IN VITRO IMMUNO-MODULATORY ACTIVITY OF AQUEOUS *Quercus infectoria* GALL EXTRACT

BY

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Author

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

BSC	Biological safety cabinet
DMSO	Dimethyl sulfoxide
EPS	Exopolysaccharides
h	Hour
IFN-γ	Interferon-gamma
IL	Interleukin
IVIg	Intravenous immunoglobulin therapy
LPS	Lipopolysaccharide
mg	Milligram
ml	Milliliter
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PAMPs	Pathogen-associated molecular pattern
PHA	Phytohaemagglutinin
PS	Phosphatidylserine
RPMI	Roswell Park Memorial Institute Medium
TGF-β	Transforming growth factor beta
Th	T-helper
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor α
ul	Microliter
ug	Microgram

ABSTRAK

Modulasi immuno kini telah menjadi bahagian yang penting dalam rawatan kanser, penyakit berjangkit dan kekurangan imun. Terdapat banyak tumbuhan tempatan di mana kompaun bioaktif boleh diekstrak. Salah satu tumbuhan herba penting yang telah menarik perhatian ramai penyelidik ialah *Q. infectoria* (manjakani). Kajian kesan ekstrak manjakani pada makrofaj dapat meningkatkan pengetahuan kita tentang aktiviti immuno-modulasinya. Dalam kajian ini, makrofaj J774A.1 dirawat dengan *Q. infectoria* ekstrak manjakani dengan kepekatan 62.5, 31.25 dan 15.625 ug/ml. Proliferasi sel telah dikaji menggunakan ujian MTT setelah sel masing-masing dirawat dengan kepekatan ekstrak berbeza pada 24, 48 dan 72 jam. Selain itu, aktiviti fagositik diukur menggunakan kaedah *cytometer* dan hasilnya dianalisis selepas 72 jam rawatan. Ujian ELISA digunakan untuk pengesanan sitokin pada makrofaj dirawat dengan 62.5 ug/ml ekstrak manjakani. Proliferasi makrofaj J774A.1 bertambah dalam semua kepekatan yang dirawat berbanding dengan makrofaj yang tidak dirawat. Dengan cara yang sama, rawatan dengan ekstrak manjakani berasaskan air juga menunjukkan peningkatan dalam aktiviti fagositik makrofaj. Walaupun makrofaj yang dirawat dengan 62.5 ug/ml ekstrak meningkatkan nilai penyerapan untuk sitokin IL-2, IL-5, IL-10, IL-17A, IL-23, TGF-β dan TNF-α tetapi adalah tidak signifikan secara statistik daripada makrofaj yang tidak dirawat. Oleh itu, boleh dicadangkan bahawa ekstrak manjakani berasaskan air dapat memberi kesan kepada aktiviti immuno-modulasi makrofaj. Lebih banyak kajian boleh dijalankan untuk memberi keputusan yang lebih tepat seperti kajian DNA atau evaluasi penghasilan sitokin secara kuantitatif.

ABSTRACT

Immuno-modulatory entities have now become an important part in the treatment of cancer, infectious diseases and immune deficiencies. There are many local plants from which the bioactive compound can be extracted. One of the important herbal plants that attracts the attention of many researchers is *Q. infectoria* (manjakani). The effect of its gall extract on macrophages can enhance our knowledge on its immuno-modulatory activity. In this study, macrophages J774A.1 were treated with aqueous *Q. infectoria* gall extract with a concentration of 62.5, 31.25 and 15.625 ug/ml. The proliferation of the cells was studied using MTT assay after treating the cells for respectively 24, 48 and 72 h. Apart from this, the phagocytic activity was measured using a flow cytometer and the results were analyzed after 72 h of treatment. Multi-analyte ELISArray was used in the detection of cytokines with 62.5 ug/ml treated macrophages. It was observed that the proliferation of J774A.1 was greater in all concentration of treated compared to untreated macrophages. In the same way, treatment with aqueous *Q. infectoria* extract also showed an increased in the phagocytic activity of the macrophages. Although treating the macrophages with 62.5 ug/ml of extract increased the absorbance value in IL-2, IL-5, IL-10, IL-17A, IL-23, TGF-β and TNF-α but they are not statistically significant from untreated macrophages. Hence it could be suggested that an aqueous *Q. infectoria* gall extract could show some effect on the immuno-modulatory activities of macrophages. More study can be carried out to give a detail and accurate result such as DNA study or quantifying the cytokine productions.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Immune system gives protection from intruders such as bacteria, viruses or parasites and it has to differentiate host cells, beneficial commensal flora and those harmful invading organism during its activation (Male *et al*., 2013). Serious impairment of any arm of this system can cause severe or life-threatening infections in a world full of potentially dangerous microbes. Innate immunity always presents as ready to attack but many pathogenic microbes have evolved to resist the defense mechanism. The emergence of earliest infection by ancient disease agent, *Mycobacterium tuberculosis* has co-evolved with the human immune system by discarding and gaining genes to escape from it (Cambier *et. al*, 2014). Macrophage plays important role in avoiding defense mechanism as it will engulf the bacterium but not destroyed it until the host becomes immune-compromised due to other infections such as cancer or diabetes type II to develop a disease. In addition, Human immunodeficiency virus 1 (HIV-1) infected patients undergo altering healthy innate immune mechanisms by viruses (Howie *et al*., 2000). This condition leads to defects of innate immunity and results in a worse outcome to the patients with HIV-1 infection making them vulnerable to other infections.

The theory of boosting immune system now become part of modern medicine ranging from preventive measures, optimization of wellness or even self-medication for mild illnesses. Plant-derived products like polysaccharides, lectins, peptides, flavonoids

and tannins have been used to modulate immune response or immune system in various *in-vitro* models (Virendra Kumar *et al.*, 2011). A medicinal plant which has an immuno-modulatory effect could provide alternative potential substance, especially in relation to host defense mechanism therapy.

In 2009, the total market size for prescription and over the counter medicine were estimated at RM4.5 billion while the traditional medicine and health supplements market was estimated at RM3 billion (MOPI, 2017). Market growth has been fairly consistent at between 8% - 10% annually for the past several years. The process of drug discovery from natural products are very challenging and takes a long time to develop (Koehn and Carter, 2005) and taking these alternatives medication could considerably at risk without proper knowledge about it. However, the increase of demands for a traditional supplement in Malaysia market has risen because of easy access and no prescription needed for traditional medicine or supplements. Natural ingredients from plants are suggested to have a good outcome without having extreme side effects but it still has a deficiency of benefit study for traditional medicine to proof its claim.

These natural ingredients could come from their leaves, stems, roots, fruits or seeds. One of the famous plants in traditional medicine in Malaysia exclusively in the treatment of women after birth is *Quercus infectoria* gall (known traditionally as manjakani). *Q. infectoria* gall extract have an anti-inflammatory activity that could suggest the immuno-modulatory activity of its properties (Kaur *et al.*, 2004); its antioxidant activity and abrogates oxidative stress was claimed to induce functional alterations in murine macrophages (Kaur *et al*., 2008) and the greater amount of phenolic

and flavonoid contents leads to more potent antioxidant that can react with wide range of molecules in living cells as shown by *Q. infectoria* (M.Asif *et al.*, 2012). This plant is demonstrated to have good benefit in treatment especially in wound healing (Umachigi and Jayaveera, 2008) (Chokpaisarn *et al.*, 2017) which support the benefit of its properties in the immune system.

1.2 *Quercus infectoria* gall and medicinal uses

Q. infectoria originates from family Fagaceae which usually known as gall oak, which is a small plant growing up from 4 to 6 feet tall, crooked, with smooth and bright leaves, acorn long and narrow, scaly and downy that prefer semi-shade to no shade or partial sun to full sun and requires moist soil that normally found in Greece, Asia Minor and Iran. They have monoecious flowers that are pollinated by wind. The galls of *Q. infectoria* are used since centuries as a home remedy to treat a sore throat and chronic diarrhea in both rural and urban areas (Shrestha *et al*., 2014). The gall can be obtained from the branches of the tree is called as ‘majuphal’ in India, ‘machakai’ in Kannada and ‘manjakani’ in Malaysia. In Malaysia ‘manjakani’ are recognized to have many benefits to the health of women’s intimate organs as well as to address numerous illnesses of women. One of the functions is to treat fungus infection and are used widely in women health after birth.

Aqueous *Q. infectoria* gall extracts are shown to exhibit widespread antimicrobial activity against food-borne pathogenic bacteria (Fathabad *et. al*, 2015). Moreover, *Q. infectoria* is widely used in some countries such as India, Malaysia and Iran in traditional medicine. It is reported to contain large amounts of bioactive constituents such as saponins, alkaloids, tannins, glycosides, triterpenes, sterols, phenolic compounds,

carbohydrates, and flavonoids in various extracts (Shrestha *et al*., 2014). *Q. infectoria* has a high concentration of gallic and tannic acid (Kaur *et al*., 2008) which might be the foundation of natural antioxidant and antibacterial activity of its bioactive compound.

Nutgall of *Q. infectoria* (see Figure 1.1) comes from primarily insect gall induced by a wasp (*Cynips sp*.) on twigs of *Q. infectoria* (Jackson, 2014). This gall is excreted from its branches when the wall of the tree is being attacked by the parasite as a defense mechanism. The production of the galls is effective in the survival of the tree from cynipid wasp larvae including increased the protection against natural enemies such as parasitoid (Zargaran *et. al*, 2011). The process of fighting against parasite produce active compound such as gallic and tannin acid in the gall which has potential benefit as antibacterial and antifungal agents. The larvae will produce a hole on the surface of the galls when its leaves the galls.

Figure 1.1 *Quercus infectoria* gall: The galls can be identified macroscopically based on their physical appearances which were globular in shape and have tuberculate surface, 0.8 cm to 2.5 cm in diameter with green-yellow in color. The galls also had an astringent smell and strongly pungent taste. When the larvae leave the galls it creates holes on the surface of the galls.

1.3 Bioactive compound derived from *Quercus infectoria* gall

The main constituents found in the galls of *Q. infectoria* are tannin (50-70%), free gallic acid and ellagic acid and have high concentration of amino acids such as tannic, quinic, gallic, mallic and protocatechuic (Hamad *et al*., 2017). Tannic acid and gallic acid, are the important products of tannins, have been reported to have antioxidant activity (Kaur *et al*, 2008) and good activity as antimicrobial (Fathabad *et al.*, 2015), antibacterial activity (Nor Amilah *et al.*, 2014), antifungal activity (Vanga *et al.*, 2017) and anti-carcinogenic property (Roshni and Ramesh, 2013).

Tannin in *Q. infectoria* gall has bitter in taste and will make the color of the extract to be darker (Baharuddin *et al*., 2015). People commonly consume *Q. infectoria* gall by immersing it in the water. Usually, they grind the dried gall and boil it in the water and take it as drink beverage. Methanol solvent is best to extract the active phytochemicals in the *Q. infectoria* gall but the differences between aqueous and methanol extracts were insignificant (Baharuddin *et al.*, 2015), furthermore the extracts of aqueous and acetone give similar results in the antimicrobial activity (Basri and Fan, 2005) thus the aqueous solvent are the best to replicate common user.

Water extracts of *Q. infectoria* also gives better yield for gallic and tannic acid extraction in which the concentration of both compounds are highest in water compared to 100% of methanol, ethanol and acetone also 70% of methanol and ethanol solvent (Ab. Rahman *et al*., 2015). By using aqueous extract, we can study the similar effect of *Q. infectoria* gall extract consumed by common people in daily life.

1.4 Problems statement

The properties of antibacterial, antifungal, anti-oxidant and anti-inflammatory by *Q. infectoria* gall have been reported by many studies but the involvement of *Q. infectoria* gall in the modulation of the immune response are still lacking.

Immune-modulators could give effect to the human system such as regulate or down-regulate the production of its property such as phagocytic activity, cytokines release and apoptotic activity in cells. By using the extract as a supplement could enhance one's wellness but could also give harm to others health condition especially people with the immune systems impairment. Proper knowledge about the impact of its bioactive compound to our immune system is necessary.

1.5 Rationale of study

Immuno-modulatory entities with additional safety and effectiveness are still in need especially for natural products of plant origin. It was essential to study the effect of extract that usually consumed by common people in daily life and to gain much knowledge about the characteristic of the compound in its immuno-modulatory activity. Immunotherapy for cancer (Khalil *et al.*, 2016), diabetes type I (Tooley *et al*., 2012) and even atopic dermatitis problems (Nahm, 2015) are still in search for the new and future immuno-modulatory therapy that gives promising result in treating patients.

At present, there was only a few experimental data on the immuno-modulatory activity of aqueous *Q. infectoria* gall extract which could be used to determine the benefit of the extract as immuno-modulators. *Q. infectoria* gall extract may be used as alternatives

medicine to substitute current medication by reason of most immuno-stimulants and immuno-suppressants in clinical use are the cytotoxic drugs which can possess serious side effect to human. Furthermore, it is important to gain additional knowledge about its immuno-modulatory activity and to provide preliminary information in the understanding of the effectiveness of the gall extract as an agent that could possibly modulate or suppress the immune response *in vitro* prior to *in vivo* study.

1.6 Objectives of study

1.6.1 General objectives

This study was conducted to evaluate the immuno-modulatory activity of aqueous *Q. infectoria* gall extract against macrophage *in vitro*.

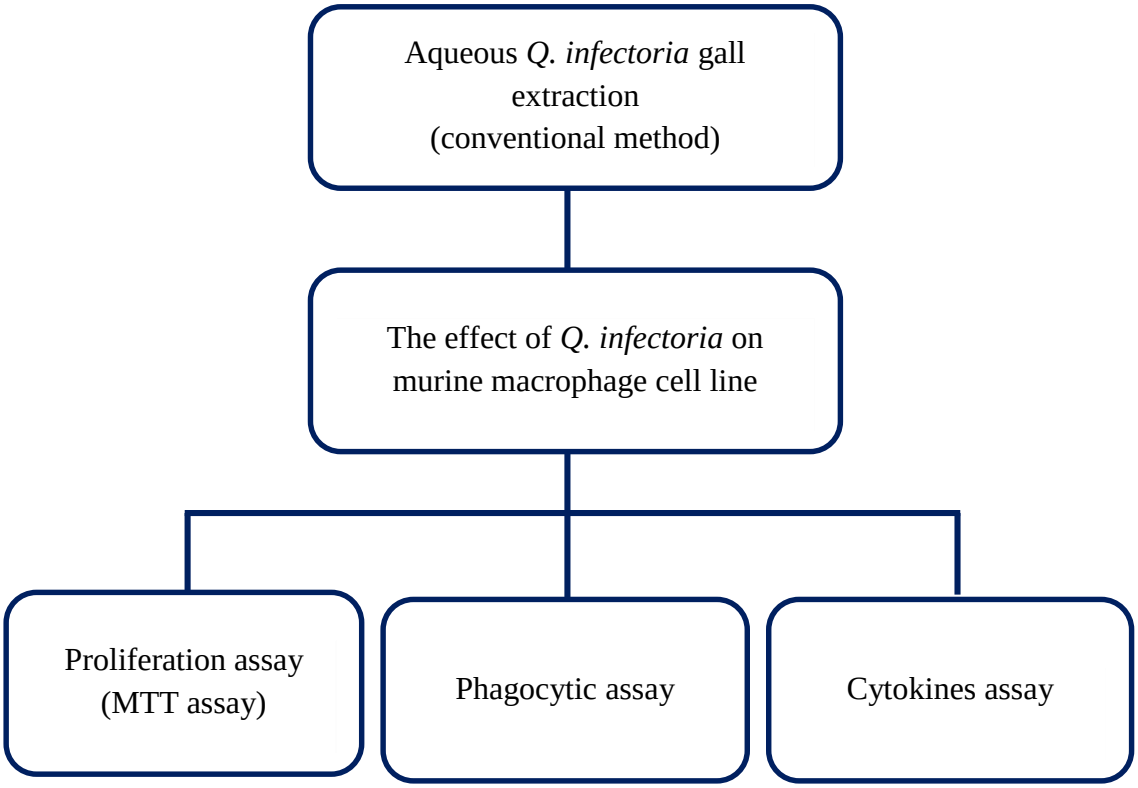
1.6.2 Specific objectives

i) To access the proliferation activity of macrophage in the presence of aqueous *Q. infectoria* gall extract.

ii) To determine the phagocytic activity of macrophages after treated with aqueous *Q. infectoria* gall extract.

iii) To evaluate the cytokines regulation of treated macrophages with aqueous *Q. infectoria* gall extract.

1.7 Flow chart of the study



CHAPTER 2

LITERATURE REVIEW

2.1 Immune system and macrophage

Human is equipped with a complex immune system that guards and protects our body from infections. An immune system is alienated into two categories which are innate and adaptive immunity. Innate immunity gives rapid response while adaptive immunity provides longer protection to the infections. Innate immunity acts as the first line of host defense, mediated by phagocytes, able to discriminate between self and non-self and alert the immune response (Spiering, 2015) with several non-specific protective mechanisms against infection. The macrophages and neutrophils detect and attack other cells carrying pathogen-associated molecular patterns (PAMS) or other small proteins that signal pathogen invasion such as cytokines and chemokine to immediately attach to and restrict microbial pathogens.

Innate immunity identifies pathogen via Toll-like receptors (TLRs). TLRs that can be classified into cell surface TLRs and intracellular TLRs based on their localization. Cell surface TLRs include TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10, whereas intracellular TLRs are localized in the endosome and include TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 (Kawasaki and Kawai, 2014) that react to different and specific antigen pathogen-associated molecular patterns (PAMPs) such as unmethylated double-stranded DNA (CpG), single-stranded RNA (ssRNA), lipoproteins,

lipopolysaccharide (LPS), and flagellin (Lim and Staudt, 2013). In addition, acquired immunity eliminates pathogen in the late phase of infection and generate the immunological memory which characterized by specific interaction between intruders and host (Bonilla and Oettgen, 2010). Immune cells are being generated from bone marrow as hematopoietic stem cells to common granulocyte or macrophage and lymphoid progenitor.

Macrophages are developed directly from multicellular organisms to perform clearance of dying cells and protect the host by phagocytic activity through innate immunity (Martinez and Gordon, 2014) containing resident tissue macrophages and monocyte-derived recruited cells during inflammation. Disease states are conditions are regularly associated with macrophage activation. Macrophages are distributed into M1 and M2 type, with typically M1 macrophages implicated in initiating and sustaining inflammation and M2 macrophages associated with resolution of chronic inflammation (Martinez *et. al*, 2009). M1 macrophage promotes T-helper 1 (TH1) response and has efficient antigen presentation capacity thus can kill intracellular pathogens and execute tumor destruction. On the other hand, M2 macrophage promotes TH2 response and responsible for clearing parasites thru encapsulation. It also regulates tumor promotion, tissue remodeling and immunity.

Through a process called phagocytosis, macrophages plays a very important role in immunological signaling, destruction or clearance of unwanted particles from tissues by internalization (Mutzke *et al.*, 2015). The process begins with the binding of the particles to the surface of the macrophage via receptors. This lead to the establishment of

a phagosome and eventually to the death of the invading cell or the degradation of the foreign particle (Razali *et al.*, 2014). In normal condition, macrophage will be at rest but could be stimulated to be active in immune response especially in phagocytosis and inflammation (Duque and Descoteaux, 2014). Phagocytosis activity will root to the inflammation at the site of infected area to enclose the infections.

2.2 Types of macrophage: for cell culture

Murine-derived cell lines often used in a study assuming that the response of cell lines is suggestive to the potential response in the primary human cell (Merly and Smith, 2017). Murine macrophages are easy to handle in laboratory compared to human macrophage cell line because it does not need any differentiation step from monocytes to become macrophage and ready to use in any experimental design.

2.2.1 J774A.1 murine macrophage

This macrophage is derived from reticulum cell sarcoma from Mus musculus, mouse Strain BALB/cN. J774A.1 is a monocyte; macrophage with the morphology of a macrophage and mostly adherent. Most J774A.1 cells were round or elliptic shape, but the number of elongated and spindle-like cells slightly increased after EPS or heat-killed bacteria (Wu *et al.*, 2010). In culture, J774A.1 appears as two distinct population which is round and monocyte-like while the other is stretched and more macrophage-like but at higher densities the round type is predominant but at 40-80% confluent the two population are highly distinct.

The difference between the morphology of macrophage in low-density and high-density culture are visualized in Figure 2.1. The cells can be distinct into a different shape, round and elongated (A) but most of the cells appear to be rounded (B). The cell should be maintained until it reaches at least 80% confluence before testing.

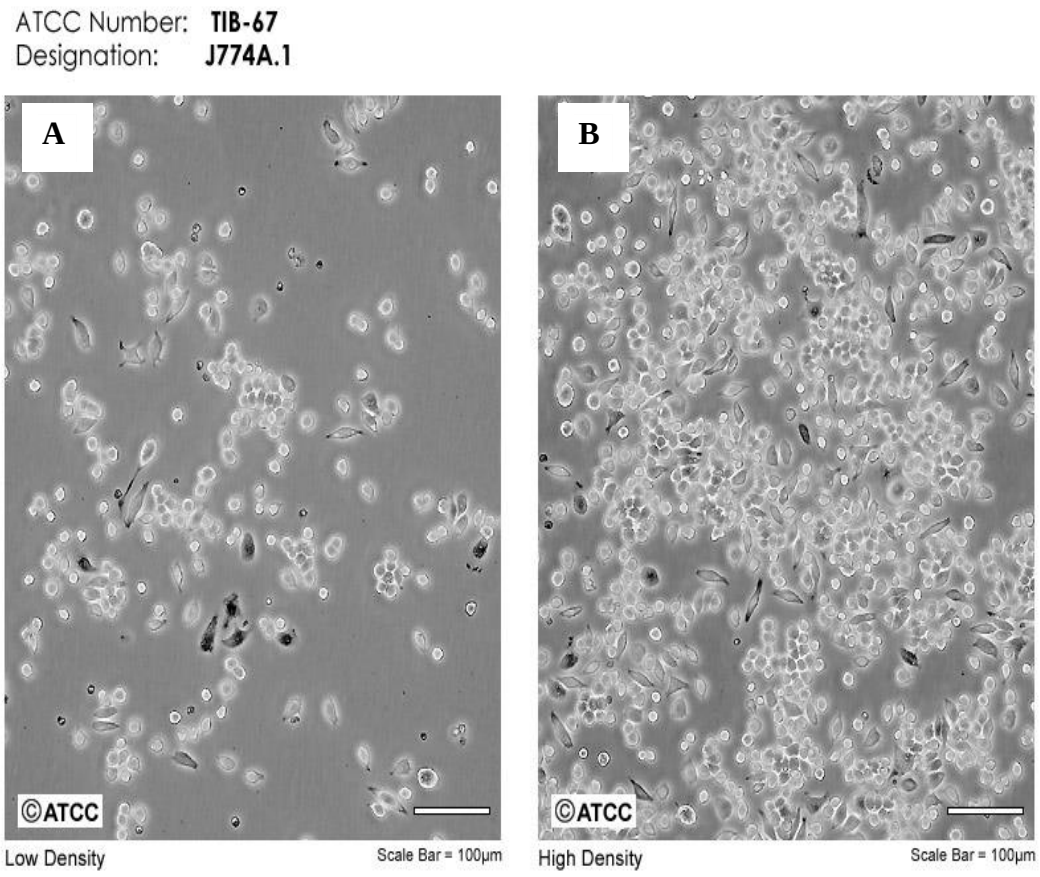


Figure 2.1 Morphology of macrophage (J774A.1). A: Low-density macrophage and B: High-density macrophage.

Source: ATCC Product Sheet J774A.1 (ATCC ® TIB-67^{TM}), (2015)

2.2.2 RAW 264.7 murine macrophage

These cells are macrophage-like, Abelson murine leukemia virus-transformed cell line derived from BALB/c mice and suitable as a transfection host. RAW 264.7 is smaller and more rounded phenotype with fewer cytoplasmic extensions (Chamberlain *et al.*, 2009), will pinocytose neutral red and will phagocytose latex beads and zymosan. This cell line is easy to propagate, high efficiency for DNA transfection, sensitivity to RNA interference, and supports replication of murine noroviruses. The morphology can be visualized in Figure 2.2.

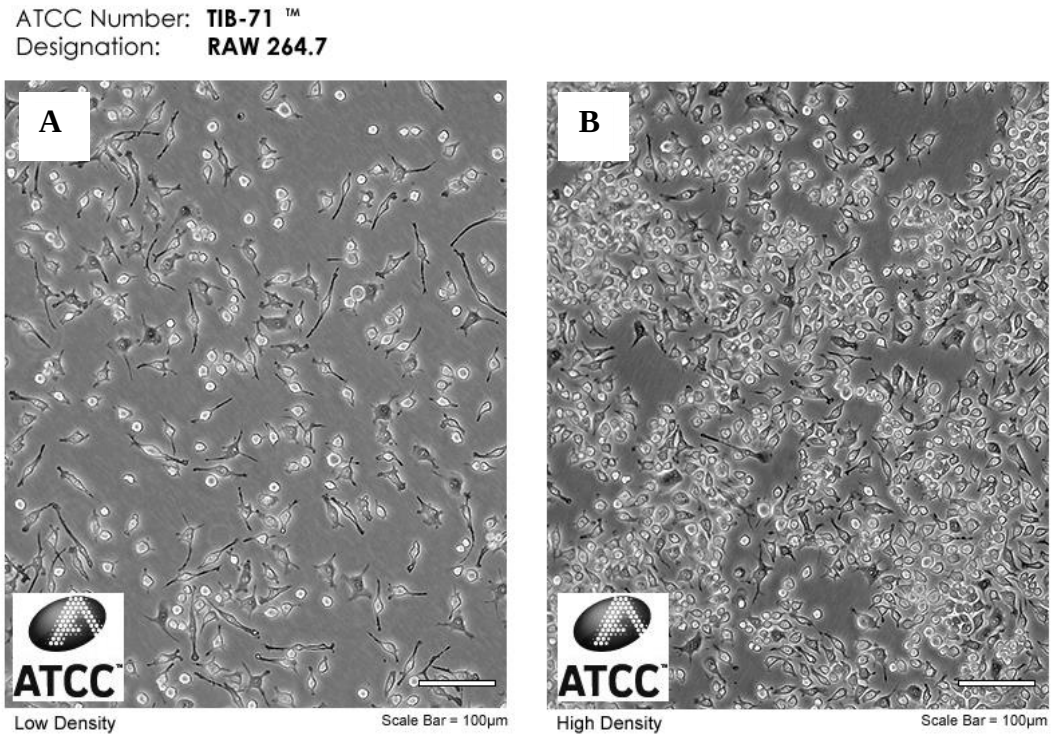


Figure 2.2 Morphology of macrophage (RAW 264.7). A: Low- density macrophage and B: High-density macrophage.

Source: ATCC Product Sheet RAW 264.7 (ATCC® TIB71™), (2017)

2.2.3 THP-1 human monocytic cell line

Human cell line THP-1 is a monocyte that derived from acute monocytic leukemia. This cell line is widely used to study monocyte/macrophage functions, mechanisms, signaling pathways, nutrient and drug transport which has become a common model to estimate modulation of monocyte and macrophage (Chanput *et al*. 2014) and also suitable to use as a transfection host. The morphology can be visualized in Figure 2.3.

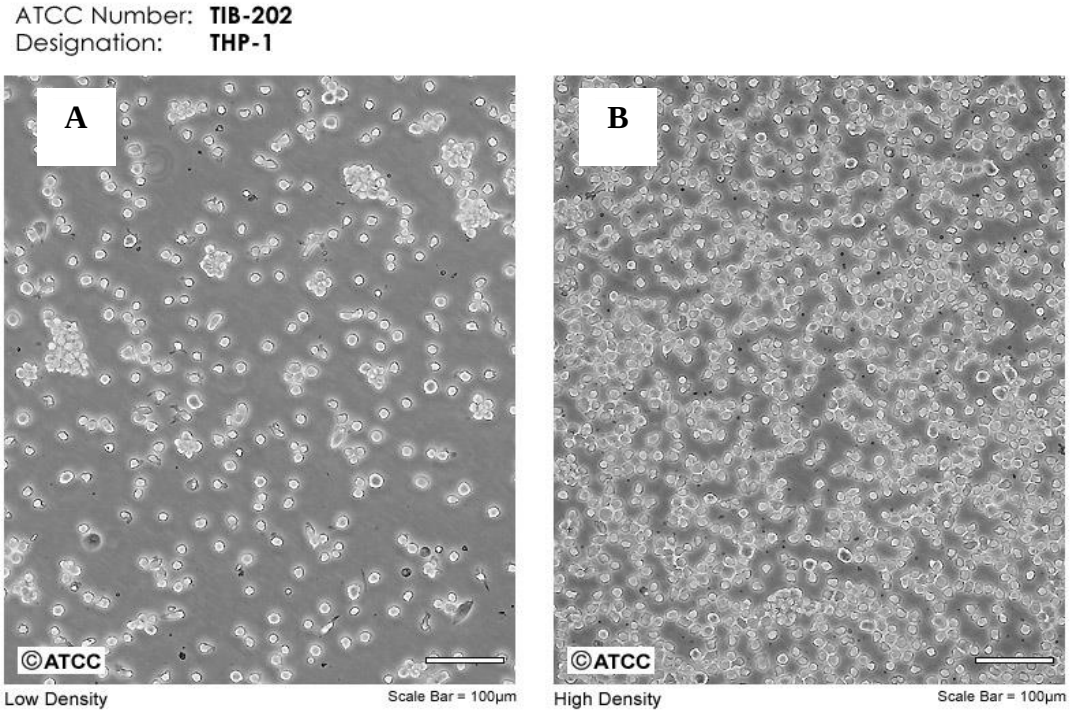


Figure 2.3 Morphology of monocytes (THP-1). A: Low-density monocytes and B: High-density monocytes

Source: ATCC Product Sheet THP-1 (ATCC® TIB-202™), (2017)

2.3 Immune system and proliferation of macrophages

Tissue macrophage goes through *in situ* proliferation in a different process in order to increase its population density. This process is being controlled by the representative TH2 cytokine interleukin-4 (IL-4) which a fundamental component of TH2 inflammation. Exogenous IL-4 was sufficient to drive the accumulation of tissue macrophages through self-renewal thus expansion of innate cells for pathogen control or wound repair does not depend on employment of potentially tissue-destructive inflammatory cells (Jenkins *et al.*, 2011).

The most commonly assay methods used to estimate the number of viable cells in multi-well plates is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT is based on the ability of metabolically active cells to reduce the yellow tetrazolium salt to form insoluble purple formazan crystals, which are solubilized by the addition of a detergent and quantified base on color (Harizi *et al.*, 2011). Although the MTT is considered to be a fast, simple and accurate *in vitro* method for investigation on the effect of different substances and nutrients, the test must be used with care because it's not correlated well with actual cell growth and viability (Lü *et al.*, 2012). It is an alternative to radiometric testing and is widely used in drug sensitivity or cytotoxicity studies.

2.4 Immune system and phagocytosis

Phagocytic removal of apoptotic cells is an important regulatory event in development, tissue homeostasis and inflammation during an immune response. The role of phagocytosis is played mostly by monocytes, macrophages, neutrophils and dendritic cells. Phagocytosis contributes to host defense and the pathogenesis of autoimmune, malignant, and metabolic disorders (Gordon, 2016).

Immune system boosts their phagocytic activity via activation of the macrophage. This action results in increased cell size, amplified production of lysosomal enzymes, enhanced cell metabolism, improved the ability to phagocytose and kill ingested unwanted particles such as pathogens (Fujiwara and Kobayashi, 2005). Microbes and dead cells could trigger inflammatory mediators to cause inflammation in the immune response. Therefore the host response of delivering leukocytes and plasma proteins to the site of infection or injury so that the offending agents could be removed or eliminated. The phagocytosis is equipped with lysosome which has anti-microbial substance such as lysozyme, nucleases, proteases, lipases and hydrogen peroxidase.

The phagocytic pathway begins when cells like macrophages engulf a solid particle to form an internal compartment known as a phagosome. The process of engulfment of solid particulate material by the macrophage occurs in a vacuole at early phagosome then it merges with a lysosome to form a phagolysosome. The pathogen is killed and digested in preparation for antigen presentation in this acidic, hydrolytic compartment (Aderem, 2003). The lysosome secretes different enzymes to lyse and digest to kill the invading microbes (see Figure 2.4) (Gordon, 2016).

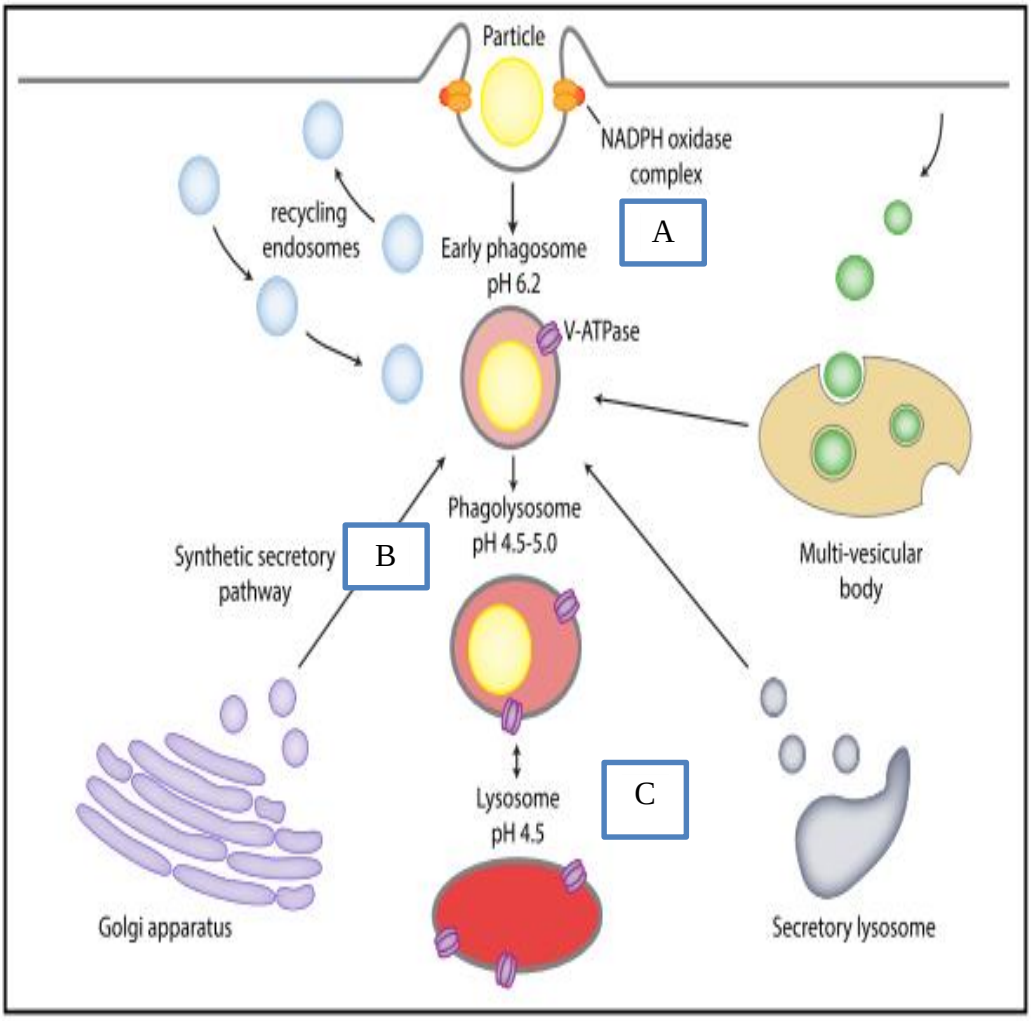


Figure 2.4 Phagocytic pathway. A: Early phagosome, B: Phagolysosome and C: Lysosome. Phagocytosis is the process which a phagocyte cell engulfs a solid particle to form an internal compartment known as a phagosome and develop phagolysosome with the secretion of enzymes by lysosome.

Adapted from: Gordon, (2016)

Q. infectoria gall extract show good anti-inflammatory in the previous study, thus support the activation of clearance of unwanted particles in the immune response. Flow cytometry analysis could be useful for detection of a wide range of particle sizes following uptake by macrophage in phagocytosis activity (Mutzke *et al*., 2015). A better understanding of how phagocytosis regulates immune responses will lead to the development of enhanced antigen-delivery tools for vaccines, new approaches for developing drugs that regulate inflammation and an improved understanding of how pathological inflammation develops during microbial infection and autoimmune diseases.

2.5 Immune system and cytokines

There are different types of cytokines, including chemokines, interferons, interleukins, lymphokines and tumor necrosis factor. Cytokines are cell signaling molecules that aid cell to cell communication in immune responses and stimulate the movement of cells towards sites of inflammation, infection and trauma. In formal with other mediators, cytokines rule macrophages into a spectrum of inflammation-promoting "classically activated," to anti-inflammatory or "alternatively activated" macrophages (Duque and Descoteaux, 2014).

Deregulated cytokine secretion is associated with several disease states ranging from chronic inflammation to allergy. During cell injury, infection, invasion, and inflammation; immune cells like monocytes, macrophages or non-immune cells like fibroblasts and endothelial cells released IL-1β (Zhang and An, 2009). Mostly, pro-inflammatory cytokines are produced by activated macrophages and involved in the up-regulation of inflammatory reactions.

Generally the sources of inflammatory cytokines are secreted from macrophages (Murray and Stow, 2014), which normally act in a protective manner. However, these same cytokines trigger many acute and chronic inflammatory diseases. In certain infection cytokines play an important role to contain infection, for example, IFN-γ produced by macrophages may tolerate for a quicker response to *Mycobacterium tuberculosis* (MTB) before T-cell specific immunity develops (Robinson *et al.*, 2010). The IFN-γ produced by macrophages may contribute greatly to effective macrophage responses that limit MTB growth.

2.6 Immune system and apoptosis

Apoptosis or programmed cell death are designed to eliminate the cells via a healthy process in the body. On the contrary, necrosis is a premature death of cells and living tissue caused by external factors such as infection, toxins or trauma. Apoptotic cell death is considered to be a toxic process where the cell is a passive victim and follows an energy-independent mode of death while necrosis refers to the degradative processes that occur after cell death thus become an inappropriate term to describe a mechanism of cell death (Elmore, 2007).

Cells require apoptosis to compensate for cell growth and balance the proliferation of new cells in the human system. In cancer, there is a loss of balance between cell division and cell death and cells that should have died did not receive the signals to do so (Wong, 2011). The problem can arise in any one step along the way of

apoptosis. Quantification of cell death using flow cytometry is widely used to features of apoptotic cells.

The benefit of using flow cytometry is it measures apoptosis by evaluating the binding of fluorescently-tagged annexin V, which is a probe for membrane phosphatidylserine (PS), in tandem with propidium iodide (PI) which enters cells with damaged membranes (Hollville and Martin, 2016). Restructuring of membrane PS early in apoptosis, apoptotic cells typically bind annexin V prior to PI uptake, although PI uptake does eventually occur during apoptosis accordingly with cells enter secondary necrosis. In contrast, necrotic cells typically exhibit annexin V binding and PI uptake simultaneously.

2.7 Immuno-modulatory and immunomodulator

A biological or synthetic substance that could stimulate, suppress or modulate any of the components of the immune systems including both innate and adaptive arms of the immune response (Mukherjee *et al.*, 2014). Any substances either extrinsic or intrinsic that have the ability to change immune response by modifying the scope, type, duration or competency of immune response (Lebish and Moraski, 1987) are immuno-modulators. In the presence of infection, cytokines will produce soluble proteins that ensure communication between immune and non-immune cells and will change the pathway from innate to adaptive immunity, thus promoting the inflammation, activating the anti-inflammatory response or macrophage.

Studies have demonstrated that plant extracts possess various biological activities including antitumor and immuno-modulatory activity. Medicinal plants can be

a modulator that provides an alternative to conventional chemotherapy for a variety of diseases, especially in the condition of the immune compromised host which has impaired immune response or similarly in autoimmune disorders when a selective immunosuppression is desired (Amirghofran *et al.*, 2009). Screening of 20 medicinal plants in Malaysia showed that methanolic extracts of *Alpinia galangal*, *Orthosiphon aristatus* and *Annona muricata* are able to modulate innate immune system had been demonstrated using CD18/11a expression and phagocytosis activity of leukocytes (Harun *et. al*, 2015). The immune-modulatory effect of a plant-derives substance can be a discovery of therapeutic agent for modulating the immune system.

The mechanism of immunomodulation is very complex (Figure 2.5) because it requires activation, proliferation and differentiation of immune response such as macrophage, T-helper cell, B-cell and others. Two major armies which are macrophages and lymphocytes have the ability to guard and activate cellular defense with the help of molecular detectors, such as TLRs (Spiering, 2015). Specific antibodies generated to the specific antigen or toxin is released to initiate the destruction of a foreign organism to contain the infections.

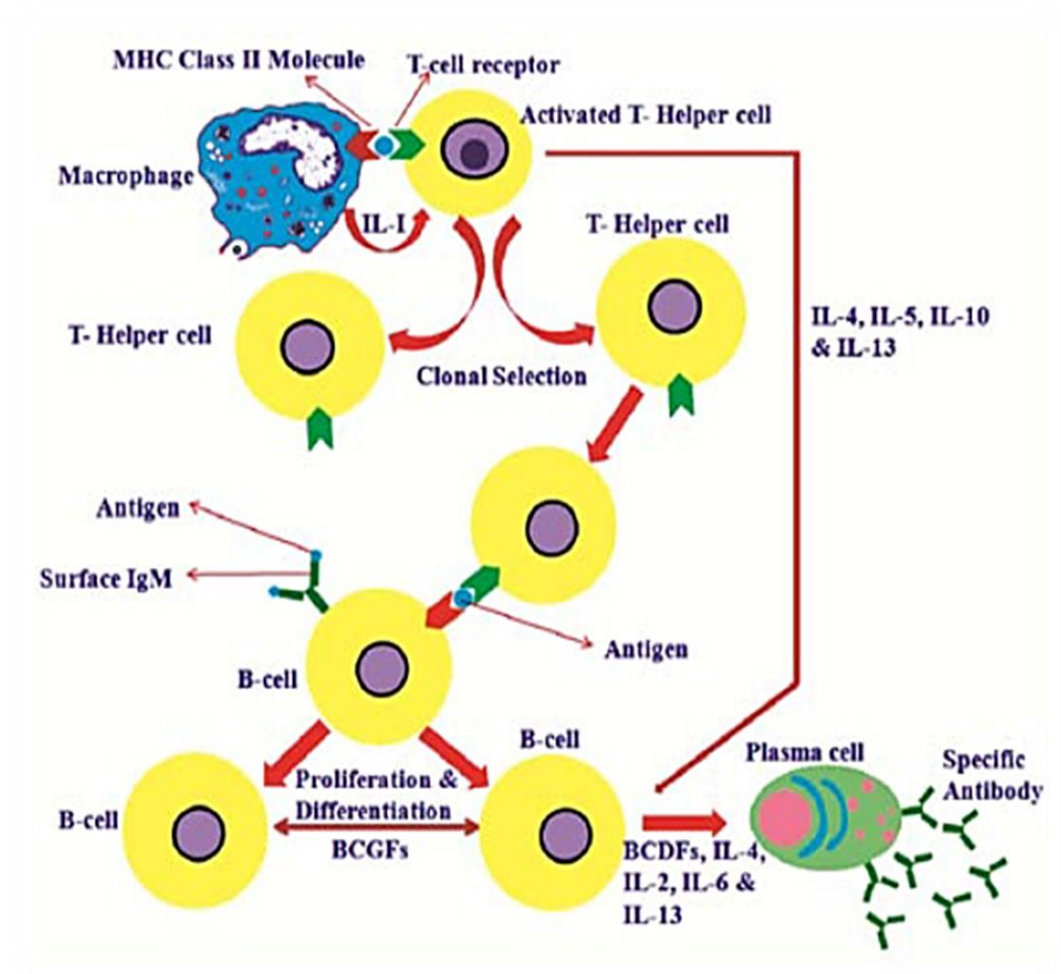


Figure 2.5 Mechanism of immunomodulation; antigen and antibody binding to specific receptor, proliferation and differentiation of immune cells with the regulations of cytokines or chemokines are the fundamental part in the immune system.

Adapted from: Mukherjee *et al.*, (2014)

2.8 Disease associated with immune system

Diseases associated with impairment of immune systems such as autoimmune disease like systemic lupus erythematous (SLE), diabetes type I, atopic dermatitis, arthritis, multiple sclerosis and inflammatory bowel disease become more challenging to clinicians. Autoimmunity conditions cause general other illnesses such as liver inflammation, kidney failure, and other organ deficiency.

Diabetes type I has become one of the important diseases that effects young people where this autoimmune disease targets the insulin secreting β cells of the pancreas make it lose the ability to normalize blood glucose level and other metabolites (Tooley *et al.*, 2012). While in atopic dermatitis, pathogenetic mechanism of it is complex which related to hypersensitivity that causes by genetic abnormalities, environmental factors, skin barrier defects and immune dysfunction (Nahm, 2015). Meanwhile, systemic lupus erythematosus (SLE) that is a heterogeneous autoimmune disease that are hard to be identified in the early phase and involve many organs such as liver, joint and kidney (Kuhn *et al.*, 2015). To diagnose this immune-related disease are very difficult and to gives precise treatment also very challenging. The individual immune condition differs from one and another and specific approach or personalized treatment is the best choices to be uncovered. Immunotherapy are the treatment that needs to be discovered with new and safe drug with low cost that could be widely available in many countries.