

IMMUNOMODULATORY PROPERTIES OF TUALANG HONEY
ON BALB/C MICE

by

TAN TIONG KIT

THESIS SUBMITTED IN FULLFILMENT OF BACHELOR (HONS)
OF HEALTH SCIENCE (BIOMEDICINE)

2013

School of Health Science,
Kampus Kesihatan Universiti Sains Malaysia

CERTIFICATE

This is to certify that the dissertation entitled
Immunomodulatory Properties of Tualang Honey on BALB/c Mice
is the bona fide record of research work done by
Tan Tiong Kit during the period from September 2012 to May 2013 under my
supervision.

Supervisor



(Dr. Nurul Asma Abdullah)

Senior Lecturer,
School of Dental Science,
Health Campus,
Universiti Sains Malaysia

Date : 17/06/2012 .

ACKNOWLEDGEMENT

This thesis is the result of endeavor, efforts and cooperation upon which many people have contributed. I am very grateful as I was surrounded by group of helpful and generous people which helped me, guided me and taught me in making this thesis a reality. First and foremost, I would like to express my gratitude to my supervisor, Dr. Nurul Asma Abdullah who has given me the chance to carry out this research and fully supported me throughout the study. Her patience, motivation, enthusiasm, encouragement and immense knowledge made my study smoother and easier, not forget to mention that I have learnt a lot throughout this research under her guidance.

I would also like to express my gratitude to my senior, Mr. Johnathan Malagobadan for his guidance, assistance and support as well as his willingness to share his knowledge with me in Immunology field. Besides, not to forget to express my appreciation to Mr. Jamaruddin Mat Asan from Immunology Department, School of Medical Science, USM for guiding me in running cell subset analysis by using flow cytometry as well as the way to analyze the results, without him, my work would be much difficult. My sincere thanks also goes to School of Dental Sciences, School of Health Sciences and Animal Research and Service Centre (ARASC) which have provided me equipment, animals and laboratory to carry out and complete my study as well as funding for my research.

Last but not least, I would like to thank my family members and my god brother, Koo for supporting me throughout my studies, providing me unconditional and hundred percent cares and support. Without you all, it would not be possible for me to complete this thesis. Thank you.

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LIST OF SYMBOL, ABBREVIATION AND ACRONYMN

ADCC	Antibody Dependent Cell Cytotoxicity
APC	Antigen presenting cells
BMDC	Bone marrow-derived dendritic cells
CD	Cluster of differentiation
Con-A	Concanavalin A
CTL	Cytotoxic T lymphocytes
FITC	Fluorescenseisothiocynate
GM-CSF	Granulocyte macrophage colony-stimulating factor
H ₂ O ₂	Hydrogen peroxide
HL-60	Human promyelocytic leukemia cells
IFN- γ	Interferon- γ
Ig	Immunoglobulin
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin-1
kD	kilo Dalton
LPA	Lysophosphatidic acid
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MDR	Multi-drug resistant
MRSA	Methicilin-resistant <i>Staphylococcus aureus</i>
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF κ B	Nuclear factor kappa B
NK	Natural killer
NK T	Natural killer T
NO	Nitric oxide
OD	Optical density
PBMC	Peripheral blood macrophages

PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PGE ₂	Prostaglandin E ₂
PHA	Phytohemagglutinin
PMN	Polymorphonuclear cells
TGF-beta1	Tumour growth factor- beta 1
Th	T-helper
TLR-4	Toll like receptor 4
TNF- α	Tumour necrosis factor α
%	percent
©	copyright
®	registered
°C	degree Celcius
μ	micro
m	milli
n	nano
SD	standard deviation
™	trademark
α	alpha
β	beta
γ	gamma
δ	delta

ABSTRAK

Ekstrak produk semulajadi tempatan semakin mendapat tumpuan dalam bidang kajian serta terapeutik. Salah satu produk semulajadi yang terkenal di negara Malaysia iaitu madu Tualang (*Koompassia excelsa*) telah menunjukkan kesannya sebagai anti-inflamasi, anti-oksida dan anti-bakteria menerusi kajian saintifik. Kajian ini memfokuskan kesan immunomodulasi madu Tualang ke atas mencit BALB/c. Madu Tualang diberi secara oral selama 14 hari kepada mencit jantan jenis BALB/c (5 /kumpulan) dalam dos yang berlainan iaitu 0.5 g/kg, 1.5 g/kg dan 3.0 g/kg yang dinamakan sebagai kumpulan dos Rendah, Sederhana dan Tinggi. Berat badan sebelum rawatan serta selepas rawatan ditimbang. Setelah mencit tersebut dikorbankan, limpa telah dikeluarkan serta ditimbang. Limpa tersebut telah dihancurkan untuk mendapat ampaiian sel limpa. Ampaiian sel limpa tersebut kemudian ditandakan dengan pelbagai antibodi penanda permukaan iaitu CD3⁺CD4⁺, CD3⁺CD8⁺, CD14⁺ dan CD19⁺ dan populasi sel-sel immunisasi ini telah ditentukan dengan menggunakan sitometri aliran. Selain itu, asai proliferasi limfosit telah dijalankan menggunakan CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS). Mencit BALB/c yang diberi madu Tualang menunjukkan peningkatan dalam populasi sel CD3⁺/CD4⁺ (sel T pembantu), CD3⁺/CD8⁺ (sel T pembunuh), CD14⁺ (makrofaj) and CD19⁺ (sel B) berbanding dengan kumpulan kawalan negatif di mana peningkatan tersebut telah meningkat mengikut dos madu Tualang yang diberi. Selain itu, analisa proliferasi limfosit juga menunjukkan peningkatan tindakbalas proliferasi limfosit mengikut dos madu Tualang yang diberi. Berat organ limpa mencit yang diberi madu Tualang juga menunjukkan peningkatan mengikut dos madu Tualang yang diberi. Kesemua keputusan telah menunjukkan kesan

immunostimulasi madu Tualang dalam mencit BALB/c melalui peningkatan populasi limfosit terutamanya sel T pembantu, sel T pembunuh dan sel B.

ABSTRACT

Local natural product extracts has been gaining great focus from research and therapeutic aspect. Similarly, the outcome of studies on one of nation's renowned Tualang honey (TH) (*Koompassia excelsa*) has profoundly anchored its board aptitude in anti-inflammatory, anti-oxidant and anti-microbial properties. This research focuses on the study of the immunomodulatory properties of TH in BALB/c. TH was orally administered daily for 14 days to BALB/c male mice (5/group) in dose ranging from 0.5 g/kg, 1.5 g/kg and 3.0 g/kg namely Low group, Medium group and High group. Pre-treatment and post-treatment body weights were measured. Upon sacrificing, the spleen was weighed and then homogenized. The splenocytes were stained with various surface markers antibodies namely CD3⁺/CD4⁺ (T helper), CD3⁺/CD8⁺ (T cytotoxic), CD14⁺ (macrophage) and CD19⁺ (B lymphocyte) and immune cell populations were obtained by using flow cytometer. On the other hand, proliferation assay of splenocytes were done by using CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS). Body weight in Control, Medium and High groups showed increment after the treatment but body weight of mice in Low group decreased upon completion of TH treatment. BALB/c mice treated with TH showed increased populations of CD3⁺/CD4⁺, CD3⁺/CD8⁺, CD14⁺ and CD19⁺ compared to control group. Proliferation analysis of splenocytes as well as spleen weight obtained from TH-treated mice also showed an increment. The results revealed the immunostimulant potential of TH in mice by enhancing lymphocyte populations especially T helper (CD3⁺/CD4⁺), cytotoxic (CD3⁺/CD8⁺) and B (CD19⁺) cells.

CHAPTER ONE

INTRODUCTION

1.1 Introduction to the study

Human immune system is a complex system consists of collection of cells, tissues and molecules that mediate resistance to infections (Abbas *et al.*, 2010). The physiologic function of immune system is to prevent infections and to eradicate established infections. Cytokines are groups of soluble protein and peptides that regulate immune system by either activating or deactivating immune cells. Our body is more prone to infections when our immune systems are down-regulated, thus, it is important to maintain or up-regulate our immune system in order to stay healthy. Nowadays, people begin to gain their interest and confidence on natural products and foods for their immunomodulatory effect as food and natural products contain many substances that can control the physiological functions of the body and modulating immune response (Shuichi *et al.*, 2004). These food and natural products can regulate and boost our immune system to fight against pathogens. Researches on carrots, celery, coriander, fennel and parsley found that five of these vegetables from Umbelliferae significantly stimulated the proliferation of human PBMC and secretion of IFN- γ (Cherng *et al.*, 2008). It is also found that alignan compound, Arctigenin may regulate immune responses in activated macrophages and lymphocytes including TNF- α and NO production and lymphocyte proliferation (Cho *et al.*, 1999). Another natural product *Echinacea* which is commonly used as traditional remedy to treat cold and enhance immune system also been proven for its immunomodulatory effect (Block and Mead, 2003). The *Echinacea* extracts shorten the duration and severity of colds and other upper

respiratory infections (URIs) when given as soon as symptoms become evident (Block and Mead, 2003).

Besides the example given above, honey is also commonly used to promote general health and boost immune system since ancient times. Honey was first used in Egypt, Syrians, Chinese, Greeks, and Romans since 4000 years ago (Zumla and Lulat, 1989). Honey as an excellent adjuvant for acceleration of wound healing is widely accepted in folk medicine. Russian soldiers used honey for this purpose in World War I, and scientific support for these beliefs is beginning to appear. A few studies proved that honey can be used in wound healing by using the animal model (Bergman *et al.*, 1983b). The typical composition of honey is: moisture, 17.7%; total sugars, 76.4%; ash, 0.18%; and total acid (as formic acid), 0.08%. Use of honey in open wound from radical vulvectomy showed less bacterial colonization and faster wound healing is reported (Cavanagh *et al.*, 1970). Topical application of honey was observed in isolated instances to be effective in decubitus ulcers, infected wounds, and burns (Bergman *et al.*, 1983a; Gupta *et al.*, 1992; Oryan and Zaker, 1998). Honey, for the most part, is made up of simple sugars and is an excellent source of energy. Honey has been proved to be highly bacteriocidal (Estevinho *et al.*, 2008; Irish *et al.*, 2008; Deshpande and Kulkarni, 2010; Aparna *et al.*, 2012; Bizerra *et al.*, 2012). Several aspects of its use indicate that honey also functions as a food preservative (Krushna *et al.*, 2007; Ricordel *et al.*, 2003). Honeys contain a number of components to act as preservatives; these include α -tocopherol, ascorbic acid, flavonoids, and other phenolics and enzymes such as glucose oxidase, catalase, and peroxidase (Cerutti, 1994).

One of the most precious types of honey that can be found in Malaysia is Tualang honey (TH). TH is collected from wild honey bees' hives on Tualang trees

found in the Malaysian rain forest. It has been used traditionally for the treatment of various diseases (Mohamed *et al.*, 2010). The majestic Tualang tree (*koompassia excelsa*) usually grows to an astonishing height of more than 250 metres. The best TH is usually the honey harvested from February-March where the rainforest blooms with flowers. The color of freshly harvested honey is liquid amber. The local honey-gatherers usually perform a ritual honey harvest rite by chanting ancient mantra to cajole charm and calm the bees. Studies on immunomodulatory properties of TH have not been reported yet.

In this study, a quantitative experimental design was employed. A total of 20 male BALB/c mice (weight around 350- 400g) age 6-7 weeks were used in this study and randomly divided into 4 groups. The animals were divided into 3 treatment groups with 0.5 g/kg, 1.5 g/kg and 3.0 g/kg of TH respectively and one group served as a control. Pre-treatment body weight was measured. Upon finishing 14 days of oral gavage of TH with respective doses, bloods were drawn from mice and post-treatment body weight was again measured before mice were sacrificed to obtain spleen. The weight of freshly obtained spleen was measured and recorded. Splenocytes were then harvested freshly to prepare for MTS proliferation assay and cell subset analysis. Results were compared between treatment groups and control group. MTS proliferation assay was used to evaluate the effect of TH on proliferation of immune cells where cell subset analysis was used to determine the immune cell subtypes that were being regulated or suppressed following TH administration. The results obtained from stated analyses were used to make a summary on the effect of TH on the immunomodulatory properties in BALB/C mice.

1.2 Objectives

1.2.1 General Objective

To study the immunomodulatory properties of TH on BALB/c mouse.

1.2.2 Specific Objective

1. To determine the cell subsets population in mice administrated with TH.
2. To evaluate the proliferation of splenocytes in mice administered with TH.
3. To measure the post-treatment spleen weight in mice administered with TH.
4. To evaluate the pre- and post-treatment body weight in mice administered with TH.

1.3 Justification of study

Immunomodulatory through dietary approach had been shown to be effective. Many food and natural products have been shown to possess immunomodulatory properties in *in vivo* and *in vitro* study. Honey is one of the natural products that possess many health benefits. TH is one of the national treasures of Malaysia. Traditionally, TH has been used to promote health by the local citizen but there are no scientific studies and evidences to prove its health benefits. Many studies on various types of natural products i. e. plants, animals or microorganism showed their potential as good immunomodulators. Hence, honey is one of the natural products that presented many health benefits such as improve wound healing, anti-microbial, anti-inflammatory and anti-proliferation. However, the study on honey is general as there are many different types of honey such as Manuka honey, jungle honey, Akasia honey, TH and many more. To date, a study specifically on the immunomodulatory properties of TH has not been done. Thus, in this research, we evaluated the immunomodulatory properties of TH on BALB/c mice.

CHAPTER TWO

LITERATURE REVIEW

2.1 Immune System and Immunomodulation

Our immune system main function is to protect us from infection. Collection of cells, tissues and molecules that mediate resistance to infections is called the immune system while the coordinated reaction of these cells and molecules to infectious agent is the immune response (Abbas *et al.*, 2010). Immunity is defined as resistance to disease especially infectious disease (Abbas *et al.*, 2010). Leukocytes are main cells involved in immune system, they can be divided into phagocytes (neutrophils, basophils, eosinophils, monocytes and macrophages) and lymphocytes (T lymphocytes, B lymphocytes and natural killer cells) (Calder and Grimble, 2002). Human immune system is one of the most important physiological systems which play an important role in maintaining well-being and health. Immune system is a very complex system and its optimal functioning involves a variety of biological activities such as cell division, cell proliferation, energy metabolism and protein production. Immune system can be divided into two parts, the innate immune system and adaptive immune system (Figure 2.1). Both the innate and acquired immune responses are integrated via direct cell-to-cell interaction as well as via the aids of cytokines under the existence of particular stimulus (Calder and Grimble, 2002). Innate immunity is the first line of defense against infectious agent mainly preventing invasion of infectious agent into our body while acquired immunity is a specific immunity involving the recognition of antigen specifically and destroy them by mean or cell-mediated or humoral immunity. Innate immunity is driven by hematopoietic such as macrophages, dendritic cells, mast cells,

neutrophils, eosinophils, natural killers (NK) cells, NK T cells, plasmacytoid dendritic cells, and $\gamma\delta$ T cells (Belardelli and Ferrantini, 2002; Vega and Martin, 2008). Innate immune system made up of network of germline-encoded receptors such as Toll-like receptors (TLRs) that recognize microbial and endogenous molecules resulting from injured tissues (Vega and Martin, 2008). Innate immunity and adaptive immunity can be linked via the cytokine produced by dendritic cells such as type I IFN (α and β), GM-CSF, TNF- α , IL-12, IL-15, IL-18, IL-1, IL-2 and IFN- γ (Belardelli and Ferrantini, 2002; Le Bon and Tough, 2002). Adaptive immunity is an active immunity which is more specific in action and elicits a stronger immune response in eliminating pathogen. Adaptive immunity made up of two major types; cell mediated immunity and humoral immunity. Cell mediated immunity involved via the activation of T cells. Matured T cells are activated via interaction between T cell receptors with antigenic peptides presented with MHC molecules by APCs (Bonilla and Oettgen, 2010). T helper cells are able to produce range of cytokines known to be in T cells repertoire (Coffman *et al.*, 1991). T helper cells are capable to activate B cells which involve in humoral immunity. It is found that a 39 kD T cell membrane protein is responsible in inducing T-cell-dependent B- cell activation leading to involvement of humoral immunity (Noelle and Snow, 1992). Humoral immunity is the principle protective immune response against extracellular bacteria via the production of antibodies (Abbas *et al.*, 2010).

According to Miller-Keane Encyclopedia and Dictionary of Medicine, Nursing and Allied Health (2003), immunomodulation is defined as the adjustment of the immune response to a desired level, as in immunopotential, immunosuppression or induction of immunologic tolerance. Well balanced immune function is vital as decrease in immune function increases chance of infection while an over reactive immune system

lead to some disorders such as allergies and autoimmune diseases (Abbas *et al.*, 2010). Modulating immune system at optimal state is important to prevent or control many diseases such as neoplastic formation and metabolic syndrome (Despres and Lemieux, 2006; Kabingu *et al.*, 2007). The control of diseases by means of immunological approaches involves either the development of immunity by stimulation of the immune system or suppression of unwanted immune reaction (Saravanan *et al.*, 2012). Immunomodulation not only plays important roles in combating chronic diseases but also atopic diseases; previous studies showed that the incidence of tuberculosis was inversely proportional to the incidence of asthma and rhinoconjunctivitis (von Mutius *et al.*, 2000). This is due to mycobacterium elicited strong Th1 responses which reduced allergic reaction such as asthma (von Mutius *et al.*, 2000). Neuroendocrine-immune interactions such as stress hormone could cause sufficient immunosuppression to adverse health effect (Pruett, 2001).

By means of modulating patient's own anti-tumour immune response, a beneficial therapeutic approach can be utilized in fighting cancer as chemotherapeutic and radiation treatments do not show great results (Ehrke, 2003). Immunomodulation can be achieved by several approaches such as vaccination. Vaccination modulates immune system in preventing diseases. Research on Hepatitis B showed that IL-12 could induce virus specific T helper and cytotoxic T lymphocytes which improved the efficacy of therapeutic vaccine (Rigopoulou *et al.*, 2005).

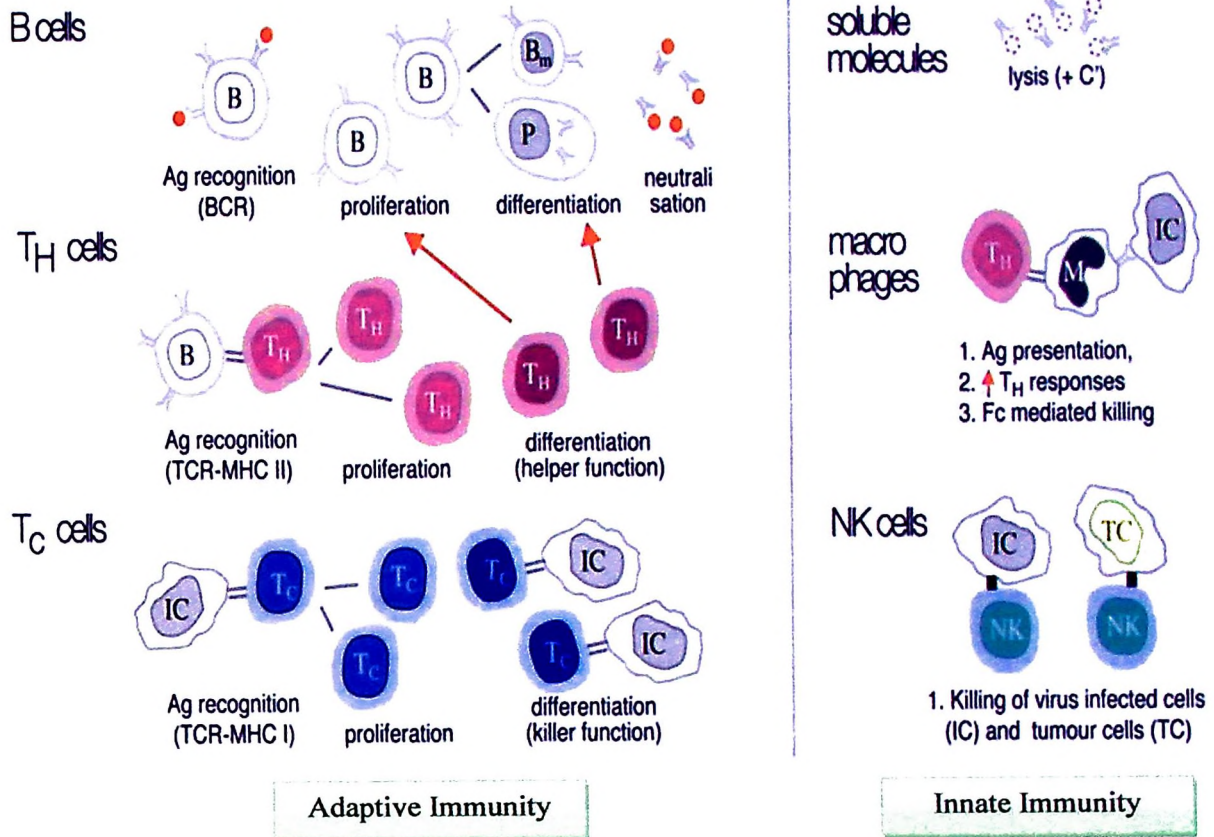


Figure 2.1 The interfacing of adaptive and innate immunity. (Source: http://nfs.unipv.it/nfs/minf/dispense/immunology/lectures/files/images/interface_innate_adaptive.jpg)

2.2 Immunomodulation by Foods and Natural Products

Many natural products especially foods hold promising effect for maintaining immune homeostasis in the healthy population (Wichers, 2009). Foods contain many substances that can control physiological functions as well as modulating immune response (Shuichi *et al.*, 2004). Food-derived substances incorporated into the body via various routes modulate immune functions (Amati *et al.*, 2003). Research showed that foods improved immune functions helped to reduce infection rates and mitigated the severity of infectious diseases (Braga *et al.*, 1996). Protein nutritional status strongly affects the functioning of T-cell and B-cell subsets as well as innate immunity (Wichers, 2009). Some micronutrients are said to be important to immunomodulation includes vitamins A, C, D, E, B12 and B6, folate, iron, zinc, selenium and beta-carotene (Yehia *et al.*, 2011). Fatty acids, particularly polyunsaturated long-chain fatty acids (PUFAs) are able to regulate cellular functions and immunity (Calder and Grimble, 2002). Besides, linoleic acid increased superoxide release from neutrophils and macrophages, and also promoted adhesion of neutrophils adhesions to endothelial cells (Badwey *et al.*, 1984; Bates *et al.*, 1995). Animal studies showed that essential fatty acids deficiency reduced the ability of mice to produce IgG and IgM against sheep red blood cells suggesting fatty acids is important in regulating immune function (DeWille *et al.*, 1989). Fish oil supplement for pregnant mothers showed to influence the capacity of neonatal immune function (Dunstan *et al.*, 2003). Evidence based research found that PUFAs could regulate the expression of genes coded for cytokines, adhesion molecules, nitric oxide synthase and many other inflammatory proteins, furthermore, they are able to regulate transcription factor nuclear factor kappa B (NF- κ B) where vitamin E also found to exhibits such effect (Chen *et al.*, 2000; Lavrovsky *et al.*, 2000; Wallace *et al.*, 2001).

Other than fatty acids, various vitamins found in foods also involved in immunomodulation. Vitamin A is found to be playing important role in reducing morbidity and mortality rate in measles in infants and children (Markowitz *et al.*, 1989). Vitamin C inhibited effect result from the expression of pro-inflammatory cytokines such as IL-6 and TNF α in adult *in vitro* (Hartel *et al.*, 2007). *In vivo* study on lab animals showed that Vitamin E supplement in diet had increased antibody production, lymphocytes proliferation, improved NK cell activity and phagocytosis ability of macrophages (Meydani and Beharka, 1998). Vitamin B6 has immunomodulatory effect as it is needed in the formation of cysteine which is a type of amino acids that serves as precursors of glutathione which in turn associated with lymphocyte proliferation (Grimble, 1997). Vitamin B9 or folate supplement was associated with decreased rate of infection, positive effect on blastogenic response and proliferation of T lymphocytes, enhanced delayed type hypersensitivity response, and enhanced phagocytosis and immunoglobulin production in *in vitro*, animal and human studies (Yehia *et al.*, 2011). Several amino acids also found to be immunomodulators, *in vitro* studies found out that *arginine* is needed for lymphocytes proliferation beside increasing macrophages and NK cell cytotoxicity (Bergeret *et al.*, 1987; Efron *et al.*, 1991). Another amino acids *glutamine* is highly utilized by neutrophils, macrophages and lymphocytes which suggests the importance to immune function (Reynolds *et al.*, 1990). Besides, *glutamine* also improved nitrogen retention and lowered incidence of bacteremia in patients with trauma (Houdijk *et al.*, 1998).

Minerals and trace element found in food naturally also found to be immunomodulating through several researches. Iron, one of the most abundant minerals directly inhibited IFN- γ activity, iron load in macrophages inhibited IFN- γ -mediated pathways in macrophages like formation of TNF- α and expression of MHC class II antigen (Weiss *et al.*, 1992). Iron also interfered with cytokine activities and the cell-mediated immune effector mechanisms of macrophages (Weiss *et al.*, 2004). Selenium has immunostimulatory effect, they increased expression of the α and β subunits of the IL-2 receptor, which are expressed on T and B lymphocytes (Roy *et al.*, 1993). Stimulation of IL-2 in turns potentiated killer cells, lymphocytes numbers and antibody productions as well as increased responsive of bone marrow to produce immune-cell precursors (McKenzie *et al.*, 2002). Selenium supplement was found to increase number of NK cells in elderly (Ravaglia *et al.*, 2000). A daily 200 μ g sodium selenite supplement significantly increased the T-lymphocyte-mediated immune response in healthy people with depleted selenium stores (Roy *et al.*, 1994). Another important minerals zinc also found to be important in T lymphocyte activation and signal transduction via involving in the non-covalent interaction of cytoplasmic tails of CD4 and CD8 cell with the tyrosine kinase p56^{lck} which is an important process in early activation of T-cell (Turner *et al.*, 1990). Furthermore, T-cell proliferation in response to mitogens, cytotoxicity and delayed-type hypersensitivity were suppressed in acrodermatitis enteropathica (AE) children with zinc deficiency (Prasad, 2000).

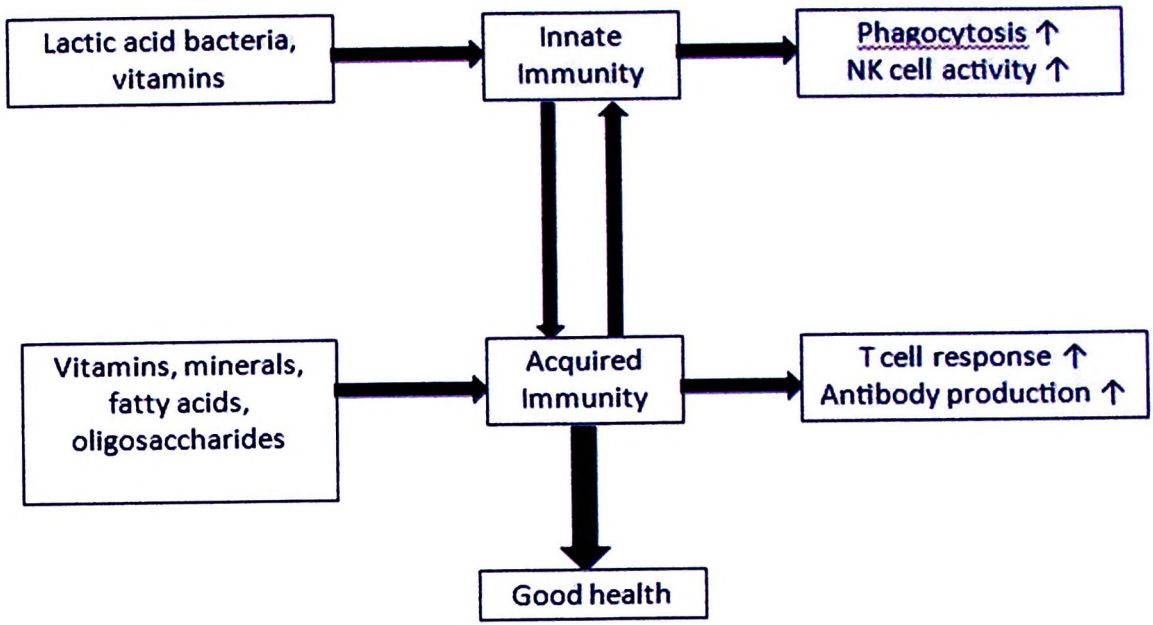


Figure 2.2 Diagram illustrated modulation of immune function by foods. (Source: Shuichi *et al.*, 2004 Modulation of Immune Functions by Foods)

Many foods that being taken daily by human have been found to be immunomodulating through researches. Besides, fruits such as strawberries was found to contain high phenolics, including flavonoids which able to stimulate splenocyte proliferation (Lin and Tang, 2007). It is proved that flavonoid quercetin and coumarinisopimpinellin might contributed to the immuno-enhancing activity of lymphocytes activation and flavonoids (quercetin and rutin) and coumarins (bergapten, xanthotoxin) also stimulate IFN- γ secretion (Cherng *et al.*, 2008). Soybeans contain many phytonutrients including flavonoids and saponins (Yamaki *et al.*, 2002). Soybean's extracts significantly stimulated the proliferation of human PBMC and the secretion of IFN- γ (Cherng *et al.*, 2007). Besides, water extraction from green soybean also found to be immunomodulating, they inhibited IgE production and regulated B cell apoptosis by suppressing BAFF and APRIL expression (Katayanagi *et al.*, 2013). Another study also showed that fermented soybean, chungkookjang was able to suppress apoptosis of immune cells (Kim *et al.*, 2007). Soy bean peptides had increased lymphocyte and granulocytes count and also CD11b⁺ and CD56⁺ cells in healthy human volunteer (Yimit *et al.*, 2012). Soybean proteins significantly increased the phagocytic activity of macrophages in rats (Yamauchi and Suetsuna, 1993). Garlic's active substance allicin significantly inhibited the secretion of IL-8, IFN- γ -inducible protein of 10 kD (IP-10), monokine induced by INF- γ (MIG) and IL-1 β from intestinal epithelial cells (Lang *et al.*, 2004). IL-8, IFN- γ -inducible protein of 10 kD (IP-10), monokine induced by INF- γ (MIG) and IL-1 β are chemokines involved in inflammatory bowel diseases and thus allicin may serve as a potential immune-mediating therapy for inflammatory bowel diseases (Lang *et al.*, 2004). Feruloylated oligosaccharides (FOs) which can be found in cereal bran also showed promising immunomodulatory effects

(Fang *et al.*, 2012). FOs induced TNF- α , IL-1b, IL-6, NO and PGE₂ production in unstimulated macrophages and inhibit their production in LPA-stimulated macrophages (Fang *et al.*, 2012). FOs consumption can protect against cardiovascular diseases and enhance innate immunity (Fang *et al.*, 2012). Besides, lipopolysaccharides (LPS) or LPS-like components of cereal induced production of IL-10 from PBMCs (Yamazaki *et al.*, 2008). Apples extract at 5 mg/ml significantly inhibited the TNF- α -induced NF-kB activation in cancer cells (Yoon and Liu, 2007). Arabinoxylan rice bran (MGN-3/Biobran) which is a modified form of arabinoxylan from rice bran was found to be a potent stimulant of phagocytic function of macrophage and induced high levels of TNF- α and IL-6 (Revilla *et al.*, 2013). Research showed that marine oligopeptide preparation (MOP) from Chum Salmon (*Oncorhynchus keta*) had immunostimulant effect by improving the cellular immunity, humoral immunity and NK cell function in mice. It also stimulated Th cells that led to secretion of Th1 (IL-2, IFN- γ) and Th2 (IL-5, IL-6) cytokines (Yang *et al.*, 2009). Some phenolics from emblica were found to significantly increase splenocytes proliferation and inhibit growth of MCF-7 and HELF suggesting the anti-tumour properties might arise by immunomodulatory properties (Liu *et al.*, 2012).

Besides dietary foods, many natural products or herbs also serve as immunomodulators. *Echinacea purpurea* is one of the natural products that has shown immunomodulatory properties. *Echinacea purpurea* was originally used by native Americans as an immune stimulant to increase resistance to viral, bacterial and fungal infections of upper respiratory tract (Goel *et al.*, 2002; Sharma *et al.*, 2010). Root extracts of *Echinacea purpurea* had increased the expression of MHC class II, CD86 and CD54 surface biomarkers as well as IL-6 and TNF- α cytokine secretion in murine

bone marrow-derived dendritic cells (BMDC) (Benson *et al.*, 2010). *Echinacea purpurea* plant extract was found to be immunomodulating by increasing phagocytic activity of alveolar macrophages of rats and enhanced release of cytokines such as TNF- α and IFN- γ (Goel *et al.*, 2002). *In vitro* study on *Echinacea purpurea* found that it significantly increased secretion of IL-1, TNF- α , IL-6 and IL-10 by human peripheral blood macrophages (Burger *et al.*, 1997). *Echinacea purpurea* was able to stimulate *de novo* production of NK cells and enhanced their cytolytic function in advanced age animals (Currier and Miller, 2000). Polysaccharides purified from *Echinacea purpurea* enhanced the spontaneous motility of polymorphonuclear leukocytes (PMN) and increased their ability to kill staphylococci. Besides, monocytes also being activated to secrete TNF- α , IL-6 and IL-1 (Roesler *et al.*, 1991). Ginseng is another natural product that has many health benefits including improving immune system and function. Research found that one of the component of *Panax ginseng*'s root ginsenoside S-IIA was a potent inducer of IL-8 production by human monocytes and THP-1 cells followed by increasing IL-8 mRNA expression (Sonoda *et al.*, 1998). Besides, *Panax ginseng* has immune homeostasis effect where it restores lymphocytes in secondary lymphoid organs (Hong *et al.*, 2012). A study on *Echinacea* and ginseng found that both of them significantly increased NK-function and Antibody Dependent Cell Cytotoxicity (ADCC) of peripheral blood macrophages in normal individuals and patients with depressed cellular immunity (See *et al.*, 1997). *Panax ginseng* was found to be able to modulate pro-inflammatory cytokine production (IL-1 β and TNF- α) in mice via the increased of macrophage toll-like receptor 4 expression during physical stress in mouse (Pannacci *et al.*, 2006). *Panax ginseng* significantly increased IL-2 and IFN- γ productions in spleen cells induced by Con-A (Wang *et al.*, 2004). The major saponin component of

Vietnamese ginseng, majonoside-R2 was found to be able to increase the phagocytic index evaluated by carbon clearance test in mice (Huong *et al.*, 1997).

2.3 Honey and General Health Properties of Honey

Honey is a food product which is collected from various plants and processed by honey bees (Ghashm *et al.*, 2010). Honey was first used in Egypt some 4000 years ago, and that it had also been used by the Syrians, Chinese, Greeks, and Romans (Zumla and Lulat, 1989). The typical composition of honey is: moisture, 17.7%; total sugars, 76.4%; ash, 0.18%; and total acid (as formic acid), 0.08%. Traditionally, its use in food has been as a sweetening agent. However, several aspects of its use indicate that honey also functions as a food preservative. In Japan, their consumption amount is up to about 40,000 tons per year. The Japanese most frequently use honey on items such as sandwiches and toast. Honeys contain a number of components to act as preservatives; these include α -tocopherol, ascorbic acid, flavonoids, and other phenolics and enzymes such as glucose oxidase, catalase, and peroxidase (Cerutti, 1994). It was suggested that any of these substances owed their preservative properties to their antioxidative activity (Cerutti, 1994). Honey also contained approximately 200 substances, including a mixture of sugars (fructose, glucose, maltose, and sucrose) and small amounts of other constituents, such as minerals, proteins, vitamins, organic acids, flavonoids, phenolic acids, enzymes, and other phytochemicals (Aljadi and Kamaruddin, 2004). Phenolic acids can be further divided into substituted benzoic acids and cinnamic acids. Flavonoids in honey include flavonols, flavones and flavanones. These compounds are important as they determine the colour, taste, aroma and health effect of honey (Khalil *et al.*, 2011).

Besides serving as food products, honey also found to exhibit some healing properties including anti-oxidant, anti-inflammatory, anti-bacterial, anti-viral, anti-ulcerous, anti-lipid and anti-cancer properties (Gheldof and Engeseth, 2002; Swellam *et al.*, 2003; Temaru *et al.*, 2007; Boukraa and Amara, 2008; Estevinho *et al.*, 2008; Irish *et al.*, 2008). In addition, honey as an excellent adjuvant for acceleration of wound healing is widely accepted in folk medicine. A few studies were made before by using a none specific honey which had proved that honey can be used in wound healing on animal model (Bergman *et al.*, 1983b). Use of honey in open wound from radical vulvectomy was noted to have less bacterial colonization and faster wound healing (Cavanagh *et al.*, 1970). Topical application of honey was observed in isolated instances to be effective in decubitus ulcers, infected wounds, and burns.

Studies on anti-proliferative effect of honey also gave promising results where honey inhibited the growth of some cancer cells *in vitro* including colon cancer and bladder cancer (Rao *et al.*, 1995; Swellam *et al.*, 2003). The phytochemical component of honey is responsible for its antioxidant activity which protects cells from the damage caused by free radicals thus decreasing the inflammation process (Molan, 1999). It was reported that the free radical scavenging activities of honey was mainly due to the contents of flavonoids and phenolic acids (Henriques *et al.*, 2006). Review on data suggested that honey either administered alone or in combination with conventional therapy has the potential to be a novel antioxidant in the management of chronic diseases commonly associated with oxidative stress (Erejuwa *et al.*, 2012). A strong correlation between the antioxidant activities of honeys and their total phenol contents had been discovered (Alzahrani *et al.*, 2012).

Honey, for the most part, is made up of simple sugars and is an excellent source of energy. It is hypertonic and had been shown to be sterile and highly bacteriocidal (Estevinho *et al.*, 2008; Irish *et al.*, 2008; Deshpande and Kulkarni, 2010; Aparna *et al.*, 2012; Bizerra *et al.*, 2012). Honey at a concentration of 40% was found to be bactericidal to various gram-negative and gram positive bacteria (Al-Waili *et al.*, 2001). Laboratory studies had demonstrated the significant antibacterial activity of honey (Molan, 1999). Hydrogen peroxide is the major contributor to the antimicrobial activity of honey, and the different concentrations of this compound in different honeys resulted in their varying antimicrobial effects (Molan, 1992). Hydrogen peroxide concentration in honey is determined by the rate of its production by glucose oxidase and its destruction by catalases and hydrogen peroxidase is one of the compound in honey that is antibacterial (Bizerra *et al.*, 2012). The hydrogen peroxide content in honey is about 900-fold lower than the concentration commonly used for disinfecting medical equipments (Bizerra *et al.*, 2012). The hydrogen peroxide in honey involved in oxidative damage which leads to inhibition of bacterial growth and DNA degradation with the help by other honey components (Brudzynski *et al.*, 2011). Studies reviewed that methylglyoxal and the antimicrobial peptide bee defensin-1 were identified as important compound that contributed to antibacterial action in honey (Kwakman and Zaat, 2012). Study on honey showed that the diameters of the zone of inhibition (ZDI) of honey at various concentrations proved that honey possess antibacterial activity against *Staphylococcus aureus* and *Streptococcus pyogenes* and also inhibited the formation of biofilm by *Streptococcus pyogenes* (Maddocks *et al.*, 2012). Honeys were found to be effective for their antimicrobial activity on sensitive, non-MDR, MDR and ATCC strains of *Staphylococcus aureus* (Deshpande and Kulkarni, 2010). Honey is also found

to be a natural, non-toxic product that has activity against *Pseudomonas aeruginosa* (Mullai and Menon, 2007). Honey mouthrinse effectively inhibited six oral microorganisms and possesses antiplaque action (Aparna *et al.*, 2012). Research on antimicrobial effect of honeys against pathogenic bacteria showed that honey able to inhibit the growth of *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enteric subsp, Enterica*, *Streptococcus pyogenes*, *Bacillus cereus* and *Bacillus subtilis* (Voidarou *et al.*, 2011).

Honey also showing anti-inflammatory effect which aids in speeding up wound healing. Monocytes are stimulated by honey in vitro to produce both pro-inflammatory and anti-inflammatory cytokines (Tonks *et al.*, 2003). Monocytes react to honey due to the release of inflammatory and anti-inflammatory cytokines and a reduced formation of reactive oxygen intermediaries (Tonks *et al.*, 2003). Honey appears to draw fluid from the underlying circulation, providing both a moist environment and topical nutrition that may enhance tissue growth (Molan, 1999). Honey has also been shown to be effective in other medicinal applications. Honey has proved effective in the treatment of infantile gastroenteritis (Jeddar *et al.*, 1985). Studied showed honey reduced cytokine (TNF- α , IL-1 β , and IL-10) and nitric oxide levels and increased heme oxygenase-1 levels which support anti-inflammatory properties of honey. Research found that honey inhibited peroxynitrite synthesis in LPS-treated rats which can inhibit inflammatory responses that lead to cell damage and death (Kassim *et al.*, 2012).

Another famous and well known health benefit of honey is that honey promotes wound healing. Many research on the effect of honey in wound healing have been done across many different type of honeys found in all over the world. One of the Malaysian honey, Gelam honey, was shown to accelerated the rate of wound healing by increasing

wound contraction when applied topically (Tan *et al.*, 2012). Honey is a good agent as topical treatment for superficial burns as it favours fast re-epithelialization and decreases inflammatory reaction (Maghsoudi *et al.*, 2011). Besides, honey dressings improved wound healing, sterilized wound, reduce hypertrophic scars and postburn contractures (Gupta *et al.*, 2011). For research on treatment for chronic recurrent pilonidal sinus wound, honey dressing therapy showed a good results (Thomas *et al.*, 2011). Honey is able to stimulate tissue growth, synthesis of collagen and trigger angiogenesis in wounds (Bergman *et al.*, 1983a; Gupta *et al.*, 1992; Suguna *et al.*, 1992; Subrahmanyam, 1998). Honey also improved formation of granulation tissue and epithelialization of wounds (Oladejo *et al.*, 2003). Rabbits' wounds treated with topical honey showed less edema, fewer polymorphonuclear and mononuclear cell infiltrations, less necrosis, better wound contraction, better epithelialization and lower glycosaminoglycan and proteoglycan concentrations (Oryan and Zaker, 1998). Honey appeared to be effective in reducing bacterial contamination and promoting wound healing (Mphande *et al.*, 2007).

2.4 Tualang Honey and General Health Benefits of Tualang Honey

TH is collected from wild honey bees' hives from the comb of Tualang trees found in the Malaysian rain forest, and is produced by *Apis dorsata* or Asian rock bees (Tan *et al.*, 2009). It has been used traditionally for the treatment of various diseases (Mohamed *et al.*, 2010). The major components of TH are furfural derivatives such as 5-(hydroxymethyl)-furfural, furfural 2-furyl methyl ketone, 5-methyl furfural, and fatty acids such as palmitic acid, ethyllinoleate, and ethyl oleate (Muthiah *et al.*, 2012). The main phenolic and flavonoid compounds in TH include kaempferol, quercetin, ellagic acid, gallic acid, hesperetin, and catechin (Hussein *et al.*, 2011). TH contains 26.51% of

moisture, 0.19 % of ash and has pH of 3.30 and conductivity of 1.37 mS/cm (Chua *et al.*, 2012). A study on the antioxidative compounds of TH has found that TH had the highest total phenolic and protein content compared to other types of honey; Gelam, Indian forest and Pineapple honey (Mohd Effendy *et al.*, 2012). Among Malaysian honey, TH was shown to exhibit the highest content of phenolics, flavonoids and DPPH radical-scavenging activities (Khalil *et al.*, 2011). Among Malaysian honey, selenium only can be found in TH, selenium is a micronutrient is very important for proper immune system particularly thyroid function in humans (Chua *et al.*, 2012).



Figure 2.3 Tualang tree (*koompassia excelsa*) (source: www.rainforestoutdoor.com)

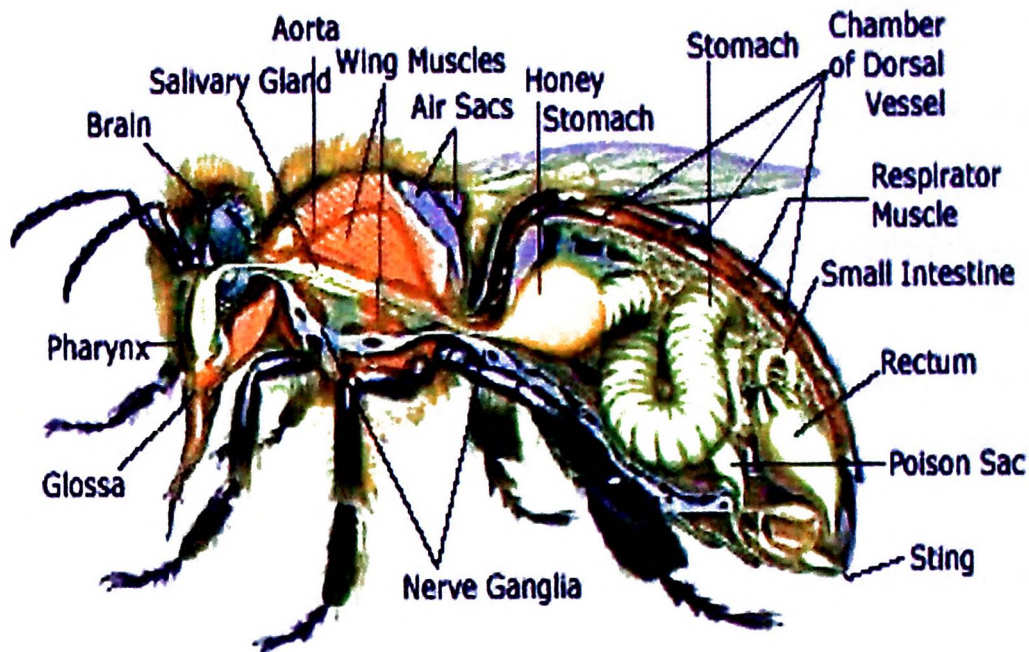


Figure 2.4 Diagram of *Apis dorsata* (Asian Rock Bee) (Source: mymaa-madutualang.blogspot.com)

TH also shows good antimicrobial properties. TH could potentially be used as an alternative therapeutic agent against certain microorganisms, particularly *A. baumannii* and *S. maltophilia* (Tan *et al.*, 2009). TH has been reported to have antibacterial properties against various microorganisms, including those from burn-related diagnoses such as *Pseudomonas* spp. and MRSA (Tan *et al.*, 2009; Pieper *et al.*, 2009; Molan, 1999). Research also showed that TH demonstrated bacteriostatic and bacteriocidal effect on bacteria isolated from wounds such as *Staphylococcus aureus*, *Coagulase-negative Staphylococcus aureus*, *Streptococcus* spp., *Enterobacter cloacae*, *Klebsiella pneumonia*, *Pseudomonas* spp., and *Acinetobacter* spp. (Nasir *et al.*, 2010).