs are often used to study the mechanisms involved in human immune system because these cells can mimic the *in vivo* system of immune responses very closely (Mihajlovic *et al*., 2014).

Based on literature review, it was reported that 0.1 % Multifloral honey was sufficient to activate phagocytes and to stimulate proliferation in peripheral blood B and T lymphocytes (Abuharfeil *et al*., 1999). Besides immuno-activating effect, it was also reported previously that honey may possess immuno-suppressive activity to treat allergic reactions (Bogdanov, 2011a). For example, Ashaari *et al.* (2013) reported previously that daily ingestion of 1 g/kg body weight of MTH for 4 weeks can improve the symptoms of allergic rhinitis (hay fever) in Malaysian patients. In their study, it was postulated that MTH could possibly suppressed B cells activity and lgE-mediated hypersensitivity reaction in these patients (Ashaari *et al*., 2013).

Based on these previous findings, it was speculated that this immunomodulatory effect may also present in the MTH used in present study but with variation depending on the botanical origin of the honey. To date, the immunomodulatory effect of MTH in regulating cell activation in PBMCs has not been reported. Based on the findings obtained in Chapter 4, microarray analysis showed that 0.125 % MTH significantly regulated the expression of 44 immune related genes in PBMCs. Hence, it was hypothesized that cell activation in PBMCs subpopulations (B cells, cytotoxic T cells, helper T cells and NK cells) may be regulated differently by MTH, potentially leading to cell proliferation. On the other hand, it was also hypothesized that MTH may possibly possess immuno-suppressive effect in suppressing cell activation in PBMCs subpopulations as reported in previous study using MTH. Hence, the percentage of cell populations and cell activation in MTH-treated PBMCs was determined in this chapter using flow cytometry.

The flow cytometer is a powerful detection tool that utilizes antibodies conjugated with fluorochromes to detect the presence of specific cell markers and simultaneously determine multiple characteristics of cells within a mixed population based on size, phenotype and structural complexity. Immunophenotyping using flow cytometry is also rapid and accurate because the different PBMCs subpopulations can be quantified within minutes and any debris or dead cells in the samples can be

excluded during data analysis (Rahman, 2006). However, there are several limitations in using flow cytometry for immunophenotyping. One of the major limitations is the possibility of spectral overlap from the multicolour fluorescence emitted from the fluorochromes which can contribute to high background reading. This can be reduced or avoided by selecting fluorochromes at very different ends of the spectrum. Another way to reduce this spectral overlap is to carry out fluorescence compensation during data analysis. Briefly, fluorescence compensation is a method used to calculate and subtract the percentage of any fluorochromes in a particular detection channel that is not assigned specifically to it. Another limitation of flow cytometry is the availability of specific cell surface markers with desired fluorochromes that can be detected by the lasers in the flow cytometer. In some cases, different cell surface markers are only available with particular fluorochromes. Hence, data analysis for these cell populations will need to be carried out separately using the same sample (Rahman, 2006).

Besides flow cytometry, any changes in cell population for each PBMCs can also be determined using DNA synthesis cell proliferation assay or metabolic cell proliferation assay. The DNA synthesis cell proliferation assay measures the amount of DNA synthesized in dividing cells by incorporating 5-bromo-2'-deoxyuridine (BrdU) into the newly synthesized DNA during cell division. This BrdU molecule can be detected using specific antibody conjugated with a reporter dye and quantified using colorimetric approach. As for the metabolic cell proliferation assay, this assay can be used to measure cell proliferation based on the metabolic activity in a population of cells. One of the most commonly used metabolic cell proliferation assay is MTT assay. As mentioned previously, the MTT assay utilizes the increase in the activity of lactate dehydrogenase in mitochondria during cell division to reduce tetrazolium salt into formazan dye which can be subsequently measured using spectrophotometer (Smith, 2012). However, both the DNA synthesis cell proliferation assay and metabolic cell proliferation assay cannot specifically sort and quantify the

different PBMCs subpopulations separately. Therefore, flow cytometry remains the preferred choice to study the immunomodulatory effect of MTH in regulating cell activation in different PBMCs subpopulations.

During experimental setup, it was initially planned to treat the PBMCs with 0.125 % MTH for 24 hours. However, due to time limitation in analyzing the MTH-treated PBMCs using the flow cytometer BD LSR Fortessa in University Putra Malaysia, the incubation duration was reduced to 16 hours. Based on a previous study, it was reported that PBMCs can be activated rapidly within an hour after mitogen stimulation and can reach maximal expression between 16 to 24 hours (Simms & Ellis, 1996). The different PBMCs subpopulations treated with MTH were determined and quantified using lineage specific markers and activation marker (CD69) antibodies conjugated with fluorochromes. In order to study the immunomodulatory activity of MTH in regulating cellular activation in PBMCs, the PBMCs were pre-stimulated with PMA and ionomycin followed by MTH treatment. The PMA is a small organic compound that can diffuse through cell membrane into the cytoplasm to directly activate protein kinase C (PKC) which will subsequently affects gene expression, biosynthesis of protein, cell differentiation and enzymatic activity in different immune cells (Goel *et al.*, 2007). The ionomycin was also used in PBMCs stimulation to trigger calcium release for the signalling of NFAT which is expressed in most cells of the immune system (Goel *et al.*, 2007). Hypothetically, if MTH possessed immuno-activating activity, the expression of cell activation marker (CD69) in PBMCs subpopulations will be expected to increase after MTH treatment. In contrast, if MTH possessed immuno-suppressive activity, the expression of cell activation marker (CD69) in PBMCs subpopulations will be expected to decrease after MTH treatment.

Objectives:

1. To investigate changes in PBMCs subpopulations (B cells, helper T cells, cytotoxic T cells and NK cells) after MTH treatment

2. To investigate changes in cellular activation (CD69+) in PBMCs subpopulations (B cells, helper T cells and cytotoxic T cells) after MTH treatment

5.2 Materials and methods

5.2.1 Cell seeding and mitogen stimulation

The PBMCs were isolated from blood samples collected from 6 healthy donors. The freshly prepared PBMCs were seeded into 96-well U bottom plates containing 5 x 10^5 cells in 100 µl of R5 medium per well. In order to study the immunomodulatory activity of MTH in regulating cell activation in PBMCs, the PBMCs were pre-stimulated with PMA and ionomycin diluted in R5 medium to give a final concentration of 5 ng/ml and 500 ng/ml respectively. The concentrations of PMA and ionomycin were optimized from 3 different combinations. The first combination was 5 ng/ml PMA with 500 ng/ml ionomycin, the second combination was 25 ng/ml PMA with 750 ng/ml ionomycin and the third combination was 50 ng/ml with 1000 ng/ml ionomycin. Non-stimulated PBMCs (with R5 medium only) and PBMCs stimulated for 4 hours (untreated) were used as negative controls. The non-stimulated and pre-stimulated PBMCs were incubated for 4 hours at 37 °C in a humidified atmosphere of 5 % carbon dioxide prior to 0.125 % MTH treatment for 16 hours. As mentioned above, the incubation duration was reduced from 24 hours to 16 hours due to time limitation.

In addition, PBMCs stimulated with PMA and ionomycin for prolong duration (20 hours) was used as positive control. The purpose of this positive control was to ensure that the PBMCs subpopulations were viable and capable of being activated. The incubation duration of 20 hours was selected based on a previous study conducted by Draganova *et al.* (2010), where the immunomodulatory effect of Bulgarian propolis in PBMCs subpopulations (helper T cells, cytotoxic T cells, B cell and NK cells) were determined using flow cytometry. In their study, the PBMCs stimulated with PMA for 24 hours was used as positive control (Draganova *et al.*, 2010).

5.2.2 MTH preparation

The 0.125 % MTH was serially diluted from stock MTH solution as described in **Section 3.2.2** using R5 medium and filtered using 0.45 µm minisart filter prior to treatment.

5.2.3 MTH treatment

After 4 hours of pre-stimulation using PMA (5 ng/ml) and ionomycin (500 ng/ml), the mitogen-stimulated PBMCs were centrifuged at 1800 rpm for 5 minutes at 23 °C and the supernatant was discarded. The cell pellet was washed using R0 medium and subsequently resuspended with 100 µl of 0.125 % MTH. The MTH-treated PBMCs were then incubated at 37 °C in a humidified atmosphere of 5 % carbon dioxide for 16 hours.

5.2.4 Staining of PBMCs

After 16 hours of incubation, the MTH-treated PBMCs were centrifuged at 1800 rpm for 5 minutes at 23 °C. The supernatant was discarded by flicking the 96-well U bottom plates and patted dry onto paper towel to remove any residue. The cell pellet was washed again using 100 µl FACS buffer and subsequently resuspended with 30 µl FACS buffer. Next, the PBMCs subpopulations in MTH-treated PBMCs were identified and quantified using lineage specific and activation marker antibodies conjugated with fluorochromes such as APC/CD3 (125 µg/ml), FITC/CD8 (25 µg/ml), PE-Cy7/CD19 (200 µg/ml), PE/CD56 (100 µg/ml), PE/CD69 (10 µg/ml) and 7AAD (50 µg/ml) (BioLegend, California, USA).

Basically, 1 µl of the antibodies was added into the MTH-treated PBMCs and mixed well using pipette. The stained PBMCs were covered with aluminium foil and incubated at 4 °C for 20 minutes. After 20 minutes of incubation, the PBMCs were washed with 100 µl FACS buffer to remove unbound antibodies. The PBMCs were then centrifuged at 1800 rpm for 5 minutes at 23 °C and the supernatant was

discarded. Next, 200 µl FACS buffer was added into each well to resuspend the cell pellet. Finally, the stained PBMCs were transferred into individual FACS tube and additional 300 µl FACS buffer was added into each FACS tube to make the final volume up to 500 µl. All stained PBMCs were kept on ice and in dark until immunophenotyping analysis within 2 hours. For each immunophenotyping analysis, single colour tubes (APC, FITC, PE-Cy7 and PE) and unstained cells were used for compensation.

5.2.5 Immunophenotyping

The stained PBMCs were analyzed using flow cytometer BD LSR Fortessa and software BD FACS Diva. Based on the side-scatter channel (SSC) and forward-scatter channel (FSC) plot, the PBMCs were gated and 10,000 cells were collected for each immunophenotyping analysis.

5.2.6 Statistical analysis

All data entry was obtained from 6 biological replicates and statistical analysis were conducted using GraphPad Prism version 5.02. The values were expressed as mean ± SEM, as a method to estimate the population mean. In this study, PBMCs activation was determined in PMA/I stimulated PBMCs treated with 0.125 % MTH for 16 hours and compared to non-stimulated PBMCs and PMA/I stimulated PBMCs. Hence, one-way ANOVA followed by Tukey posttest was used to compare overall difference between group means. The level of statistical significance was set at $p < 0.05$.

The Tukey posttest is a multiple comparison posttest commonly used to compare and determine any significant differences between the mean of different groups. In present study, the percentage of cytotoxic T cells, helper T cells, B cells and NK cells after PMA/I stimulation and 0.125 % MTH treatment were compared to their respective controls.

5.3 Results

5.3.1 Immunomodulatory effect of MTH on PBMCs subpopulations

The immunomodulatory effect of MTH on the phenotypes of different PBMCs subpopulations (B cells, cytotoxic T cells, helper T cells and NK cells) was determined using flow cytometry and shown in **Figure 5.1** below. Upon 0.125 % MTH treatment for 16 hours, it was found that the percentages of PMA/I stimulated CD3+ CD8+ cytotoxic T cells and CD3+ CD8- helper T cells were slightly decreased by 2.7 % (**Figure 5.1A**) and 1.9 % (**Figure 5.1B**) respectively when compared to untreated populations. In contrast, the percentage of PMA/I stimulated CD3- CD56+ NK cells was slightly increased by 0.4 % when compared to untreated population (**Figure 5.1C**). The percentage of PMA/I stimulated CD3- CD19+ B cells remained unchanged after MTH treatment (**Figure 5.1D**). Nevertheless, these changes were not statistically significant, suggesting that MTH did not stimulate cell proliferation in PBMCs subpopulations.

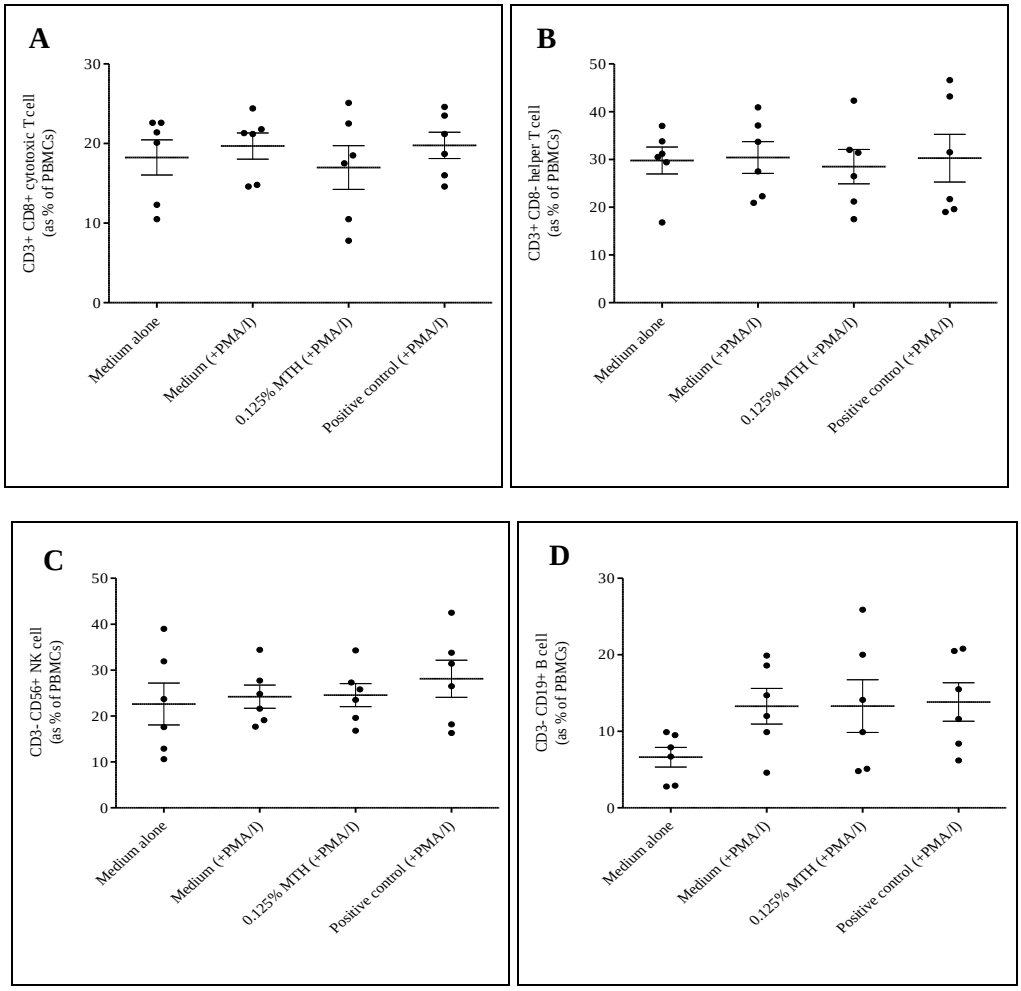


Figure 5.1 Immunomodulatory effect of MTH in CD3+ CD8+ cytotoxic T cells (**A**), CD3+ CD8- helper T cells (**B**), CD3- CD56+ NK cells (**C**) and CD3- CD19+ B cells (**D**). All values were represented as mean ± SEM from 6 biological replicates and analyzed using one-way ANOVA followed by Tukey posttest. 'Medium alone' represented basal level in non-stimulated PBMCs, 'Medium (+PMA/I)' represented basal level in stimulated PBMCs, '0.125 % MTH (+PMA/I)' represented stimulated PBMCs treated with 0.125 % MTH and 'Positive control (+PMA/I)' represented stimulated PBMCs.

Based on **Table 5.1** below, the percentages of CD3+ CD8+ cytotoxic T cells, CD3+ CD8- helper T cells, CD3- CD56+ NK cells and CD3- CD19+ B cells in non-stimulated and untreated PBMCs were 18.3 ± 2.2 %, 29.8 ± 2.8 %, 22.6 ± 4.5 % and 6.6 ± 1.3 % respectively. Upon PMA/I stimulation, the percentages of CD3+ CD8+

cytotoxic T cells, CD3+ CD8- helper T cells, CD3- CD56+ NK cells and CD3- CD19+ B cells were slightly increased to 19.7 ± 1.6 %, 30.4 ± 3.3 %, 24.2 ± 2.5 % and 13.3 ± 2.3 % respectively. As for positive control, the percentages of PMA/I stimulated CD3+ CD8+ cytotoxic T cells, CD3+ CD8- helper T cells, CD3- CD56+ NK cells and CD3- CD19+ B cells were found to be 19.8 ± 1.6 %, 30.3 ± 5.0 %, 28.1 ± 4.0 % and 13.8 ± 2.5 % respectively after incubated for 20 hours. However, these changes were not statistically significant when compared to non-stimulated PBMCs.

Table 5.1 Immunomodulatory effect of MTH and PMA/I stimulation effect in CD3+ CD8+ cytotoxic T cells, CD3+ CD8- helper T cells, CD3- CD56+ NK cells and CD3- CD19+ B cells

Type of PBMCs subpopulations	Percentage of cells			
	Medium alone	Medium (+PMA/I)	0.125 % MTH (+PMA/I)	Positive control (+PMA/I)
CD3+ CD8+ cytotoxic T cell	18.3 ± 2.2	19.7 ± 1.6	17.0 ± 2.7	19.8 ± 1.6
CD3+ CD8- helper T cell	29.8 ± 2.8	30.4 ± 3.3	28.5 ± 3.6	30.3 ± 5.0
CD3- CD56+ NK cell	22.6 ± 4.5	24.2 ± 2.5	24.6 ± 2.5	28.1 ± 4.0
CD3- CD19+ B cell	6.6 ± 1.3	13.3 ± 2.3	13.3 ± 3.4	13.8 ± 2.5

All values were represented as mean ± SEM from 6 biological replicates in 3 independent experiments.

5.3.2 Immunomodulatory effect of MTH on PBMCs activation

The activation of CD3+ CD8+ cytotoxic T cells, CD3+ CD8- helper T cells and CD3- CD19+ B cells in MTH-treated PBMCs were evaluated using CD69 cell surface marker. The CD69 is a signaling transmembrane glycoprotein commonly expressed on T cells, B cells, NK cells, neutrophils and eosinophils upon activation. Functionally, CD69 plays a crucial role as signal transmitting receptor in lymphocytes during lymphocytes activation and proliferation. Taking T cells as an example, the expression

of CD69 is initially very low in resting T cells (5 % – 10 %). However, the expression of CD69 is rapidly upregulated within an hour after PMA stimulation and can reach maximal expression between 16 to 24 hours before decline gradually towards 72 hours (Simms & Ellis, 1996). In present study, the cell activation in CD3- CD56+ NK cells was not determined because both CD56 and CD69 were conjugated with PE and hence cannot be differentiated during analysis.

When gated from cytotoxic T cells, helper T cells and B cells respectively in non-stimulated PBMCs (**Figure 5.1**), the basal percentages of activated CD 69+ CD3+ CD8+ cytotoxic T cells, CD69+ CD3+ CD8- helper T cells and CD69+ CD3- CD19+ B cells were found to be 7.2 ± 2.2 %, 2.0 ± 0.7 % and 35.6 ± 1.8 % (**Figure 5.2** and **Table 5.2**). Upon PMA/I stimulation, the percentages of activated CD69+ CD3+ CD8+ cytotoxic T cells, CD69+ CD3+ CD8- helper T cells and CD69+ CD3- CD19+ B cells were significantly ($P < 0.0001$) increased to 97.2 ± 1.3 %, 98.5 ± 0.5 % and 99.1 ± 0.3 % respectively, suggesting that the PMA/I stimulation on PBMCs activation was successful. Upon 0.125 % MTH treatment for 16 hours, the percentage of PMA/I stimulated CD69+ CD3+ CD8+ cytotoxic T cells, CD69+ CD3+ CD8- helper T cells and CD69+ CD3- CD19+ B cells were slightly increased to 97.8 ± 2.5 %, 99.0 ± 0.7 % and 99.2 ± 0.3 % respectively. Nevertheless, these increments were not statistically significant when compared to PMA/I stimulated PBMCs without MTH treatment (**Figure 5.2** and **Table 5.2**).

As for positive control, the percentages of PMA/I stimulated CD69+ CD3+ CD8+ cytotoxic T cells, CD69+ CD3+ CD8- helper T cells and CD69+ CD3- CD19+ B cells were found to be 99.4 ± 0.3 %, 99.7 ± 0.1 % and 99.0 ± 0.2 % respectively (**Table 5.2**). These increments were statistically significant ($p < 0.0001$) when compared to unstimulated PBMCs.

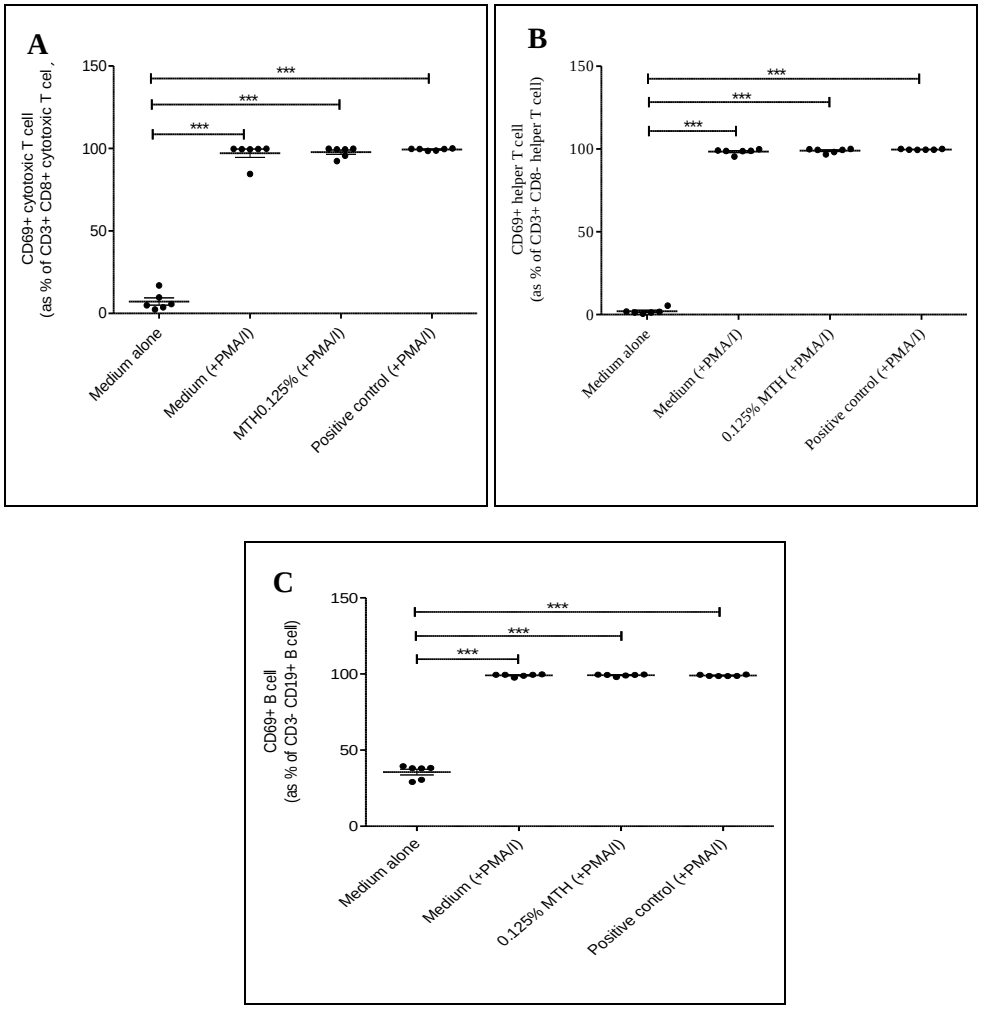


Figure 5.2 Immunomodulatory effect of MTH in activated CD69+ CD3+ CD8+ cytotoxic T cells (**A**), activated CD69+ CD3+ CD8- helper T cells (**B**) and activated CD69+ CD3- CD19+ B cells (**C**). All values were represented as mean ± SEM from 6 biological replicates. (***) represented $p < 0.0001$ analyzed using one-way ANOVA and Tukey posttest. 'Medium alone' represented basal level in non-stimulated PBMCs, 'Medium (+PMA/I)' represented basal level in stimulated PBMCs, '0.125 % MTH (+PMA/I)' represented stimulated PBMCs treated with 0.125 % MTH and 'Positive control (+PMA/I)' represented stimulated PBMCs.

Table 5.2 Immunomodulatory effect of MTH in activated CD3+ CD8+ cytotoxic T cells, activated CD3+ CD8- helper T cells and activated CD3- CD19+ B cells

Type of PBMCs subpopulations	Percentage of cells			
	Medium alone	Medium (+PMA/I)	0.125 % MTH (+PMA/I)	Positive control (+PMA/I)
CD69+ CD3+ CD8+ cytotoxic T cell	7.2 ± 2.2	97.2 ± 1.3	97.8 ± 2.5	99.4 ± 0.3
CD69+ CD3+ CD8- helper T cell	2.0 ± 0.7	98.5 ± 0.5	99.0 ± 0.7	99.7 ± 0.1
CD69+ CD3- CD19+ B cell	35.6 ± 1.8	99.1 ± 0.3	99.2 ± 0.3	99.0 ± 0.2

All values were represented as mean ± SEM from 6 biological replicates in 3 independent experiments.

5.4 Discussion

The immunomodulatory effect of MTH on PBMCs subpopulations was evaluated using flow cytometry. In this study, it was found that the basal percentages of CD3+ CD8+ cytotoxic T cells, CD3+ CD8- helper T cells, CD3- CD19+ B cells and CD3- CD56+ NK cells in non-stimulated and untreated PBMCs were 18.3 ± 2.2 %, 29.8 ± 2.8 %, 6.6 ± 1.3 % and 22.6 ± 4.5 % respectively. These results fall within the normal range as reported by Dhaliwal *et al.* (1995). In their study, 152 Malaysian donors were recruited and the reference range for cytotoxic T cells, helper T cells, B cells and NK cells were showed in **Table 5.3** below (Dhaliwal *et al.*, 1995).

Table 5.3 Reference range of PBMCs subpopulations in Malaysian donors

Cells	Dhaliwal *et al.* (1995)	Present study
Cytotoxic T cells	10.5 – 31.4 %	18.2 %
Helper T cells	16.8 – 37.0 %	29.8 %
B cells	2.8 % - 9.9 %	6.6 %
NK cells	10.6 % - 39.0 %	22.6 %

(Dhaliwal *et al.*, 1995)

In present study, it was found that the expression of CD69 in PMA/I-stimulated cytotoxic T cells, helper T cell and B cells treated with 0.125 % MTH were only slightly increased with no significant difference when compared to untreated populations. This suggested that MTH did not possessed notable immune activating effect in regulating cell activation in these PBMCs subpopulations. Nevertheless, these results also indicated that MTH possibly did not possessed immune suppressive effect because the expression of CD69 was not reduced in these PBMCs subpopulations after MTH treatment. Additionally, the PBMCs used in present study were isolated from healthy donors. This suggested that 0.125 % MTH can potentially maintain the health without suppressing the immune system in healthy individuals. This was consistent with traditional belief that honey can naturally maintain good health (Bogdanov, 2011b).

As compared to other honey, present findings contradicted with the findings reported by Abuharfeil *et al.* (1999). In their study, it was found that the proliferation of B lymphocytes and T lymphocytes were enhanced by 60.8 % and 54.4 % respectively in the presence of 0.1 % and 0.2 % of Multifloral honey respectively after 72 hours of incubation (Abuharfeil *et al*., 1999). In contrast, present findings showed that cell population in cytotoxic T cells, helper T cells, B cells and NK cells were not significantly enhanced after 0.125 % MTH treatment for 16 hours. As mentioned previously, the incubation duration in this study was reduced from 24 hours to 16 hours due to time limitation in analyzing the MTH-treated PBMCs using the flow cytometer BD LSR Fortessa in University Putra Malaysia. When compared to previous study, it was speculated that these differences may be possibly due to difference in incubation durations where Abuharfeil *et al.* (1999) incubated the B lymphocytes and T lymphocytes for 72 hours allowing more time for the immune cells to proliferate. Additionally, it was also speculated that these differences may be possibly due to different composition in MTH and Multifloral honey which contributed to different biological activity. This speculation was supported by Tonks

et al. (2003), who reported that Australian Jelly Bush honey, New Zealand Manuka honey and Pasture honey had different degree of immuno-stimulating effect in enhancing TNF-α, IL-1β and IL-6 production in MM6 cells and human monocytes due to different composition in these honeys (Tonks *et al*., 2003).

In terms of experimental setup, it was speculated that any changes in the percentage of cytotoxic T cells, helper T cells, B cells and NK cells after PMA/I stimulation and 0.125 % MTH treatment were possibly due to the activation and proliferation of memory cells because the incubation duration was relatively short (16 hours). In future, experimental design can be improved by identifying these memory cells using human memory cell markers such as chemokine receptor 7 (CCR7) and CD45RA for T cells. For example, naive T cells normally express high level of CCR7 and CD45RA. These naive T cells can differentiate into effector memory T cells and central memory T cells with various levels of CCR7 and CD45RA. In brief, effector memory T cells are CCR7- CD45RA- and central memory T cells are CCR7+ CD45RA- (Sallusto *et al.*, 2004).

In present study, the experimental setup was designed to determine cell activation through the expression level of CD69 in PBMCs subpopulations treated with 0.125 % MTH for 16 hours. Any changes in cell population for each PBMCs subpopulations were not successfully determined. This was because 16 hours of incubation was too short to provide any distinct changes as the cells required more time to undergo several cell divisions. In future, the incubation time of 0.125 % MTH can be further extended to 48 or 72 hours in order to study the immunomodulatory effect of MTH in regulating cell proliferation in PBMCs subpopulations. With longer incubation duration, it was hypothesized that the MTH-treated PBMCs can potentially undergo several cell proliferations, resulting in higher percentages of PBMCs subpopulations after MTH treatment. This hypothesis was supported by Lee *et al.* (2002), who reported that little proliferation was observed in antigen-stimulated helper T cells cultured in RPMI medium supplemented with 10 % FBS at the first 36 hours of

incubation. Rapid cell division was only observed after 36 hours with majority of the helper T cells undergone 3 or 4 cell divisions by 60 hours of incubation (Lee *et al.*, 2002). As for B cells, clonal expansion and differentiation into effectors B cells normally take approximately 4 to 5 days upon activation (Martini *et al.*, 2005).

5.5 Concluding remarks

Overall, present findings suggested that MTH did not suppressed cell activation in PBMCs subpopulations. Based on the microarray data obtained in Chapter 4, it was found that the expression of *IFNG* and *IL10* were significantly regulated in MTH-treated PBMCs. This suggested that MTh can potentially regulate the expression of immune-related genes in PBMCs without suppressing cell activation. In order to verify that these gene expressions were translated into proteins, the production of these cytokines in MTH-treated PBMCs were subsequently quantified using specific sandwich ELISA. In addition, the production of IL-2 and IL-4 were also quantified to serve as negative controls.

CHAPTER 6: IMMUNOMODULATORY EFFECT OF MTH ON Th1 AND Th2 CYTOKINES PRODUCTION IN PBMCs

6.1 Introduction

In living organisms, proteins are synthesized from amino acids based on the genetic information encoded in genes. These protein molecules are often macromolecules which consist of one or more chains of amino acid folded into specific 3-dimentional structure that determines its activity. Functionally, these protein molecules are involved in various biological activities such as DNA replication, transportation of biomolecules and catalysing metabolic reactions within the organisms. In the human immune system, different immune cells can produce various soluble proteins, known as cytokines which can both promote and suppress immune responses (Popa *et al.*, 2012).

Based on literature review, it was reported previously that the production of IL-6 was enhanced in MM6 cells treated with 1 % Manuka honey (831 ± 146 pg/ml) and 1 % Danish honey (316 ± 61 pg/ml) when compared to artificial honey (17 ± 7 pg/ml) (Timm *et al*., 2008). In another study, it was also reported that the production of TNF-α was significantly ($p < 0.001$) enhanced in human monocytes treated with 1 % Manuka honey (Tonks *et al.*, 2001). These findings suggested that honey can generally promote the production of various cytokines in different immune cells. Hence, it was hypothesized that similar immunomodulatory effect may be observed in MTH to regulate the production cytokines in PBMCs.

In this study, the immunomodulatory effect of MTH in regulating the expression of various immune related genes in PBMCs was previously determined using Agilent microarray. Based on the microarray findings, 2 genes (*IFNG* and *IL10*) which involved in the production of type 1 and type 2 cytokines were selected for further study. In addition, *IL2* and *IL4* were also selected to serve as negative controls to validate the results obtained in Chapter 4. In order to verify that the expressions of these genes were translated into proteins, the production of IFN-γ, IL-10, IL-2 and IL-

4 were quantified in MTH-treated PBMCs using sandwich ELISA. As mentioned in **Section 2.4.4**, ELISA is a technique commonly used to quantify the concentration of specific proteins in supernatant solutions using enzyme-linked antibodies (Lequin, 2005). The sandwich ELISA has several advantages over the conventional direct ELISA. For example, the sandwich ELISA uses a matched pair of protein-specific capturing antibodies and detection antibodies which can significantly improve its specificity and sensitivity as compared to the conventional direct ELISA (Lequin, 2005).

In order to examine the immunomodulatory effect of MTH on the production and accumulation of these Th1 and Th2 cytokines, the incubation duration was extended to a wider range (24 hours, 48 hours and 72 hours). The PBMCs were also stimulated with PMA/I mitogen to enhance the production of cytokines (IFN-γ and IL-10).

Objective:

1. To determine the secretion of cytokines (IFN-γ and IL-10) after MTH treatment in PBMCs

6.2 Materials and methods

6.2.1 Cell seeding

The PBMCs were isolated from blood samples collected from 6 healthy donors. The freshly prepared PBMCs were seeded into 96-well U bottom plates containing 2 x 10^5 cells in 100 µl of R5 medium per well and rested at 37 °C in a humidified atmosphere of 5 % carbon dioxide for 2 hours prior to mitogen stimulation and MTH treatment.

6.2.2 MTH preparation

The PBMCs were treated with 0.125 % MTH. During MTH preparation, the 0.125 % MTH were serially diluted from stock MTH solution as described in **Section 3.2.2** using R5 medium and filtered using 0.45 µm minisart filter prior to treatment.

6.2.3 MTH treatment and mitogen stimulation

After 2 hours resting, the PBMCs were collected from the incubator and centrifuged at 1800 rpm for 5 minutes at 23 °C. Supernatant were discarded and PBMCs were concurrently stimulated with PMA and ionomycin to give a final concentration of 5 ng/ml and 500 ng/ml respectively as well as treated with 100 µl of 0.125 % MTH.

The mitogen stimulated and MTH-treated PBMCs were incubated at 37 °C in a humidified atmosphere of 5 % carbon dioxide for 24 hours, 48 hours and 72 hours. As stated previously, a wider range of incubation time (24 hours, 48 hours and 72 hours) were selected in order to examine the immunomodulatory effect of MTH on the production and accumulation of IFN-γ and IL-10 cytokines in MTH-treated PBMCs. In this experimental setup, non-stimulated PBMCs with R5 medium only and PBMCs stimulated with PMA/I for each respective incubation time (24 hours, 48 hours and 72 hours) were used as controls. After each incubation time, the treated PBMCs were centrifuged at 1800 rpm for 5 minutes at 23 °C. The PBMCs supernatant was harvested into 500 µl microcentrifuge tubes and stored in -80 °C freezer.

6.2.4 ELISA

Production of IFN-γ, IL-10, IL-2 and IL-4 cytokines from MTH-treated PBMCs was quantified separately using ELISA Max standard set kit (BioLegend, California, USA) according to manufacturer's instruction with slight modification. Initially, 100 µl diluted capturing antibody was added into 96-wells plates (Nunc Maxisorp) and the plates were sealed and incubated overnight in 4 °C fridge. After overnight, the plates were washed 5 times with 200 µl wash buffer per wells to remove the excess capturing

antibodies. For each washing step, the plates were flicked and tapped gently onto paper towel to remove any residual buffer.

In order to block non-specific binding and reduce the background reading, 200 µl assay diluent was added into each well and the plates were sealed and incubated at room temperature for 1 hour with gentle shaking on orbital shaker (BioSan, Riga, Latvia). After blocking, the plates were washed 5 times with 200 µl wash buffer per wells to remove the assay diluent. Next, 75 µl PBMCs supernatants and standard solutions were added into the respective wells (assay diluent served as zero standard). The plates were sealed and incubated at room temperature for 2 hours with gentle shaking. After 2 hours of incubation, the plates were washed 5 times with 200 µl wash buffer per wells to remove the excess PBMCs supernatant and standard solutions. Next, 100 µl Avidin-HRP solution was added into each well and the plates were sealed and incubated at room temperature for 30 minutes with gentle shaking. After 30 minutes, the plates were washed 6 times with 200 µl wash buffer per wells to remove the excess Avidin-HRP solution. For the final wash, the plates were soaked for 1 minute to minimize the background. Next, 100 µl TMB substrate solution was added into each well and the plates were incubated in the dark for 30 minutes (positive wells will turn blue in colour). After 30 minutes, 100 µl stop solution was added into each well to stop the reaction (positive wells will turn from blue to yellow colour). Finally, colorimetric absorbance measurements were read at 450 nm and 570 nm within 30 minutes using Varioskan Flash Multimode Reader. The absorbance readings at 570 nm were subtracted from the absorbance readings at 450 nm and corrected with the absorbance readings of zero standard. The standard curves for IFN-γ and IL-10 were plotted using Microsoft Excel and the absorbance readings for each sample were compared with the standard curve using $y = mx + c$ formula to determine the cytokine concentration in each sample.

6.2.5 Statistical analysis

All data entry was obtained from 6 biological replicates and statistical analysis were conducted using GraphPad Prism version 5.02. The values were expressed as mean and SEM, as a method to estimate the population mean. In this study, the effect of two categorical independent variables (MTH treatment and incubation time) on one continuous dependent variable (IFN-γ and IL-10 production) were determined. Hence, two-way ANOVA followed by Bonferroni posttest were used to compare overall difference between group means. The level of statistical significance was set at $p < 0.05$.

In this study, the production of cytokines (IFN-γ and IL-10) were compared between non-stimulated PBMCs, PMA/I-stimulated PBMCs, non-stimulated PBMCs treated with 0.125 % MTH and PMA/I-stimulated PBMCs treated with 0.125 % MTH. Functionally, the production of IFN-γ is regulated by IL-10 and vice versa (Fukao *et al.*, 2000). Hence, the Pearson correlation was used to calculate the correlation coefficient (r) between the production of IFN-γ and IL-10 in non-stimulated PBMCs treated with 0.125 % MTH for 24, 48 and 72 hours. The Pearson correlation is a statistical test used to investigate the relationship between two quantitative variables. The Pearson correlation coefficient (r) is used to measure the strength of the relationship between the two variables. The Pearson correlation coefficient (r) can take a range of values from "+1" to "-1". A value of "0" represents no relationship between the two variables. A value towards "+1" represents a positive relationship where an increase in one variable will cause an increase in the other variable. A value towards "-1" represents a negative relationship where an increase in one variable will cause a decrease in the other variable (Lane, 2013).

6.3 Results

6.3.1 Immunomodulatory effect of MTH on type 1 cytokines (IFN-γ and IL-2) production in PBMCs

Based on **Figure 6.1A** below, the IFN-γ production was significantly increased in non-stimulated PBMCs treated with 0.125 % MTH for 24, 48, and 72 hours of incubation by 5.28 fold, 2.92 fold and 4.29 fold respectively when compared to untreated PBMCs (medium only). As the incubation time increased from 24 hours to 72 hours, the highest production of IFN-γ under non-stimulated and MTH-treated condition was observed at 72 hours of incubation (1012.36 pg/ml) compared to 866.48 pg/ml at 24 hours of incubation and 942.47 pg/ml at 48 hours. This indicated that IFN-γ was secreted relatively late upon MTH treatment.

As for IL-2 (**Figure 6.1B**), the production of this cytokine was slightly increased in non-stimulated PBMCs treated with 0.125 % MTH for 24, 48 and 72 hours of incubation by 0.06 fold, 0.07 fold and 0.26 fold respectively when compared to untreated non-stimulated PBMCs. Nevertheless, these increments were not statistically significant. In the presence of PMA/I, the production of IL-2 in untreated PBMCs was significantly increased by 28.92 fold (24 hours), 27.58 fold (48 hours) and 28.13 fold (72 hours) when compared to untreated non-stimulated PBMCs. This showed that PMA/I successfully stimulated the PBMCs to enhance the production of IL-2. Upon 0.125 % MTH treatment for 24, 48 and 72 hours, the production of IL-2 in PMA/I stimulated PBMCs was found to be reduced by 0.24 fold, 0.16 fold and 0.07 fold respectively when compared to untreated PMA/I stimulated PBMCs. Nevertheless, these reductions were also not statistically significant. Hence, it was speculated that MTH possibly showed no immunosuppressive effect in regulating the production of IL-2 in PBMCs.

Overall, PMA/I significantly increased levels of IFN-γ and IL-2 in PBMCs cultures. However, no significant differences were observed in the production of IFN-γ and IL-2 tested from PMA/I stimulated PBMCs treated with 0.125 % MTH for all

incubation durations when compared to PMA/I stimulated PBMCs not treated with MTH.

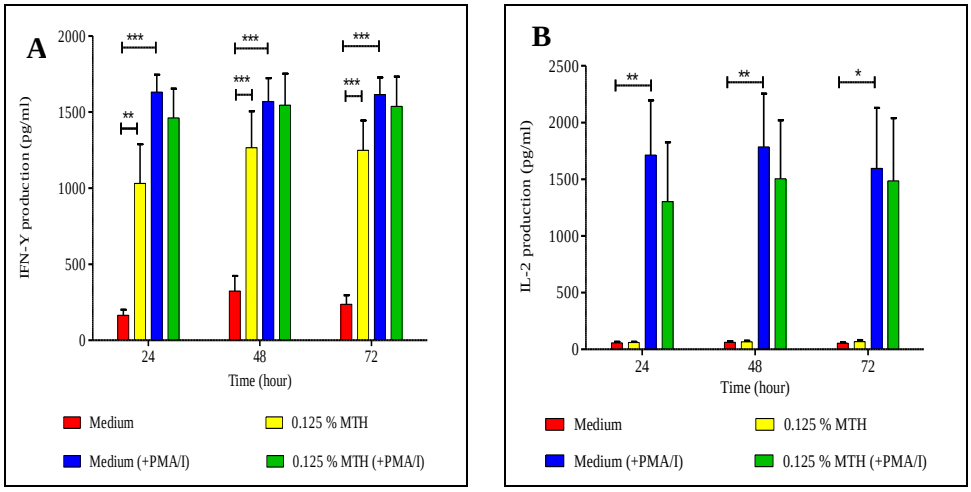


Figure 6.1 Immunomodulatory effect of MTH on IFN-γ (**A**) and IL-2 (**B**) production in PBMCs. All values represented mean ± SEM from 6 biological replicates. (*) represented $p < 0.05$, (**) represented $p < 0.01$ and (***) represented $p < 0.001$ analyzed using two-way ANOVA and Bonferroni posttest. 'Medium' represented basal level in non-stimulated PBMCs, 'Medium (+PMA/I) represented basal level in stimulated PBMCs, '0.125 % MTH' represented non-stimulated PBMCs treated with 0.125 % MTH and '0.125 % MTH (+PMA/I)' represented stimulated PBMCs treated with 0.125 % MTH.

6.3.2 Immunomodulatory effect of MTH on type 2 cytokines (IL-10 and IL-4) production in PBMCs

Based on **Figure 6.2A**, the production of IL-10 was significantly increased in non-stimulated PBMCs treated with 0.125 % MTH for 24 hours, 48 hours and 72 hours of incubation by 5.69 fold, 4.04 fold and 4.59 fold respectively when compared to non-stimulated and untreated PBMCs (medium only). As the incubation time increased from 24 hours to 72 hours, the highest production of IL-10 under non-stimulated and MTH-treated condition was observed at 24 hours of incubation (277.09 pg/ml)

compared to 242.14 pg/ml at 48 hours of incubation and 211.25 pg/ml at 72 hours. This indicated that IL-10 was secreted relatively early upon MTH treatment. In addition, it was also found that the use of PMA (5 ng/ml) and ionomycin (500 ng/ml) as stimulant resulted in low IL-10 production in stimulated PBMCs.

As for IL-14 (**Figure 6.2B**), the production of this cytokine in both PMA/I stimulated and non-stimulated PBMCs were below the sensitivity of detection of the ELISA assay. Therefore, no clear trend was observed in the production of IL-4 from PMA/I stimulated and non-stimulated PBMCs upon 0.125 % MTH treatment.

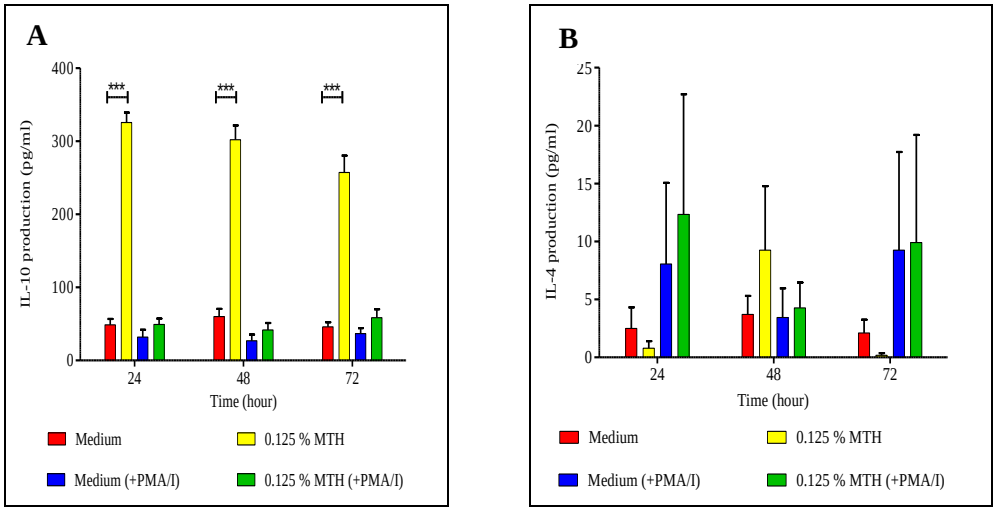


Figure 6.2 Immunomodulatory effect of MTH on IL-10 (**A**) and IL-4 (**B**) production in PBMCs. All values represented mean ± SEM from 6 biological replicates. (***) represented $p < 0.001$ analyzed using two-way ANOVA and Bonferroni posttest. 'Medium' represented basal level in non-stimulated PBMCs, 'Medium (+PMA/I)' represented basal level in stimulated PBMCs, '0.125 % MTH' represented non-stimulated PBMCs treated with 0.125 % MTH and '0.125 % MTH (+PMA/I)' represented stimulated PBMCs treated with 0.125 % MTH.

An inverse correlation (Pearson r value: -0.9999) was found between IL-10 and IFN-γ production in non-stimulated PBMCs treated with 0.125 % MTH (**Figure**

6.3). At 24 hours of incubation, the highest IL-10 production (277.09 pg/ml) (**Figure 6.2A**) resulted in lowest IFN-γ production (866.48 pg/ml) (**Figure 6.1A**). In contrast, at 72 hours of incubation time, the lowest IL-10 production (211.25 pg/ml) (**Figure 6.2A**) resulted in highest IFN-γ production (1012.36 pg/ml) (**Figure 6.1A**). This inverse correlation was statistically significant (p value: 0.0073) at 95 % confidence interval. This supported the potential immunomodulatory effect of MTH in regulating both the production of IFN-γ and IL-10 cytokines in PBMCs.

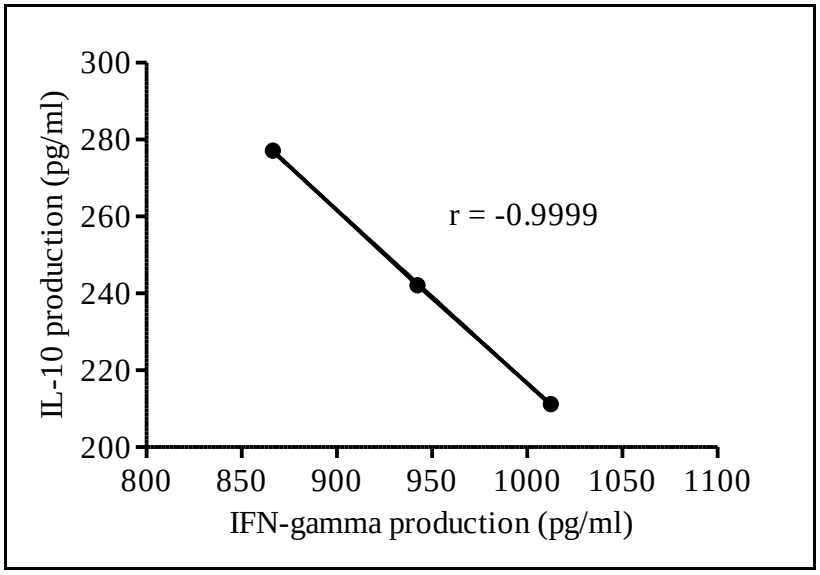


Figure 6.3 Correlation between the production of IFN-γ and IL-10 in PBMCs after 0.125 % MTH treatment for 24 hours, 48 hours and 72 hours of incubation. This correlation was analyzed using Pearson correlation analysis and all values were represented as mean obtained from 6 biological replicates.

6.4 Discussion

The immunomodulatory effect of MTH on cytokines production was measured using ELISA. In this study, it was found that MTH possessed potential immunomodulatory effects on type 1 cytokine (IFN-γ) and type 2 cytokine (IL-10) production in PBMCs. Overall, 0.125 % MTH showed immunoactivating effect on non-stimulated PBMCs by significantly elevating the levels of IFN-γ (pro-inflammatory cytokine) and IL-10

(immunoregulatory cytokine). In contrast, 0.125 % MTH appeared to show no significant immunomodulatory effect in regulating the production of IL-2 and IL-4 in PBMCs (**Figure 6.4**).

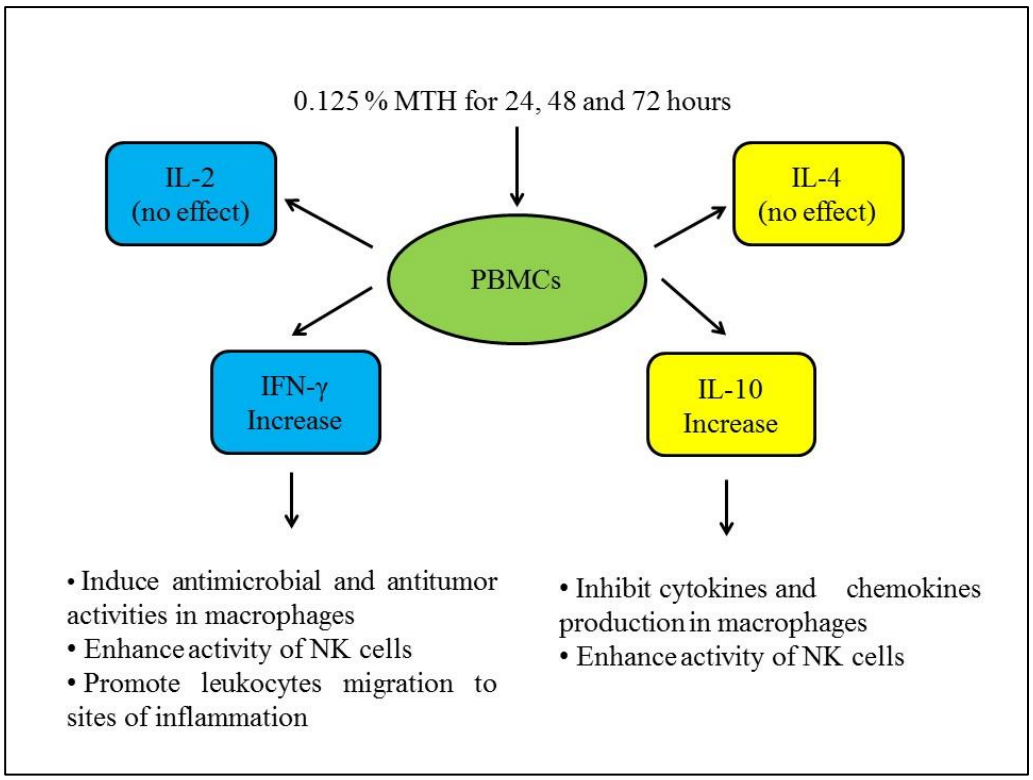


Figure 6.4 Immunomodulatory effect of MTH on the production of type 1 (IFN-γ, IL-2) and type 2 (IL-4, IL-10) cytokines in PBMCs

It was found that the production of IL-10 was significantly increased by 5.69 fold in non-stimulated PBMCs treated with 0.125 % MTH for 24 hours when compared to non-stimulated PBMCs. The production of IL-10 in MTH-treated PBMCs also correlated with the gene expression of *IL10* reported in Chapter 4. In brief, the gene expression of *IL10* was previously found to be significantly upregulated by 4.07 fold in non-stimulated PBMCs treated with 0.125 % MTH for 24 hours when compared to untreated PBMCs.

Functionally, IL-10 is known to exert immunoregulatory activities in preventing inflammatory and autoimmune disease (Borish, 1998). This cytokine is

known to regulate inflammatory processes by suppressing the production of pro-inflammatory cytokines and chemokines as well as antigen-presenting and costimulatory molecules in T cells, neutrophils, monocytes and macrophages (Saraiva & O'Garra, 2010). Theoretically, the transcription of these pro-inflammatory cytokines and chemokines are commonly regulated by NF-kB transcription factor. In the NF-kB signal transduction cascade, the inhibitor of kappa B (IkBa) initially inhibits the NF-kB (inactive state) in the cytoplasm. Upon activation, the IkB kinase (IKK) will phosphorylate the IkBa, leading to the dissociation of IkBa from NF-kB. The NF-kB (active state) will then migrate from the cytoplasm into the nucleus to activate the expression of various genes (Vallabhapurapu & Karin, 2009). Based on literature review, it was reported previously that IL-10 can inhibit NF-kB transcription factor by inhibiting IKK activity to block NF-kB nuclear translocation as well as by blocking the DNA-binding of NK-kB in the nucleus (Asadullah *et al.*, 2003). Hence, this ELISA data supported the immunomodulatory potential of MTH in regulating inflammation in PBMCs. It was also speculated that MTH may exert its immunoregulatory activity in PBMCs by elevating the production of IL-10 leading to the inhibition of NF-kB.

The production of IFN-γ was also significantly increased by 5.28 fold in non-stimulated PBMCs treated with 0.125 % MTH for 24 hours when compared to non-stimulated PBMCs. Functionally, IFN-γ is known to enhance antigen process and presentation in macrophages to induce direct antitumor and antimicrobial activities (Young & Hardy, 1995). Therefore, this ELISA data supported the antitumor and antimicrobial potential of MTH in PBMCs. It was speculated that the significant increment in the production of IFN-γ in MTH-treated PBMCs may be possibly due to the accumulation of IFN-γ in the supernatant during MTH treatment. Based on a previous study, it was reported that IFN-γ was produced and accumulated in T cell culture as early as 30 minutes after stimulation (Mak & Saunders, 2005).

In terms of signal transduction, IFN-γ primarily signals through Jak-Stat tyrosine kinase pathway to regulate the gene expression of other cytokines, chemokines and growth factors. Briefly, the IFN-γ receptor comprised of Jak2 and Jak1. When IFN-γ binds to the IFN-γ receptor on the surface of immune cells, Jak2 kinase is activated (autophosphorylation) and subsequently phosphorylates Jak1 to form a docking sites for Stat1. At the docking site, Stat1 undergoes phosphorylation and migrates to the nucleus to initial the expression of IFN-γ-regulated genes such as *CXCL9* and inducible nitric oxide synthase (*iNOS*), which are involved in the host defense against bacterial infection and tumor growth (Schroder *et al.*, 2004). Therefore, it was speculated that MTH may exert its antitumor and antimicrobial activities in PBMCs by enhancing the production of IFN-γ leading to the expression of IFN-γ-regulated genes.

However, the ELISA data for IFN-γ did not correlate with the gene expression of *IFNG* reported in Chapter 4. In contrast, the gene expression of *IFNG* was significantly downregulated by 14.40 fold in non-stimulated PBMCs treated with 0.125 % MTH for 24 hours when compared to untreated PBMCs. These contradicting findings may be possibly due to the timing of extracting these cytokine transcripts as well as the accumulative effect of IFN-γ cytokine in the supernatant after 24 hours of incubation. In a previous study conducted by Fan *et al.* (1998), it was reported that the maximal mRNA accumulation of *IFNG* in PHA-stimulated PBMCs was at approximately 8 hours after stimulation and gradually decreased as the incubation time increased to 72 hours. In present study, the gene expression of *IFNG* in MTH-treated PBMCs was determined after 24 hours of incubation and therefore the expression of *IFNG* may already decreased after MTH treatment.

Based on literature review, it was also reported previously that IFN-γ was produced and accumulated T cell culture as early as 30 minutes after stimulation (Mak & Saunders, 2005). It was also reported that IL-10 can suppress the production of IFN-γ production in immune cells (Fukao *et al.*, 2000). This suggested a possibility

that the downregulation in the expression of *IFNG* may be also due to the accumulative effect of IL-10 in the supernatant which was significantly increased in PBMCs after MTH treatment (**Figure 6.5**).

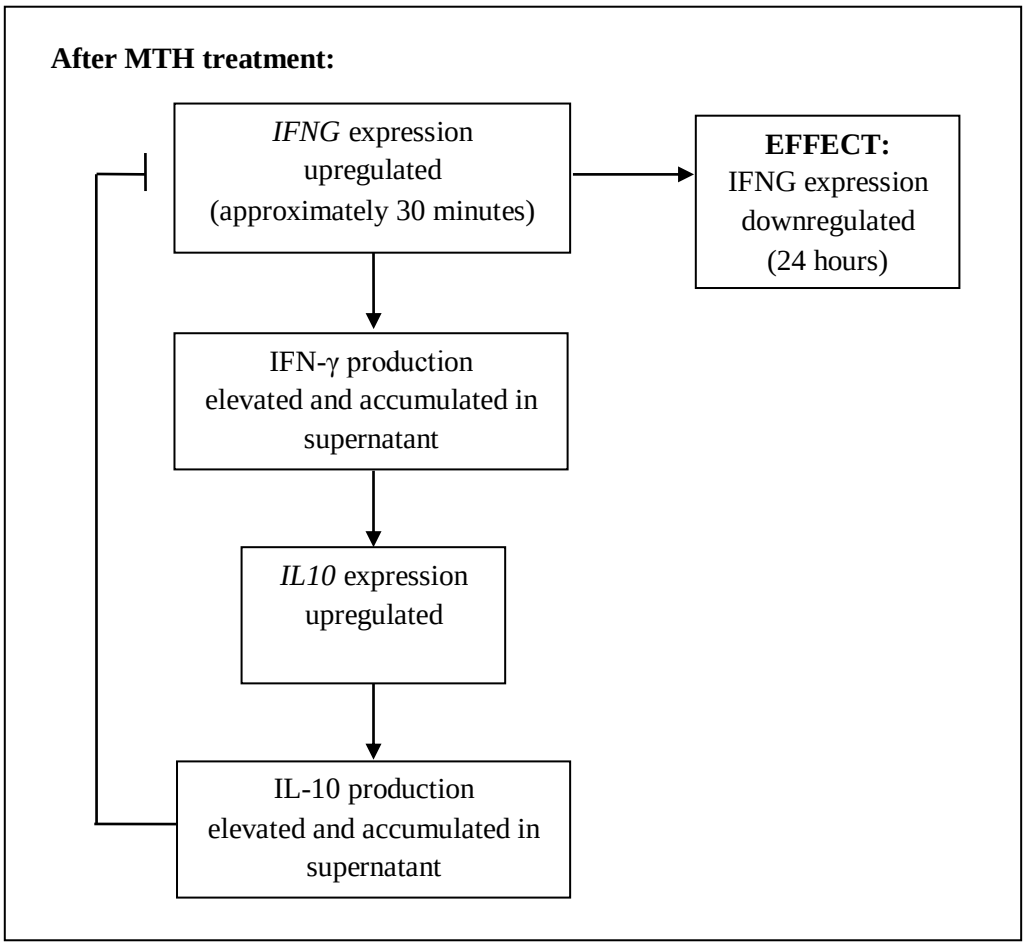


Figure 6.5 Immunomodulatory effect of MTH in regulating the expression of *IFNG* and *IL10* leading to the production of IFN-γ and IL-10 cytokines in PBMCs. (⟶) represented immunomodulatory effect and (⊣) represented inhibitory effect.

A significant inverse correlation was also observed between the production of IFN-γ and IL-10 in PBMCs treated with 0.125 % MTH for 24 hours, 48 hours and 72 hours. Functionally, IL-10 is known to play a crucial role in modulating the expression of various cytokines such as IFN-γ, IL-2, IL-4 and IL-5 in different immune cells (Moore *et al*., 2001). Therefore, this finding supported the immunoregulatory potential of MTH in regulating the production of type 1 and type 2 cytokines in PBMCs.

Based on ELISA, no significant difference was observed in the production of IL-2 in both non-stimulated and PMA/I stimulated PBMCs treated with MTH across all tested incubation durations. This ELISA data correlated with the gene expression data obtained in Chapter 4, where the gene expression of *IL2* was also not significantly regulated in non-stimulated PBMCs treated with 0.125 % MTH for 24 hours. As for IL-4, it was found that the production of IL-4 in both non-stimulated and PMA/I stimulated PBMCs were below the sensitivity of detection of the ELISA assay. Therefore, no clear trend was observed in the production of IL-4 from non-stimulated and PMA/I stimulated PBMCs upon 0.125 % MTH treatment. Nevertheless, this ELISA data still correlated with the gene expression data obtained in Chapter 4, where the gene expression of *IL4* was also not significantly regulated in non-stimulated PBMCs treated with 0.125 % MTH for 24 hours. Collectively, these findings suggested that MTH possibly did not possessed any immunomodulatory effect in regulating the production of IL-2 and IL-4 in PBMCs.

In term of experimental setup, it was found that the use of PMA (5 ng/ml) and ionomycin (500 ng/ml) as stimulant resulted in low IL-10 production in PBMCs. Similar reduction was reported by Baran *et al.* (2001), who investigated the production of IL-10 in PBMCs stimulated with PMA (5 ng/ml) and ionomycin (1 µM) (2.6 %) as compared to non-stimulated PBMCs (3.2 %) after 24 hours of incubation using flow cytometry. In another study conducted by Boehringer *et al.* (1999), it was reported that PMA did not affect the transcription of *IL10* gene and *IL-10* mRNA stability in human alveolar macrophages. Hence, it was hypothesized that the downregulation of IL-10 production upon PMA/I stimulation could possibly due to post-translational mechanisms. Therefore, it was suggested that other stimulant such as PHA can be used as alternative to stimulate PBMCs in the production of IL-10. The PHA is a lectin that binds to any glycosylated surface proteins such as T cell receptors and involves a variety of signaling pathways to stimulate cells. Therefore, PHA

stimulation tends to be more complex as compared to PMA and ionomycin which specifically stimulate cells through PKC and NFAT pathways. (Goel *et al*., 2007).

6.5 Concluding remarks

Overall, present findings showed that the production of IFN-γ and IL-10 were significantly enhanced in PBMCs treated with 0.125 % MTH, while no significant changes were observed in the production of IL-2 and IL-4. This suggested that MTH possessed immunomodulatory effect in elevating the production of IFN-γ and IL-10 in PBMCs. This also suggested that MTH may have the potential in regulating inflammation as well as promote antimicrobial activities in PBMCs. Hence, present findings can further justify the application of MTH as topical dressing in modern medical setting such as in wound healing management.

CHAPTER 7: GENERAL DISCUSSION

This present study showed that MTH significantly regulated the expression of various cytokines and chemokines genes, potentially leading to cell activation in PBMCs. For example, it was found that the expression of *IFNG* and *IL10* in PBMCs were significantly regulated by MTH. At protein level, the production of IFN-γ and IL-10 were also significantly enhanced in MTH-treated PBMCs. Collectively, these findings supported the potential immunoregulatory, antitumor and antimicrobial activities of MTH.

7.1 Potential application of MTH

7.1.1 Wound management

Traditionally, honey is commonly used as topical dressing to promote wound healing in treating burns and skin ulcers. The application of MTH as topical dressing in burn wound management has been reported previously. Rodzaian *et al.* (2011) reported that MTH (Medi®) dressing was found to be a suitable replacement for the modern silver dressing in treating burn wounds due to its effective antibacterial and rapid wound healing potential. In their study, MTH dressing was found to promote epithelization and angiogenesis in the management of burn wounds. It was also found that MTH dressing can inhibit the growth of wound-infecting bacteria due to its high osmolarity as well as through the production of H_2O_2. Besides, it was also speculated that the wound healing potential of MTH may be possibly due to its anti-inflammatory activity, whereby honey was demonstrated to decrease oxidative stress by eliminating excess free radicals produced by macrophages and hence triggering the initiation of wound recovery process (Rodzaian *et al*, 2011).

Our proliferative and immunomodulatory data on PBMCs suggested that MTH potentially involved in wound healing process from a different aspect. MTH was found to regulate the expression of cytokine genes such as *IL20* and *IL24* that can potentially stimulate the secretion of various chemokines and β-defensins

(antimicrobial peptide) in epithelial cells to facilitate the recruitment of leukocytes to the site of infection as well as to induce antimicrobial responses. Besides, the expression of *IL20* and *IL24* in PBMCs can also stimulate the proliferation of epithelial cells to strengthen the physical barrier against invading microbes (Sa *et al*., 2007). Additionally, the immunomodulatory effect of MTH in regulating the expression of chemokine genes such as *CXCL1* and *CXCL3* can potentially stimulate the recruitment of neutrophils and leukocytes to the site of infection (Addison *et al.*, 2000). The expression of these chemokine genes can also initiate the synthesis of angiogenic molecules such as VEGF-A which can promote new blood vessels formation during wound healing (Scapini *et al.*, 2004).

At protein level, present study showed that MTH significantly enhanced the production of IFN-γ and IL-10 in PBMCs. Functionally, IFN-γ is a pro-inflammatory cytokine which can induce direct antimicrobial activity in macrophages by up-regulating antigen presentation and stimulate the secretion of various cytokines to recruit other immune cells to the infected wounds (Young & Hardy, 1995). As for IL-10, the production of this immuno-regulatory cytokine can regulate inflammation process by suppressing excessive production of pro-inflammatory cytokines from T cells, macrophages and neutrophils present at the wound site. This can prevent prolonged inflammation and hence trigger the initiation of wound healing process (Saraiva & O'Garra, 2010). Overall, these present findings supported the application of MTH as a promising topical dressing in the management of wound in modern medical setting. Furthermore, these findings also provided a platform to hypothesize the *in vivo* immunomodulatory effect of MTH in regulating the production of endogenous cytokines in PBMCs.

7.1.2 Antimicrobial agent in eradicating *Mycobacterium tuberculosis* infection

The application of honey in treating infection has been well documented. To date, more scientific studies are progressively carried out to investigate the mechanisms

contributing to the antimicrobial effect of honey, particularly in Manuka honey and Tualang honey (Jenkin & Cooper, 2012; Tan *et* al, 2009).

In present study, the production of IFN-γ in PBMCs was significantly elevated after being treated with 0.125 % MTH. This *ex vivo* finding can be used to hypothesize the *in vivo* immunomodulatory effect of MTH in regulating the production of endogenous cytokines in PBMCs. High level of IFN-γ is vital for tumor surveillance and protection against infectious agents such as bacteria, fungus, virus and protozoa (Gallin *et al.*, 1995; Miller *et al.*, 2009). For example, it was found previously that aerosolized IFN-γ therapy increased the antimicrobial activity of alveolar macrophages against *M. tuberculosis*, which commonly causes tuberculosis (TB) in the respiratory system (Condos *et al.*, 2003). To date, TB is one of the most lethal infections in the world due to the discovery of antibiotics resistant *M. tuberculosis* because TB treatment normally requires the usage of multiple antibiotics over a long period of time to clear the infection. In the past, honey was recommended as one of the best remedies in treating TB (Ahmed *et al.*, 2013). With the current finding, it was proposed that MTH can be potentially used as complementary and alternative medicine (CAM) in treating TB by elevating the production of endogenous IFN-γ to increase the antimicrobial activity of macrophages. This can be achieved by enhancing the expression of MHC class II molecules in macrophages which can result in improved antigen presentation to T cells. These activated macrophages can also initiate oxidative burst to generate reactive oxygen intermediates such as H_2O_2 which can contribute to the mycobactericidal effects of mononuclear phagocytes against *M. tuberculosis* (Ahmed *et al*., 2013).

In addition, present microarray data also showed that the expression of *TLR2* was upregulated in PBMCs upon MTH treatment. As reported in a previous study, TLRs are receptors involved in cellular recognition of mycobacteria and therefore the upregulation in the expression of TLR2 conferred better responsiveness to *M. tuberculosis* (Means *et al*., 1999). When the mycobacterial cell wall glycolipids of *M.*

tuberculosis interact with TLR2 molecules on the surface of macrophages, several signalling molecules such as adaptor molecule myeloid differentiation primary response protein 88 (MyD88), IL-1 receptor-associated kinases (IRAK), TNF receptor-associated factor (TRAF) 6, TGFβ-activated protein kinase 1 (TAK1) and mitogen-activated protein (MAP) kinase are recruited in a signalling cascade in macrophages to activate transcription factor such as NK-kB. Subsequently, the activated NK-kB is translocated to the nucleus to initiate the transcription of genes involved in immune responses such as the production of pro-inflammatory cytokines and nitric oxide. The production of pro-inflammatory cytokines such as TNF and IL-12 in macrophages can stimulate the production of IFN-γ and enhance the cytotoxic activity in NK cells and cytotoxic T cells against *M. tuberculosis*. On the other hand, the production of nitric oxide in macrophages can enhance the direct antimicrobial activity of macrophages against *M. tuberculosis* (Kleinnijenhuis *et al.*, 2011).

7.1.3 CAM in cancer treatment

In traditional Chinese medication, honey (fēng mì) is often used as binding agent to harmonize the nature of other Chinese herbs in supplementing formula that are made into pills and pastes. Besides, honey is also used as booster to enhance the supplementing action of other Chinese herbs such as ginseng in order to achieve stronger medicinal effect (Brand & Wiseman, 2008). Nowadays, honey is widely used as CAM to help cancer patients to cope with their chemotherapy treatment as well as its side effects (Molassiotis *et al*., 2005).

In present study, the production of IFN-γ in PBMCs was significantly elevated after being treated with 0.125 % MTH. The presence of polyphenol in MTH may enhance the production of IFN-γ in PBMCs and possibly contribute to the anticancer activity of MTH. It was reported previously that polyphenol (caffeic acid, ferulic acid and coumaric acid) treatment significantly ($p < 0.001$) increased the production of IFN-γ in APC cells and T cells when compared to their respective controls (Yamanaka

et al., 2013). Intravesicle instillations and intraperitoneal injection of IFN-γ were found to be effective against bladder carcinoma and ovarian cancer cells by inhibiting tumor cell proliferation and sensitizing tumor cells to apoptosis in cancer patients (Giannopoulos *et al*., 2003; Pujade *et al*., 1996). This suggested that MTH can be potentially used as CAM to treat cancer in conjunction with current treatment by elevating the production of IFN-γ. Besides, it is believed that IFN-γ can promote cellular sensitivity to the pro-apoptotic effect of TNF-α by enhancing surface expression of TNF-α receptor on tumor cells as well as enhancing the cytotoxic activity of NK cells (Schroder *et al.*, 2004). Different polyphenols such as kaempferol, chrysin, caffeic acid and caffeic acid phenyl ester in honey were found to inhibit cell proliferation by down-regulating protein tyrosine kinase, which played a crucial role in phosphorylating protein molecules in living cells (Ghashm *et al.*, 2010).

7.1.4 CAM in treating allergic reaction

Traditionally, honey is used to treat allergic reactions such as hay fever due to its immuno-suppressive effect which is often related to the production of immuno-regulatory cytokine like IL-10 (Bogdanov, 2011a). High level of IL-10 is vital in preventing inflammatory and autoimmune diseases such as psoriasis and asthma (Asadullah *et al*., 2003; Moore *et al*., 2001). In present study, the production of IL-10 was significantly increased in unstimulated PBMCs treated with 0.125 % MTH. This suggested that MTH can be potentially used to treat psoriasis by suppressing an over-reactive immune system as well as inflammatory mechanisms associated with asthma via IL-10.

Psoriasis is one of the most common skin disease affecting approximately 3 % of the population. This disease is a chronic inflammatory and proliferative skin disorder due to excessive cell division in the basal skin layers and is characterized by red plaques that overlay with large adherent silvery white scales (Sindhu *et al.*, 2007). It was found that injection of 8 µg of recombinant human IL-10/kg body weight on a

daily basis successfully cleared the plaques in psoriasis patients after 24 days of treatment (Asadullah *et al*., 1998). Besides, it was also found that topical dressing of honey mixture containing honey, beeswax and olive (v/v ratio of 1:1:1) was useful in the management of psoriasis due to its anti-inflammatory and antimicrobial properties (Al-Waili, 2003).

On the other hand, asthma is a chronic respiratory disease characterized by infiltration of inflammatory cells into lung tissue, chronic airway obstruction, airway hyper-responsiveness and excessive mucus production (Kamaruzaman *et al*., 2012). Based on a previous study, it was reported that asthmatic individuals treated with inhaled corticosteroid therapy showed higher level of endogenous IL-10 production to suppress inflammatory mechanisms associated with asthma such as suppression of IL-2 and IFN-γ production from Th1 lymphocytes, IL-4 and IL-5 production from Th2 lymphocytes as well as IL-6 and IL-8 production from mononuclear phagocytes (John *et al*., 1998). Although corticosteroid is effective in managing asthma symptoms, there are still concerns regarding the side effects from prolonged usage of drugs especially among children (Li & Brown, 2009). In addition, it was also reported that 25 % of aerosolized honey treatment managed to reduce massive infiltration of lymphocytes, mononuclear cells and eosinophils into lung tissue in rabbits pre-exposed to ovalbumin inhalation, which mimic the human condition of asthma (Kamaruzaman *et al*., 2012).

7.2 Composition of MTH potentially contributing to its immunomodulatory effect

Our data suggested that MTH possessed immunomodulatory effect on PBMCs by regulating the expression of various immune related genes, stimulating cell activation in PBMCs subpopulation as well as enhancing the production of IFN-γ and IL-10. It is also important to identify the active components responsible for this effect. The presence of various phenolic compounds may have acted synergistically in regulating immuno responses in PBMCs based on the fact that honey was used as a whole

product. The total phenolic content in the MTH was found to be slightly higher (435.23 mg GAE per kg MTH) as compared to 419.86 mg GAE per kg MTH reported by Khalil *et al.* (2012) in a previous study using the same brand of MTH. Six phenolic acids, namely gallic, syringic, benzoic, trans-cinnamic, p-coumaric and caffeic acid were identified by Khalil *et al.* (2012). In another study using the same MTH, Nurul *et al.* (2012) managed to identify 35 volatile compounds in MTH (AgroMas®) using GC-MS analysis. These volatile compounds were classified into hydrocarbons, acids, aldehydes, ketones, alcohols, furans and terpenes (Nurul *et al*., 2013). According to the nutrition information provided by FAMA, it was also found that MTH (AgroMas®) contained 740 mg of carbohydrate per kg honey, 10 mg of dietary fibre per kg honey and 60 mg of sodium per kg honey.

Based on a previous study, polyphenols such as caffeic acid, quercetin, curcumin and catechin were found to modulate the immune system through their potent antioxidant and anti-inflammatory mechanisms (Mir & Agrewala, 2008). These polyphenols can directly interact with plasma membrane proteins and phospholipids leading to the stimulation of intracellular signalling pathways and transcription factors (Kim *et al.*, 2014). For example, it was reported previously that caffeic acid can regulate the production of IL-2 in T cells through the regulation of NF-kB transcription factor. Besides NF-kB transcription factor, polyphenols can also regulate enzyme related to inflammation like cyclooxygenase (COX) (Mir & Agrewala, 2008). In brief, COX is an enzyme responsible for the formation of prostaglandins (mediator of inflammatory reaction) and thromboxanes (mediator of vasoconstriction) (Marquez *et al.*, 2004).

7.3 Future work

The application of MTH as topical dressing to promote wound healing may be attributed to its immune-stimulatory properties which resulted in the expression of cytokine and chemokine genes. The expression of these gene can subsequently lead to

the initiation of various immune responses such as recruiting leukocytes to the site of injury to clear infection or stimulating the proliferation of epithelial cells during wound healing process.

Our data suggested that MTH significantly regulating the gene expression of *IL20*, *IL24*, *CXCL1* and *CXCL3* in PBMCs. Therefore, the production of IL-20, IL-24, CXCL1 and CXCL3 should be quantified in order to provide a comprehensive understanding of the immune responses involved in MTH-treated PBMCs at protein level. Besides, the expression of *IL1* and *MAP3K13* which associated with the regulation of NF-kB transcription factor activity was also upregulated. More in-depth studies should be carried out to investigate the effect of MTH on other possible biological pathways through NF-κB transcription factor activity. Overall, this study has provided preliminary finding on the genes and downstream pathways affected by MTH, a more comprehensive study using larger sample size should be done. In future, the efficiency, safety and cost of using MTH either as topical dressing or CAM should also be evaluated.

Another main concern in using MTH is batch variation. To date, a standard grading system for MTH has not been established and therefore it is difficult to determine the potency for each batch of MTH even if the samples are harvested from the same source. Hence, it was suggested that a standard grading system based on the amount of specific active components found in MTH can be used to overcome this matter. For example, MGO was identified as the main active component responsible for the prominent antimicrobial activity in Manuka honey (Jenkins *et al.*, 2011). Hence, Manuka honey is now graded based on a scale known as UMF which corresponded with the concentration of MGO found in Manuka honey (Molan, 2002). Therefore, similar approach can also be used to determine the immunomodulatory potential of different batches of MTH in future.

In addition, further investigation should also be carried out to determine whether geographical region has any impact on the composition of MTH, which can

eventually affect its biological function. The MTH used in present study was a filtered honey sample without standardized grading, hence the results obtained were only applicable to this batch of MTH. In future, the immunomodulatory properties in different batches of MTH should be tested to see if similar results can be obtained.

CONCLUSION

In conclusion of the findings in this study, MTH possess immunomodulatory effect in regulating the expression of various cytokines and chemokines genes leading to the initiation of various immune responses in PBMCs. The upregulation in cytokines such as IFN-γ and IL-10 suggested that MTH could potentially induce antitumor and antimicrobial activities and regulate inflammatory processes by suppressing the production of pro-inflammatory cytokines. Collectively, the present study played an important role in providing scientific understanding regarding the possible biological functions of MTH in the human immune system. This scientific contribution can further justify the application of MTH as topical dressing in wound management in modern medical setting.

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APPENDIX 1

Table A1 Grading of clinical inflammatory features of alkali chemical injury on cornea (Adapted from Bashkaran *et al*., 2011)

Clinical features	Grade			
	Normal	Mild	Moderate	Severe
Conjunctival hyperemia	Absent	Mild or sectoral engorgement of the conjunctival vessels	Diffuse engorgement of the conjunctival vessels	Significant engorgement of conjunctional vessels
Corneal edema	Absent	Present with visible iris details	Present without iris details	Present without visible pupil
Corneal epithelial defect	Absent	Defect involving less than one quarter of the corneal surface	Defect involving one quarter to one half of the corneal surface	Defect involving more than one half of the corneal surface

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APPENDIX 2

School of Pharmacy

The University of Nottingham
Malaysia Campus

Immunomodulatory role of Malaysian Tualang Honey

Staff Member Leading this project: Dr Wai Ling Kok
Students involved in the project: Mr Chua Chong Kuan

Information Sheet for Healthy Volunteers

You have been invited to take part in a research study. Before you decide whether to take part it is important for you to understand why the research study is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends and the lead investigator if you wish to. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. If you decide to take part you may keep this leaflet. Thank you for reading this.

Background

Honey has been used since ancient times for its nutritutional and therapeutic values. Malaysian Tualang Honey (*Koompassia excelsa*) is collected from wild honey bees' hives on Tualang trees found in Malaysian rain forest and has been reported to have antibacterial, antioxidant and wound healing property, however the immunomodulatory role has not been widely studied. The main aim of this project is to look at the immuneregulatory role of honey and to understand the mechanisms involved.

What does the study involve?

This study will involve taking blood (30-50ml) from healthy volunteers (age 19-65). The blood obtained will be use to extract the peripheral blood mononuclear cells (PBMC) after which the PBMC will be subjected to various assays to look at immunomodulatory effect of honey.

Why have you been chosen?
All healthy adults age 19-65 without any health condition are welcome to give blood for this study.

Do you have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

What do I have to do?

You have to fill in a consent form and be there at the health centre on the day of blood taking.

What is the drug or procedure that is being tested?

No drugs or procedure being tested apart from blood donation.

What are the side-effects of any treatment or procedures received when taking part? What are the possible disadvantages and risks of taking part?

No side-effects associated with the procedures involved in blood taking. In rare cases, you may get a tiny bruise on your arm due to the needle stick but the bruise will subside after a few days without any treatment.

What if something goes wrong? Who can I complain to?

In case you have a complaint on your treatment by a member of staff or anything to do with the study, you can initially approach the lead investigator, Dr Wai Ling Kok. If this achieves no satisfactory outcome, you should then contact the Ethics Committee Secretary, Mrs Louise Sabir.

Dr Wai Ling Kok, School of Pharmacy Office B1A07, The University of Nottingham Malaysia Campus. Telephone 03-89248731. E-mail WaiLing.Kok@nottingham.edu.my

Mrs Louise Sabir, Division of Therapeutics and Molecular Medicine, D Floor, South Block, Queen's Medical Centre, Nottingham, NG7 2UH. Telephone 0115 8231063. E-mail louise.sabir@nottingham.ac.uk.

In the unlikely event that you suffer injury to yourself or damage to your property as a result in taking part in this project, the University does have an insurance policy to cover harm arising as a result of the defect in the design of the study. Any side-effects experienced during or following your participation in the trial would be reported to the Ethics Committee.

Will my taking part in this study be kept confidential?

The data-sheets generated in the research study will be kept by the lead staff member for archive purposes, and any information about you which leaves the School of Pharmacy will be anonymised so that you cannot be recognised from it.

What will happen to the results of the study?

The results of this study are principally intended for use in research project development. Results will be used to inform future study protocols. You will not be identified in any report or publication which arises from this study.

Who is organising and funding the study?

This study is being supervised by Dr Wai Ling Kok and funded by the Faculty of Science.

Who has reviewed the study?

This study has been reviewed and approved by the University of Nottingham Medical School Ethics Committee.

Contact for Further Information

Contact: Dr Wai Ling kok (tel: 03-89248731; email: WaiLing.Kok@nottingham.edu.my)

Thank you for showing interest in becoming a volunteer for this study.

School of Pharmacy

The University of Nottingham
Malaysia Campus

Title of Project: **Immunomodulatory role of Malaysian Tualang Honey**

Name of investigator: **Dr Wai Ling Kok**
Mr Chua Chong Kuan

Healthy Volunteer Consent Form

Please read this form and sign it once the above-named has explained fully the aims and procedures of the study to you.

- I voluntarily agree to take part in this study.
- I confirm that I have been given a full explanation by the above-named and that I have read and understand the attached information sheet given to me.
- I have been given the opportunity to ask questions and discuss the study with the staff members on all aspects of the study and have understood the advice and information given as a result.
- I agree to the above staff member contacting my general practitioner [and teaching or university authority if appropriate] to make known my participation in the study where relevant.
- I authorise the investigators to disclose the results of my participation in the study but not my name.
- I understand that if any personal information about me is recorded during the study it will be kept in a secure database. If data is transferred to others it will be made anonymous. If appropriate, data will be kept for 7 years after the results of this study have been published.
- I understand that I can ask for further instructions or explanations at any time.
- I confirm that I have disclosed relevant medical information before the study.
- I have not been a subject in any other research study in the last three months which involved: taking a drug; being paid a disturbance allowance; having an invasive procedure (e.g. venepuncture >50ml, endoscopy); or exposure to ionising radiation.

Name: ..... **Signature:** .....

Address: .....

Telephone number: ..... **D.O.B:** ..... **Date:** .....

TO BE COMPLETED BY THE INVESTIGATOR

- I have fully explained the purpose of the study and what is involved to the above-named volunteer.
- I have given the above-named volunteer a copy of this form together with the information sheet.

Name: ..... **Signature:** .....

Study Volunteer Number: ..... **Date :** .....

Faculty of Science

APPENDIX 3

Total phenolic assay

The total phenolic content in MTH (AgroMas®) and Manuka honey (Berringa) was determined using Folin-Ciocalteu method as described by Khalil *et al.* (2012). Briefly, 1 ml of MTH solution (0.2 g/ml) was added with 1 ml of Folin-Ciocalteu's phenol reagent (Sigma-Aldrich, Missouri, USA) in a 15 ml falcon tube (Orange Scientific). Next, 1 ml of 10 % sodium carbonate (Merck, Darmstadt, Germany) was added to the mixture and mixed evenly by inverting the tube few times. Appropriate volume of distilled water was also added to make the final volume up to 10 ml. The reaction was kept in dark for 90 minutes and absorbance reading was measured at 725 nm using Varioskan Flash Multimode Reader. The absorbance results were corrected with the absorbance readings of blank (containing only distilled water, Folin-Ciocalteu's phenol reagent and sodium carbonate). Gallic acid (Sigma-Aldrich, Missouri, USA) was used as standard (25, 50, 75 100, 125 and 150 µg/ml) and results were expressed as mg of GAEs per kg honey. The results were reported as mean ± SEM from triplicates in 3 independent experiments. Statistical analysis was carried out using unpaired T-test and statistical significance was set at < 0.05 (**Figure A1**).

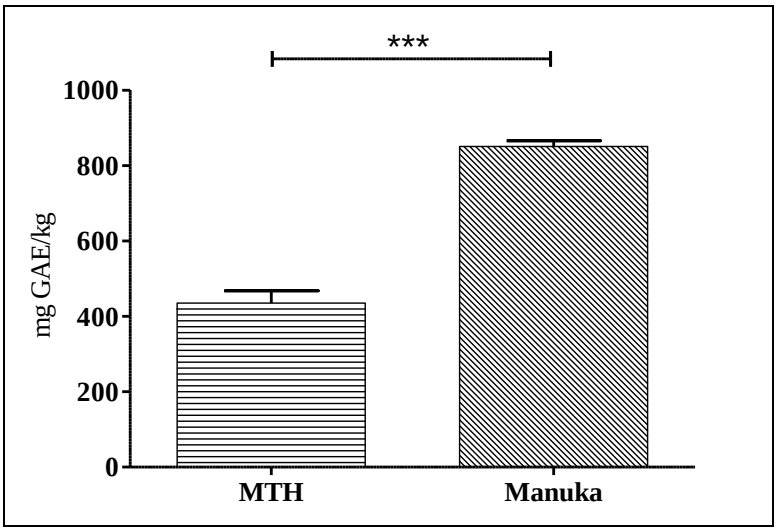

Figure A1 Total phenolic content in MTH and Manuka honey

APPENDIX 4

TRAINING COURSES

1. Demonstrating In Laboratory Practical by UNMC Graduate School (9th November 2011)
2. Getting Going On Your Thesis by UNMC Graduate School (11th January 2012)
3. Planning Your Research And Time Management by UNMC Graduate School (16th January 2012)
4. Working Effectively In Research by UNMC Graduate School (18th January 2012)
5. Mendeley Reference Manager Training by UNMC Library (4th April 2012)
6. Finishing Your Thesis by UNMC Graduate School (9th April 2012)
7. EndNote Reference Manager Training by UNMC Library (21th May 2012)
8. Critical Thinking by UNMC Graduate School (6th June 2012)
9. Marking And Assessment by UNMC Graduate School (17th September 2012)
10. Eppendorf Centrifuge 5810R Training by Mr Tan Kim Chong (6th December 2012)
11. Funding Workshop by UNMC Graduate School (4th February 2013)
12. Presentation Skills by UNMC Graduate School (18th February 2013)
13. Poster To Communicate Research by UNMC Graduate School (25th February 2013)
14. How To Write A Press Release by UNMC Graduate School (7th March 2013)
15. Viva Survivor by UNMC Graduate School (21st April 2014)
16. Microarray lab attachment with Genomax Technologies Singapore (5th August 2014-9th August 2014)

CONFERENCE / CONGRESS

1. International Conference In Chinese Medicine (ICCM) by Universiti Tunku Abdul Rahman (22 and 23th September 2012) - Poster Presentation

Abstract: Honey is a collection of nectar and sweet deposits from plants by honeybees of the genera *Apis* and *Meliponini*. The Tualang honey is collected from the honeycombs of Asian rock bees (*Apis dorsata*), which commonly build their hives on Tualang tree (*Koompassia excelsa*). In Malaysia, Tualang honey can be found mostly in lowland rainforests of Peninsular Malaysia and has been identified as one of the purest form of honeys. Recently, the Malaysian Tualang Honey has been proven to possess beneficial properties such as antimicrobial, anti-inflammatory, antioxidant, boosting the immune system and wound healing potential. However, the immunomodulatory role of this local honey has not been studied extensively and hence there is still considerable interest to investigate its immunomodulatory aspect. The objectives of this research are to investigate the immunoregulatory roles and dissect the mechanisms by which this local honey exerts its function in human. Different concentrations of honey ranging from 0.125 % to 2 % were tested against human monocytic cell lines (THP-1 and U-937) and peripheral blood mononuclear cell (PBMC) at different time points. 3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyltetrazolim bromide (MTT) assay was conducted to determine the percentage of cell viability. Cell proliferation was observed in both the U-937 cell line and PBMC in a dose and time-dependent manner. Further work will be carried out to investigate the mode of action of honey.

2. 18th Biological Science Graduate Congress by University Malaya (6th and 7th January 2014) - Oral Presentation

Abstract: The secretion of cytokines by immune cells upon infection plays a crucial role in regulating cellular and humoral immunity. It is reported earlier that honey has both the potential to boost as well as to suppress the immune system and hence this study aimed to investigate the immunomodulatory effect of Malaysian Tualang honey (TH) on cytokine profile (Th1 and Th2). PBMCs isolated from healthy volunteers were treated with 0.125% of TH for 24, 48 and 72 hours. The production of Th1 and Th2 cytokines such as IFN-γ, IL2, IL4 and IL10 were quantified using enzyme-linked immunosorbent assay (ELISA). Based on the results obtained, TH had the potential to promote the secretion of IL10 (6.6-fold increment) and IFN-γ (3.9-fold increment) in unstimulated PBMCs after 24 and 48 hours of incubation respectively as compared to unstimulated and untreated PBMCs. TH also had the potential to suppress the secretion of IL2 (0.8-fold reduction) in PBMCs stimulated with PMA and ionomycin after 48 hours of incubation as compared to stimulated and untreated PBMCs. The production of IL4 in honey-treated PBMCs was below the level of sensitivity of the ELISA assay. These data suggested that TH plays a role in activating and suppressing the secretion of Th1 and Th2 cytokines in PBMCs. Further work will be carried out to investigate its mode of action. With more understanding and scientific evidence in this aspect, local honey can be used as alternative nutraceutical to promote better health among Malaysians.

SEMINAR

1. Gibco Cell Culture Seminar by Bio-Diagnostics (15th June 2012)
2. Transcriptomics studies using microarray and RNA seq by Dr Jeffrey Wee, Chief Technology Officer, Molecular Genomics Pte Ltd (12th June 2013)
3. Post-genomics and Bio-informatics by Dr Sean May and Dr Marcos Castellanos-Uribe (4th – 5th July 2013)
4. Real time PCR Technical Seminar and Workshop by Agro-Biotechnology Institute Malaysia (ABI) and NexBio (20th – 21th May 2014)
5. Basic Knowledge for SDS-PAGE to Western Blot Seminar by Mr Katsumi Sasaki, Nacalai Tesque, Japan (28th May 2014)
6. ESCO seminar on Safety Awareness on Laminar Flow, Biological Safety Cabinet and Fume Hood by Mr Jason Tham, ESCO Malaysia (25th June 2014)
7. Women in Science and Technology (WinSET) by University of Nottingham Malaysia Campus (9th September 2014)