IMMUNOMODULATORY AND CELL CYCLE REGULATORY EFFECTS OF *CLINACANTHUS NUTANS*

WAN AFIQAH SYAHIRAH BINTI WAN GHAZALI @ WAN AZMI

UNIVERSITI SAINS MALAYSIA

August 2018

IMMUNOMODULATORY AND CELL CYCLE REGULATORY EFFECTS OF *CLINACANTHUS NUTANS*

by

WAN AFIQAH SYAHIRAH BINTI WAN GHAZALI @ WAN AZMI

Thesis submitted in fulfillment of the requirements

for the degree of

Master of Science

August 2018

ACKNOWLEDGEMENTS

First and foremost, I would like to praise Allah S.W.T for his uncounted blessings. I would like to express my deep gratitude to Associate Professor Dr. Thirumulu Ponnuraj Kannan and Associate Professor Dr. Nurul Asma Abdullah for their patient guidance, enthusiastic encouragement and useful critiques during my entire Master tenure. Thank you for your insight and wisdom in manuscripts and thesis. Nobody has been more important to me in pursuit of this research than the members of my family. I am extremely grateful to my parents, Wan Ghazali Wan Sulaiman, Wan Ainin Wan Hussin and my siblings for their unfailing love and support, mentally and spiritually throughout.

I wish to thank all my postgraduate friends from School of Dental Sciences and School of Health Sciences who helped me in my research work. Special thanks to my colleagues, A'attiyah, Hamshawagini, Wafa', Nurul Hafizah, Hii Siew Ching, Fatin Hazwani, Nur Hafizah, Nor Ainun Maziah, Najian, Nur Fathin Alia, Fuad, Nazihah, Hazrini and Faiz for the mental support during lab work. I am thankful that we could share our ups and downs together. The best kind of friends are those that enter your life and help you to see a blue sky where before there was a cloudy sky. They are the ones who have so much faith in you that you start having faith in yourself. These are the friends I should be grateful for. I would like to acknowledge the scholarship, MyBrain from Ministry of Higher Education, Malaysia, Universiti Sains Malaysia Research Universiti Grant (1001/PPSG/812182) and also Graduate Assistant Scheme from Universiti Sains Malaysia. Without these, it would not have been possible for me to pursue my Master program.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

°C	Degree Celsius
%	Per cent
µl	Microliter
µg	Microgram
ANOVA	Analysis of variance
ATCC	American type culture collection
B cells	Bursa of fabricius cells
C. nutans	*Clinacanthus nutans*
cDNA	Complementary deoxyribonucleic acid
cm^2	Centimeter square
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl Suphoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl-hydrate
EC_{50}	Half maximal effective concentration
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
HGF-1	Human gingival fibroblast-1
IC_{50}	Half maximal inhibitory concentration

IFN-γ	Interferon gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MDH	Malate dehydrogenase
Mg	Milligram
Ml	Milliliter
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase
NO	Nitric oxide
NK	Natural killer
OD	Optical density
PBS	Phosphate-buffered saline
PES	Phenazine ethosulfate
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
rpm	Revolutions per minute

Real time RT-PCR	Real-time polymerase chain reaction
T	Thymus
Th	T helper
TNF-α	Tumor necrosis factor alpha
TGF-β	Transforming growth factor beta
Tregs	T regulatory
UPS	Ubiquitin protease system

LIST OF APPENDICES

KESAN IMMUNOMODULATOR DAN PENGAWALAN KITARAN SEL TERHADAP *CLINACANTHUS NUTANS*

ABSTRAK

Kajian ini terdiri daripada analisis aktiviti proliferasi, pengujian nitrik oksida, sitokin dan ujian fagositosis untuk menilai sifat immunomodulator serta analisis ekspresi gen pengawalan kitaran sel. Turunan makrofaj murine sel (J774A.1) dirawat dengan pelbagai kepekatan (250, 125, 62.5, 31.25 and 15.625 µg/ml) ekstrak etanol dan akueus daun *C. nutans*. Ujian MTS menunjukkan pernambahan sel dengan nilai daripada EC_{50} antara 4.051 hingga 100.6 µg/ml. Ujian sitokin menunjukkan bahawa *C. nutans* mempunyai sifat antiradang dan meningkatkan pengeluaran nitrik oksida makrofaj dengan purata pengeluaran (41-130 µmol/L) yang dapat mengawal tindak balas keradangan. Peratusan pelingkungan FITC adalah antara 14.9 hingga 55.1%. Aktiviti fagositik menunjukkan peningkatan dalam peratusan pelingkungan FITC berkadar langsung dengan kepekatan ekstrak. Turunan sel fibroblast gingival manusia (HGF-1) telah dirawat dengan ekstrak daun *C. nutans* etanol dan akueus selama 1, 3 dan 7 hari. Analisis ekpresi gen menunjukkan pengawalaturan menurun *BCL-2* dalam ekstrak etanol pada hari 1 dan 7 dan pengawalseliaan pada hari ke 3 menunjukkan sifat apoptotiknya. *BAX* menunjukkan pengawalaturan menurun dalam ekstrak akueus pada hari 1 menunjukkan sifat apoptotik *C. nutans* dan pengawalaturan menaik *BAX* dalam kedua-dua ekstrak pada hari 1, 3 dan 7 menunjukkan sifat-sifat apoptosisnya. Pengawalaturan menurun *C-MYC* pada hari 1 boleh mengakibatkan penurunan jumlah sel disebabkan oleh perencatan dalam pertumbuhan sel. Pengawal aturan *C-MYC* dalam kedua-dua ekstrak di kesemua hari boleh merencat isyarat untuk kelangsungan hidup dan kematian.

Pengawalaturan menaik *CDK1* dalam ekstrak etanol pada hari ke 7 dan ekstrak akueus pada hari ke 3 dan 7 menunjukkan bahawa *C. nutans* dapat mengawal perkembangan kitaran sel dan menggalakkan mitosis. Terdapat pengawalaturan menurun *SUMO-1* dalam ekstrak etanol pada hari 1, 3 dan 7 dan dalam ekstrak akueus pada hari ke 1 dan 3 yang mengakibatkan kegagalan *SUMO-1* untuk konjugat dengan protein sel. Terdapat pengawalaturan menaik *SUMO-1* dalam ekstrak akueus pada hari 1, 3 dan 7 dan pada hari 1 dan 3 dalam ekstrak etanol. Keputusan kajian ini menyokong peranan *C. nutans* ekstrak dalam mengawal kitaran sel proses selular seperti apoptosis, kestabilan protein, transkipsi dan pengangkutan nuklear dengan merubah ekspresi gen *BCL-2*, *BAX*, *C-MYC*, *CDK1* dan *SUMO-1*, dengan itu dapat mengawal aktiviti kitaran sel.

IMMUNOMODULATORY AND CELL CYCLE REGULATORY EFFECTS OF *CLINACANTHUS NUTANS*

ABSTRACT

This research comprises analyses of proliferative activity, nitric oxide, cytokine and phagocytosis assays to evaluate its immunomodulatory properties as well as gene expression analysis of cell cycle regulatory genes. Murine macrophage cell line (J774A.1) was treated with various concentrations (250, 125, 62.5, 31.25 and 15.625 µg/ml) of aqueous and ethanol extracts of the leaves of *C. nutans*. MTS assay showed enhanced cell proliferation with an EC_{50} range between 4.051 to 100.6 µg/ml. Cytokine assay revealed that *C. nutans* possesses anti-inflammatory properties and increases the nitric oxide production in macrophages with a mean of production (41-130 µmol/L) that could modulate the inflammatory response. Percentage of latex beads IgG-FITC engulfment ranged between 14.9 and 55.1%. Phagocytic activity showed increase in percentage of latex beads IgG-FITC engulfment as the concentration of the extract increased. Human gingival fibroblast cell line (HGF-1) was treated with ethanol and aqueous extracts of *Clinacanthus nutans* (*C. nutans*) leaves for 1, 3 and 7 days. Gene expression analyses showed that *BCL-2* was down-regulated in ethanol extract on day 1 and 7 indicating an anti-apoptotic effect and an up-regulation on day 3 indicating its apoptotic property. Down-regulation of *BAX* in aqueous extract on day 1 indicated the apoptotic nature of *C. nutans* and up-regulation of *BAX* in both the extracts on all days showed its pro-apoptotic properties. Down-regulation of *C-MYC* in aqueous extract on day 1 could result in decrease in the number of cells due to arrest in growth. Up-regulation of *C-MYC* in both the extracts at all days could inhibit signals for death and survival. Up-regulation of *CDK1*

in the ethanol extract on day 7 and aqueous extract on day 3 and 7 showed that *C. nutans* could regulate cell cycle progression and promote mitosis. There was down-regulation of *SUMO-1* in ethanol extract on day 1, 3 and 7 and on day 1 and 3 in aqueous extract which results in the failure of *SUMO-1* to conjugate to cellular proteins. There was up-regulation of *SUMO-1* in aqueous extract on day 1, 3 and 7 and on day 1 and 3 in ethanol extract. The results of this study support the role of *C. nutans* extracts in regulating cellular processes such as apoptosis, protein stability, transcription and nuclear transport by modulating the gene expression of *BCL-2*, *BAX*, *C-MYC*, *CDK1* and *SUMO-1* thereby regulating cell cycle activity.

CHAPTER 1

INTRODUCTION

1.1 Research background

Plants have formed the basis of conventional medicinal systems thousands of years ago. Humans have depended on nature for their basic needs, for instance, medicines, fertilizers, clothing, flavors and fragrances, foodstuffs, transportation and shelter (Cragg and Newman, 2005). Continuous discovery of medicinal plant drug aims to give the latest and important lead towards many pharmacological targets like cancer, Human Immunodeficiency Virus (HIV)/Acquired Immune Deficiency Syndrome (AIDS), Alzheimer's, malaria, and pain (Balunas and Kinghorn, 2005). The medicinal value of the plant is derived from the chemical substances that contribute to the effect on the human body. These chemical substances consist of alkaloids, tannins, flavanoids and phenolic compounds. In addition to its medicinal value, medicinal plants are also used as spices and food.

Medicinal plants are composed of vital components of flora. Most of the medicinal plants are universally dispersed in India. According to Krishnaraju and colleagues, our mother earth boosts increasing attention towards the importance of medicinal plants and traditional health systems in solving health care problems (Krishnaraju *et al.*, 2005). Thus, research on plant's medicinal benefit is thriving phenomenally at the global level. Historically, all medicinal preparations were of plant origin, whether it was from the

simple form of raw plant materials or in the refined form of crude extracts, mixtures etc. (Farnsworth and Soejarto, 1991). To prove the effectiveness of medicinal plants, various medicinal herbs need to be extracted to get bioactive compounds which form the vital factor. Bioactive compound extraction efficiency is influenced by several factors like solvent polarity and concentration, solvent-to-feed ratio, extraction time and thermal degradation (Mustapa *et al.*, 2015).

Numerous medicinal plants can be found in Malaysia that possess high medicinal values. One of the medicinal plants of interest is Sabah Snake Grass with the scientific name *Clinacanthus nutans* (*C. nutans*) which comes from the family *Acanthaceae*. It is a small shrub native to tropical Asia. In Malaysia, the fresh leaves of this plant are used as herbal tea where the leaves are boiled with water. This plant is a very well known medicinal plant in Thai folklore medicine. Traditionally, it has been used in treating inflammation and viral infections (Charuwichitratana *et al.*, 1996; Yoosook *et al.*, 1999; Wanikiat *et al.*, 2008).

The biological or pharmaological effects of compounds is a immunomodulatory activity either on humoral or cellular aspects of the immune response (Wagner, 1990). In order to maintain a disease-free state, modulation of immune respose either through suppression or stimulation is necessary (Ghule *et al.*, 2006). Apart from being particularly suppressive or stimulatory, there are certain agents which have been shown to modulate pathophysiological process thus marking as immunomodulatory agents (Bafna, 2005). There is a huge possibility to discover more specific immunomodulators that imitate the

biological impact of cytokines and interleukins in natural medicinal herbs. Besides, it is the need for an integrated, systematic research on standardized products of a large number of plants with the aim of developing commercially viable phytomedicines (Hussain *et al.*, 2013).

Cell cycle phases play an important role in cell division and inappropriate cell division can cause cancer. Each gene and protein has influence on the different phases of cell cycle in cell division (Zhaohua Tang and Hickey, 2014). A research done by Teoh and colleagues reported apoptosis in MCF-7 cell line induced by the down-regulation of *BCL-2* treated with *C. nutans* leaves (Teoh *et al.*, 2017). It is clearly shown that each gene plays a vital role in cell cycle regulation. Therefore, the current study envisaged on the immunomodulatory and cell cycle regulatory effects on *C. nutans* leaves which will help in exploration on the potentials of this herb.

The research aim was to assess the proliferative activity of murine macrophage cell line when treated with with the extracts of *C. nutans* leaves. Besides, current study will find out the ability of *C. nutans* in modulating the immune parameters like nitric oxide, cytokines and phagocytosis in normal condition. Also, the research aims to analyse the expression of cell cycle regulatory genes on human gingival fibroblast (HGF-1) cell line.

1.2 Problem statement

C. nutans has been used in traditional medicine, but the full scientific potential has not been explored yet. Even though *C. nutans* has been documented to possess antimicrobial, antiviral and antioxidant scavenging properties, yet, much is not known on its immunomodulatory effects which necessitates the current research to be undertaken. Furthermore, information on the gene expression analysis of cell cycle regulatory genes related to *C. nutans* treatment on cell line is still lacking except for a previous study on *BAX* and *BCL-2*. Moreover, an earlier study focused on the effect of *C. nutans* in determining the apoptotic effect and not on the cell cycle regulation.

1.3 Justification of study

Immunomodulators are becoming well known worldwide because it is a substance that helps our body to regulate the immune system and have a tendency to normalize our immunity. It is crucial to study the immunomodulation since it is an immunological change in the body which can control the infections and other adverse health effects. Apart from that, it is important to understand the fundamental mechanism towards cell cycle and to comprehend its effect on the regulation of cell cycle since uncontrolled cell growth is one of the aetiologies of a cancerous cell. These things remain unanswered until now pertaining to the effect of *C. nutans* leaf extracts which implores the necessity to undertake the current research that will unfurl the potential of *C. nutans* to be exploited in future.

1.4 Objective

1.4.1 General objective

To study the effect of *Clinacanthus nutans* in immunomodulation and cell cycle regulation.

1.4.2 Specific objectives

1 To determine the cell proliferation of murine macrophage cell line treated with *C. nutans* using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay.

2 To assess the immunomodulatory properties of *C. nutans* using cytokine, nitric oxide and phagocytosis assays on murine macrophage cell line (J774A.1).

3 To determine the expression of cell cycle regulatory genes treated with *C. nutans* on human gingival fibroblast cell line (HGF-1).

1.5 Research hypothesis

C. nutans possesses immunomodulatory properties and regulates cell cycle regulatory genes.

CHAPTER 2

LITERATURE REVIEW

2.1 *Clinacanthus nutans*

The nomenclature and taxonomic classification of *C. nutans* are as below (Yahaya *et al*., 2015).

Plantae Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Lamiales

Family: Acanthaceae

Genus: *Clinacanthus*

Species: *nutans* - *Lindau*

This herb is extensively grown in tropical Asia and has been used as valuable medicinal herb in Malaysia, Thailand and China (Shiuan *et al.*, 2012). Generally, *C. nutans* can be found distributed throughout the tropical regions, China and Southeast Asia. *C. nutans* is a tall, growing up to 1 m in height and erect herbaceous perennial shrub. This plant is mainly native to Malaysia, Thailand and Indonesia (Nesheim *et al.*, 2006; Arullappan *et al.*, 2014). As it is found in Sabah of East Malaysia, *C. nutans* is well known with the name of "Sabah snake grass". Alternatively, in Bahasa Melayu, it is known as Daun Belalai Gajah (elephant's trunk) because of its slightly curved stem supporting the leaves

that looks like the curve of an elephant's trunk (Shim *et al.*, 2013). Figure 2.1 shows *C. nutans* whole plant, leaves and stem with leaf.

Figure 2.1: *Clinacanthus nutans* A. whole plant B. stem with leaves C. leaf

2.2 *Clinacanthus nutans* as potential herbal

Malaysia has more than 23, 000 recorded plant species that makes the country known as one of the most bio-diverse countries on the globe (Aman, 2006). Overall, each component of the plant is conventionally used in medicine, fragrance as well as for flavor in food. Apart from the interest targeting the properties in *C. nutans*, it was chosen based on 12 National Key Economic Areas (NKEAs). Malaysian government has highlighted a few potential herbs during the launching of the Economic Transformation Programme (ETP). Entry Point Project 1 (EPP1) is one of NKEA-identified areas where it focusses on high value herbal products. Initially, EPP only focussed on five local herbs, namely, Tongkat Ali (*Eurycoma longifolia* Jack), Misai Kucing (*Orthosipon aristatus* (Blume) Miq.), Hempedu Bumi (*Andrographis paniculata* (Burm.f.) Nees), Dukung Anak (*Phyllanthus niruri* L.) and Kacip Fatimah (*Marantodes pumilum* (Blume) Kuntze (*syn. Labisia pumila* (Blume) Mez). Later, six more herbs were added to the EPP. The six herbs include Mengkudu (*Morinda citrifolia* L.), Roselle (*Hibiscus sabdariffa* L.), Ginger (*Zingiber officinale*), Mas Cotek (*Ficus deltoidea* Jack), Belalai Gajah (*Clinacanthus nutans* (Burm.f.) Lindau) and Pegaga (*Centella asiatica* (L.) Urb) (Hashim *et al.*, 2016).

2.3 History of Sabah snake grass

C. nutans or well known as Sabah snake grass is a medicinal plant belonging to the family *Acanthaceae* that widely grows in the tropical region, mainly in Southeast Asia. Traditionally, *C. nutans* is used as a herbal medicine for treatment of *Herpes* infection, insect and snake bites as well as allergic responses (Tuntiwachwuttikul *et al.*, 2004; Sakdarat *et al.*, 2009). In Thailand, the plant has been authorized as a crucial medicinal

herb for fundamental healthcare by the Ministry of Public Health (Wanikiat *et al.*, 2008). Besides, *C. nutans* has been reported to be successfully used as a cream or lotion for the relief of minor skin inflammation and insect bites and in the treatment of patients with genital *Herpes* and *Varicella-zoster* lesions.

Many studies on *C. nutans* have found that drying techniques are vital to prepare and preserve the sample. Besides, drying enriches the quality of dried yield like aroma and physical appearance by delaying any biochemical changes (Rabeta and Lai, 2013). Furthermore, in Thailand, the leaves are mixed with other juices (apple, green tea or sugarcane) or served as fresh drink or refreshing drink and also consumed as raw vegetable (Shim *et al.*, 2013). In Malaysia, *C. nutans* is consumed by blending the leaves of the grass and served as normal juice. This has created a rapid growing market for herbal-based products like 'Sabah snake grass tonic' (Yahaya *et al.*, 2015).

2.4 Compounds in *Clinacanthus nutans*

Wanikiat and colleagues reported the presence of flavonoids like belutin, isovitexin, vitexin, isomollupentin-7-o-b-glucopyranoside, shaftoside, orientin and isoorientin in *C. nutans* (Wanikiat *et al.*, 2008). Besides, other chemical compounds were also reported such as glycoglycerolipids, glucosides, cerebrosides, sterols and pheophytins (Teshima *et al.*, 1998; Sakdarat *et al.*, 2009). Leaf samples were said to possess more phytochemicals that exhibited glucosidase and antioxidant activities. The leaf samples also contained high amount of carbohydrates that could probably act as one of the barriers for the stem extracts to have a low glucosidase and antioxidant activities (Khoo *et al.*, 2015).

2.5 Immunity

Immunity is the term derived from the Latin word *immunitas*. *Immunitas* in Latin refers to Roman senators during their tenures in office. According to the historical theory, immunity can be defined as protection from disease; also, can be specified as protection from infectious disease. On the other hand, cells and molecules are the ones responsible in immunity and constitute the immune system. Immune response properties are collective and coordinated response to the introduction of foreign substances (Abbas *et al.*, 2014). The immune response of diverse species to a given stimulus differs considerably. The *in vitro* evaluation of immunomodulatory properties of test compounds hence prompts the use of human cells (Hartung *et al.*, 1995).

The leaves are used as immunomodulatory, adaptogenic, diuretic, toothpaste, lithoritic, antiscorbitic, sailagogine, antibacterial, tonic and digestive (Yoganarasimhan and Jadhav, 1996). The leaves contain alkaloids, carbohydrates, pungent amide tannins, steroids, carotenoids, provitamin A, α-carotene and β-carotene, essential oils, sesquiterpenes, and amino acids (Nakatani and Nagashima, 1992). In spite of immunomodulatory agents, plant and animal origin reinforce the immune receptivity of the body against pathogens by activating basically the wide-ranging immune systems like stimulation of the function and efficiency of macrophages and other complement (Rajesh *et al.*, 2011).

2.5.1 Types of immunity

Immunology is the study of immune responses in a wider sense; also, it comprises of the study of molecular and cellular events that occur after an organism confronts other foreign macromolecules and microbes (Abbas *et al.*, 2014). Immunity basically can be divided into two types; innate and adaptive/acquired immunity as shown in Figure 2.2.

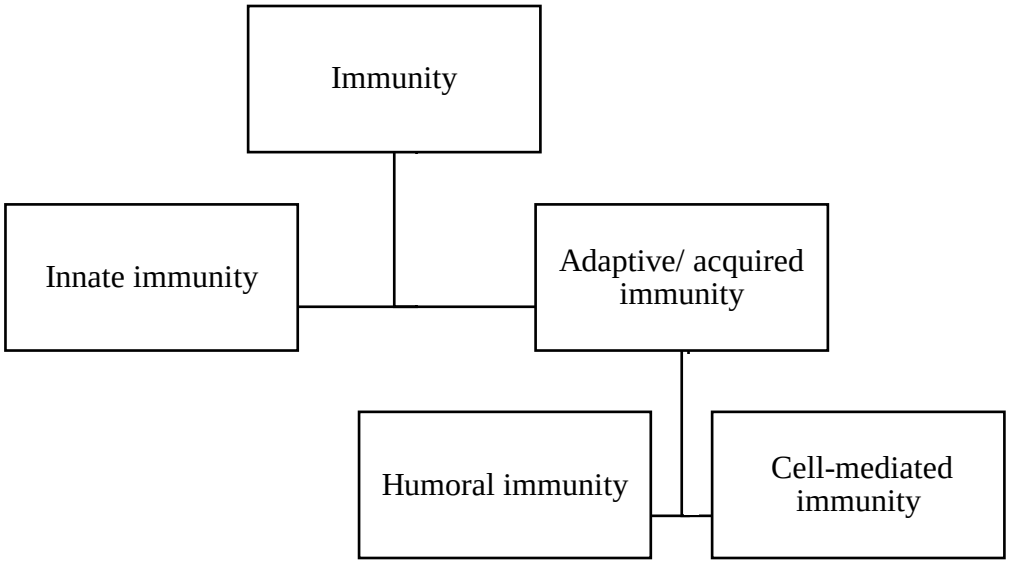

Figure 2.2: Types of immunity

Innate immunity also known as native or natural immunity provides first line of defense against microbes. It consists of two types of defense mechanisms; biochemical and cellular. Innate immunity consists of three major principal components; firstly, chemicals and physical barriers like antimicrobial and epithelial chemicals that are produced at epithelial surface. Secondly, dendritic cells, phagocytic cells (i.e. neutrophils, macrophages) and natural killer (NK) cells and other innate lymphoid cells. Thirdly, blood

proteins which comprise members of the complement systems and also other mediators of inflammation (Abbas *et al.*, 2014).

On the contrary, adaptive immunity is stimulated by exposure to infectious agents. Adaptive immunity consists of two types of different functions as stated in figure 2.2. Humoral immunity is resolved by molecules in mucosal secretion and blood, known as antibodies. These antibodies are products from cells known as B lymphocytes or B cells (Bursa of fabricius cells). However, cell-mediated immunity which is also known as cellular immunity is mediated by T (Thymus) lymphocytes or T cells (Abbas *et al.*, 2014). The mechanism on how long both types of immunity take place is shown in figure 2.3.

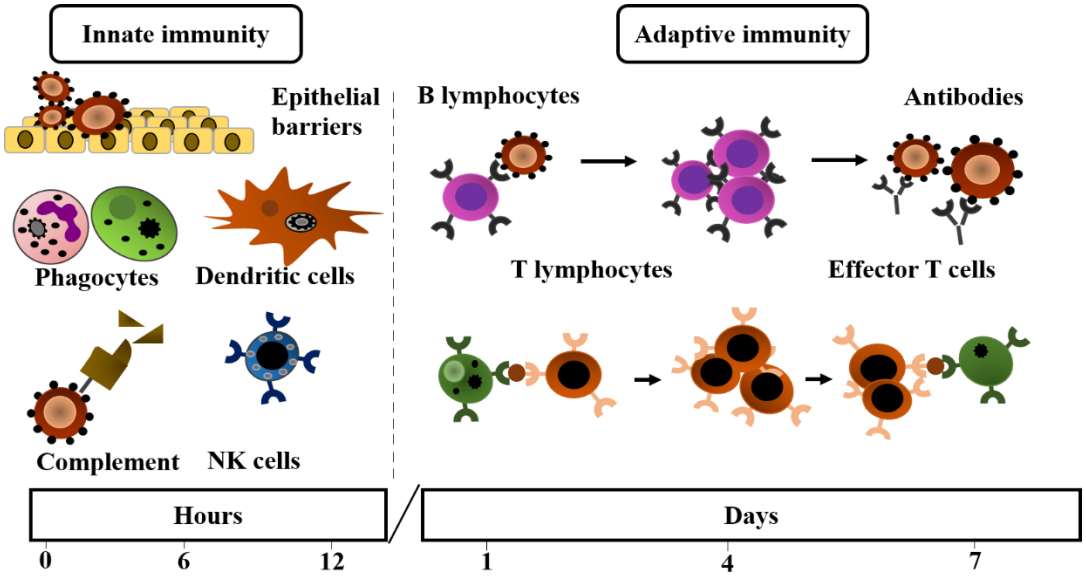

Figure 2.3: Mechanism of innate and adaptive immunity. Innate immunity gives the early defense against infections while adaptive immunity develops after the activation of lymphocytes

Disclosing the body to an antigen is known as active immunity which can generate an adaptive immune response. To develop the immune response, it may take days/weeks. Natural or acquired is considered as active immunity. Normally, a lifelong protection will occur when exposed to wild infection like hepatitis A virus (HAV) and a subsequent recovery gives rise to a natural active immune response.

On the other hand, the process of contributing IgG antibodies to protect against infection is passive immunity. Contrary with active immunity, passive immunity gives immediate but short-lived protection (i.e. several weeks to 3 or 4 months). Generally, passive immunity can be classified as acquired or natural immunity (Baxter, 2007).

2.5.2 Cytokines

Large group of secreted proteins are known as cytokines. Cytokines possess various structures and functions that can control and coordinate a lot of activities of the cell of innate and adaptive immunities (Abbas *et al.*, 2014). It consists of family of proteins like chemokines, interleukins, lymphokines, monokines and interferons. Family of cytokine is a vital component of immune system (Tayal and Kalra, 2008). Cytokines play a vital role to maintain inflammatory conditions and lymphocyte homeostasis under both steady states. Autoimmunity which occurs during inflammation can cause excessive tissue damage due to unregulated lymphocytes in steady state conditions (Sanjabi *et al.*, 2009).

The immune cells and tissues will return to their homeostatic state when infection is cleared. To clear the infection, our body needs a healthy immune system. Thus, our body

maintains the tolerance against auto reactive T cells and commensal bacteria under steady state conditions to achieve healthy immune system. Besides, it will clear the infection by mounting an effective immune response against foreign antigens (Sanjabi *et al.*, 2009). Various pathological disorders will occur due to imbalance in cytokine production of cytokine receptor expression or known as dysregulation of cytokine process. Many cell populations produce cytokines; however, macrophages and T cells (Th) are predominant producers (Tayal and Kalra, 2008). The functions of cytokines are listed in table 2.1.

Table 2.1: Cytokines and their functions (Abbas *et al*., 2014)

Cytokines	Functions
Interleukin-2 (IL-2)	Involves in proliferation of T cells, Natural killer cells (NK), and B cells
	Promotes T cell development and involves in differentiation
	Synthesis of antibody in B cells
Interleukin-4 (IL-4)	T cells involve in differentiation and proliferation of Th2
	Isotype switching to IgE in B cells
	Alternative activation and inhibition of IFNγ in macrophages
Interleukin-5 (IL-5)	Activator of eosinophil
	Produces IgA (*in vitro*) and involves in proliferation of B cells
Interleukin-6 (IL-6)	Synthesis of acute-phase proteins in liver
	In B cells, it involves in proliferation and antibody-producing cells
Interleukin-10 (IL-10)	Macrophage and dendritic cells inhibit IL-12 expression and production of co-stimulators
Interleukin-12 (IL-12)	Th1 differentiation occurs in T cells
	Both NK cells and T cells involve in IFNγ synthesis and increase cytotoxic activity
Interleukin-13 (IL-13)	Isotype switching to IgE in B cells
	Increases mucus production in epithelial cells
	Increases collagen synthesis in fibroblast
	Acts as an alternative activation in macrophages
Interleukin-17A (IL-17A)	Increases cytokine production in macrophages and chemokine production in both macrophages and endothelial cells
Interleukin-23 (IL-23)	Involves in expansion and differentiation of Th17 in T cells
Interferon-γ (IFN-γ)	Increases microbicidal functions in macrophages (classical activation)
	Th1 differentiation in T cells
Tumor necrosis factor-α (TNF-α)	Activation of inflammation and coagulation in endothelial cells
Transforming growth factor-β (TGF-β)	Inhibition of activation also stimulation of angiogenic factors in macrophages
	Inhibition of proliferation and effector functions; also differentiation of Th17 and T regulatory (Tregs) in T cells

2.5.2(a) Pro-inflammatory and anti-inflammatory cytokines

Cytokines play role as regulators of host responses to immune responses, infection, inflammation and trauma. Pro-inflammatory activity occurs when cytokines act to make disease worse. On the contrary, anti-inflammatory effect will promote healing and reduces inflammation. Anti-inflammatory effects are also applied by several of endogenous agents like soluble and membrane –bound IL-1 decoy receptors, IL-1 receptor antagonist and soluble TNF decoy receptors (Opal and DePalo, 2000). Hence, the number of anti-inflammatory substances may potentially exhibit therapeutic effects (Milligan *et al.*, 2005). IL-10 has been classified as the most powerful anti-inflammatory cytokine due to its function in down-regulating TNF, IL-1 and IL-6 production and release. Moreover, IL10 can also down-regulate pro-inflammatory cytokine receptor and also up-regulate endogenous anti-cytokines (Opal and DePalo, 2000). Pro-inflammatory cytokines that are produced in response to bacteria and fungi stimulate the development of Th17 (Abbas *et al.*, 2014). Cytokines are grouped as anti-inflammatory and pro-inflammatory (Cavaillon, 2001) (Figure 2.4).

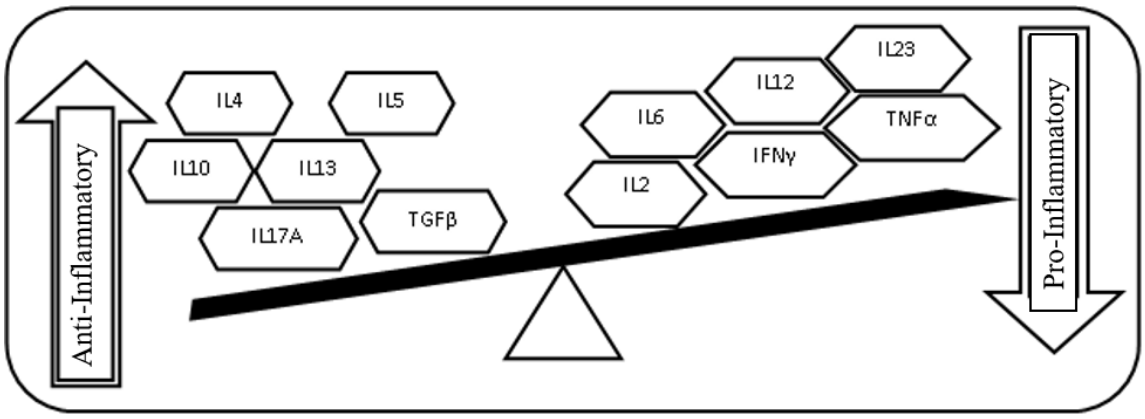

Figure 2.4: Classification of anti-inflammatory and pro-inflammatory cytokines. (Cavaillon, 2001)

2.5.3 Nitric oxide production

Nitric Oxide (NO) is derived during the oxidation by nicotinamide adenine dinucleotide phosphate oxidase (NADPH)-dependent enzyme NO synthase towards terminal guanidine nitrogen atom of L-arginine (Moncada *et al.*, 1991). NO is vital in many physiological pathways in the body, but, reactivity of NO gives it the potential that causes considerable damage to tissues and cells in its vicinity. Likewise, capacity of NO to mediate the tumoricidal and bactericidal actions of macrophages was one of its first established roles (Hibbs *et al.*, 1988). In addition, increase of NO production in macrophage is important in modulating the inflammatory response (Ohshima and Bartsch, 1994). Figure 2.5 shows the mechanism of NO production where macrophages produce reactive nitrogen species i.e. NO. NO is produced by the action of inducible nitric oxide synthase enzyme (iNOS). iNOS catalyzes the conversion of arginine to citrulline and citrulline finally lead to production of NO gas (Abbas *et al.*, 2014).

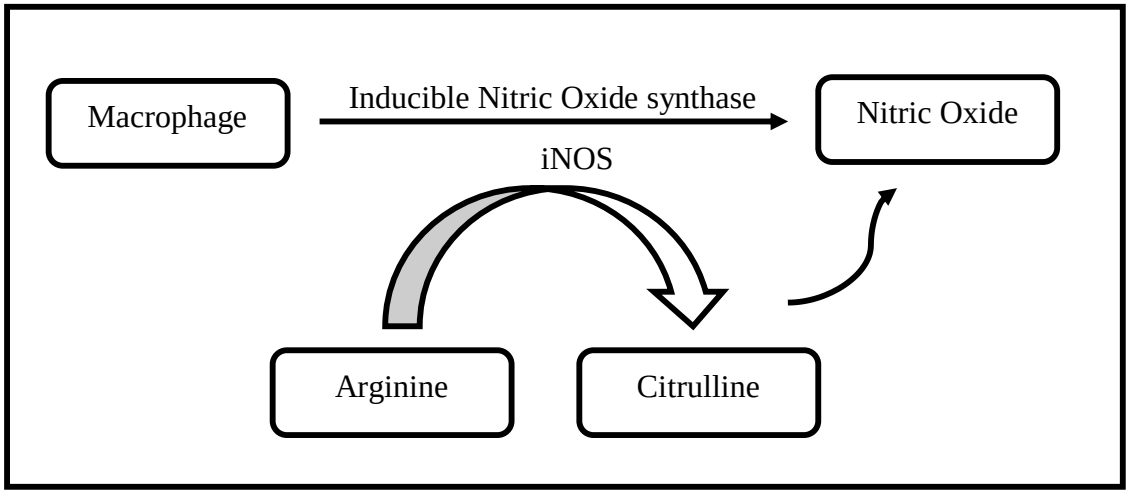

Figure 2.5: Mechanism of nitric oxide production (Abbas *et al.*, 2014)

2.5.4 Phagocytosis

Phagocytosis is a process of engulfment of large particles (>0.5 μm in diameter) into vesicles. It is energy-dependent and active process (Abbas *et al.*, 2014). Generally, phagocytosis occurs by sequential interactions between opsonic ligands and macrophage surface receptors on surface particles. Phagocytosis continues as zipper like engagement between the particle surface and macrophage membrane with the help of pseudopod advance (Araki *et al.*, 1996).

Innate immune response begins when phagocytosis of pathogens by macrophages lead to adaptive response. To obtain nutrients, lower organisms primarily use phagocytosis. Besides, in Metazoa, phagocytosis usually occurs in phagocytic cells like macrophages and neutrophils (Aderem and Underhill, 1999). Hence, phagocytosis by macrophages is important for degradation and uptake of senescent cells and infectious agents. Besides, it is also important due to involvement in development, the immune response, tissue remodeling and inflammation (Aderem and Underhill, 1999).

Corradin *et al*., claimed that phagocytosis with the ingestion of inert particles like latex beads could shift the effect of LPS in the induction of NO synthase activity in murine macrophages (Corradin *et al.*, 1991). In addition, combination of latex beads with Interferon- γ (IFNγ) can induce NO synthesis (Corradin *et al.*, 1991).

Ingestion and killing of microbes occur by activated phagocytes. There are many receptors of phagocytes that are responsible in microbes binding. Microbes may be ingested by

various membrane receptors of phagocytes. Some directly bind to microbes while others bind to opsonized microbes. Microbes that enter phagosomes which fuse with lysosome will form phagolysosomes where the microbes are killed by nitrogen species, proteolytic enzymes and reactive oxygen species (ROS) (Abbas *et al.*, 2014).

2.5.5 Immunomodulation

Immunomodulation is an immunological change in the body. Usually, drugs are immunomodulatory agents that may be an immunostimulator or immunosuppressant according on its effect on the immune system. Besides, immunomodulation is one of the vital alternatives to control diseases (Chauhan *et al.,* 2001). Medicinal plants and their products have been used to modulate the immune functions. It is a possible therapeutic measure and has become an accepted therapeutic approach. Apart from that, plants and minerals have been used since ancient times for treatment of many diseases and ailment. However, immunomodulation of immune response in medicinal plants are able to implement an alternative to conventional chemotherapy for a various of diseases (Singh *et al.*, 2016). In addition, previous study showed *C. nutans* role in modulating cell-mediated immune response on human immunocompetent cells (Sriwanthana *et al.*, 1996). Besides, *C. nutans* has also been used as an anti-inflammatory agent (Alam *et al.*, 2016).

2.5.6 Immunomodulatory activity of *Clinacanthus nutans*

A previous study reported the anti-inflammatory and immunomodulatory activities of *C. nutans* ethanol extract. According to their findings on anti-inflammatory activity, at 10 μg/ml of ethanol extract, the strongest elastase release inhibitory effect of 68.33% was observed. Also, based on immune-modulating finding, 0.1 μg/ml of 80% ethanol extract showed up-regulation of IFN-γ. However, down-regulation of IFN-γ exhibiting immunomodulatory activity occurred when a high concentration of 80% ethanol extract (100 μg/ml) was used (Tu *et al.*, 2014).

2,2-diphenyl-1-picrylhydrazyl (DPPH) is a radical scavenging activity which has been widely used to measure the antioxidant properties of various samples. Samples that are normally used for this experiment are plant extracts, fruits and beverages. Yong *et al.*, have conducted few immunomodulatory studies on *C. nutans*. Yong and his colleagues used three different extracts of *C. nutans* (chloroform, methanol and aqueous extract) for their experiment. They found that chloroform extract exhibited highest DPPH scavenging activity compared to other extracts. On the other hand, aqueous extract possessed lowest activity of antioxidant and the antioxidant activity showed a decrease in the following manner; chloroform > methanol > aqueous (Yong *et al.*, 2013).

In addition, NO scavenging activity showed that aqueous extract of *C. nutans* had the ability to scavenge NO radical in a dose dependent manner. However, methanol extract showed significantly higher NO scavenging activity compared to aqueous and chloroform extracts (Yong *et al.*, 2013).

2.6 Ethanol and aqueous based extracts of *Clinacanthus nutans* leaves

Previous researchers have been conducted on *C. nutans* using various types of extracts. Teoh and his colleagues investigated on ethanol and ethyl acetate extracts of *C. nutans* roots. From their study, both extracts promoted apoptosis by down-regulating *BCL-2* (Teoh et al., 2017). On the other hand, Huang *et al*., has studied on anti-tumor and immunomodulatory activity of ethanol extract of *C. nutans* leaves. They reported that ethanol extract of *C. nutans* leaves displayed anti-tumor properties by up-regulating the immune response (Huang *et al.*, 2016). Also, Yong *et al*., investigated on three different extracts, namely, chloroform, methanol and aqueous extracts. Based on their study, chloroform showed highest anti-proliferative effects while, there was cell proliferation in aqueous extract and not in methanol extract (Yong *et al.*, 2013). According to Zakaria *et al*., aqueous extract of *C. nutans* has higher polarity index when compared to methanol extract. Thus, the percentage yield of aqueous extract was much lower as compared to methanol extract. This may signify that the extracted compounds from *C. nutans* are mostly semi polar or non-polar (Zakaria *et al.*, 2017). The result is consistent with our study where percentage yield of aqueous extract was lower than ethanol extract in all tests. As mentioned, ethanol and aqueous extracts have been studied on different part of *C. nutans*. Since it clearly shows that different extracts of plants might yield in diverse outcomes, thus, we finally came out in investigating ethanol and aqueous extracts of *C. nutans* leaves.

2.7 Murine macrophage cell line

Murine macrophage cell line (J774A.1) (Figure 2.6) comes from *Mus musculus*, mouse organism. Tissue-based phagocytes that are derived from monocytes are known as macrophages. They are involved in both the innate and adaptive immune responses (Peakman and Vergani, 2009). Microbial metabolites like endotoxin by molecules (e.g. CD40 ligand) and by T cell cytokines (e.g. interferon-γ) activates macrophages (He *et al.*, 2002).

Macrophage and monocytes are crucial components of the inflammatory response (Pierce *et al*., 1995). The major duty of the macrophage is in host defense such as production of cytokines and phagocytosis of apoptotic cells (Hume, 2006). Besides, the diversity of plant polysaccharide has been claimed to display the advantageous pharmacological effects by way of its competency in modulating macrophage function (Schepetkin and Quinn, 2006). Previous study used murine macrophage cell line as a model to investigate the action of six cytokines and LPS on lipoprotein lipase (Tengku-Muhammad *et al.*, 1996). Recently, researchers used murine macrophage cell line to assess the potential of anti-inflammatory by evaluating NO production with iNOS, Griess reagent method and COX-2 expression by cytofluorimetric analysis (Abdallah *et al.*, 2018). Hence, the murine macrophage cell line was chosen in current study.

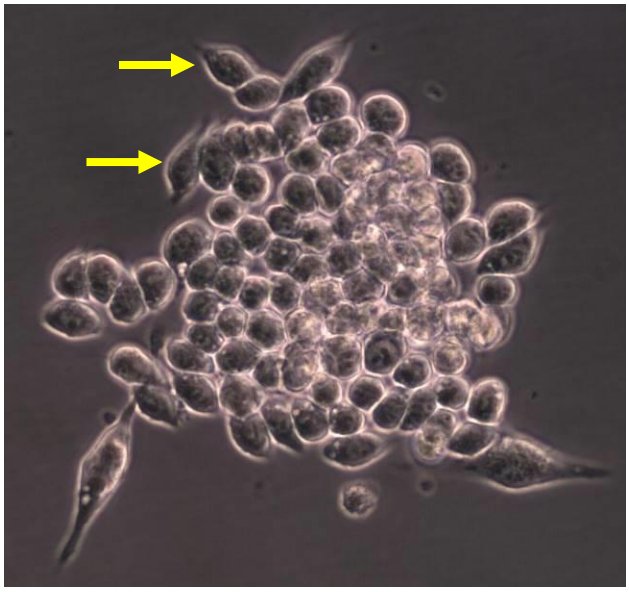

Figure 2.6: Murine macrophage (J774A.1) cell line under 400x magnification. Arrow indicates the murine macrophage cell line

2.8 Human gingival fibroblast cell line

Human gingival fibroblasts (HGF-1) (Figure 2.7) used in the study were prepared from explants of normal human gingival tissues (Takada *et al.*, 1991). There are plenty of HGFs in periodontal tissue. Inflammatory cytokines like IL6 and IL8 can be produced from HGFs upon stimulation of LPS (Bartold and Haynes, 1991; Takada *et al.*, 1991; Tamura *et al.*, 1992). In addition, it also expresses cell surface CD14 (Wang *et al.*, 1998). On the other hand, excessive production of inflammatory cytokines seems to be needed for the pathogenesis of periodontitis (Ara *et al.*, 2009). On the other hand, gingival fibroblasts form the main cell population of gingival connective tissue. It is accountable for the production of extracellular matrix of tissue in disease and health (Bartold *et al.*, 2000; Poggi *et al.*, 2003). HGF is one of the uncomplicated types of cells to culture because it

were cultured through the enzymatic digestion method as describe by Supraja and his colleagues (Supraja *et al.*, 2016). Hence, HGF-1 was considered in the current study.

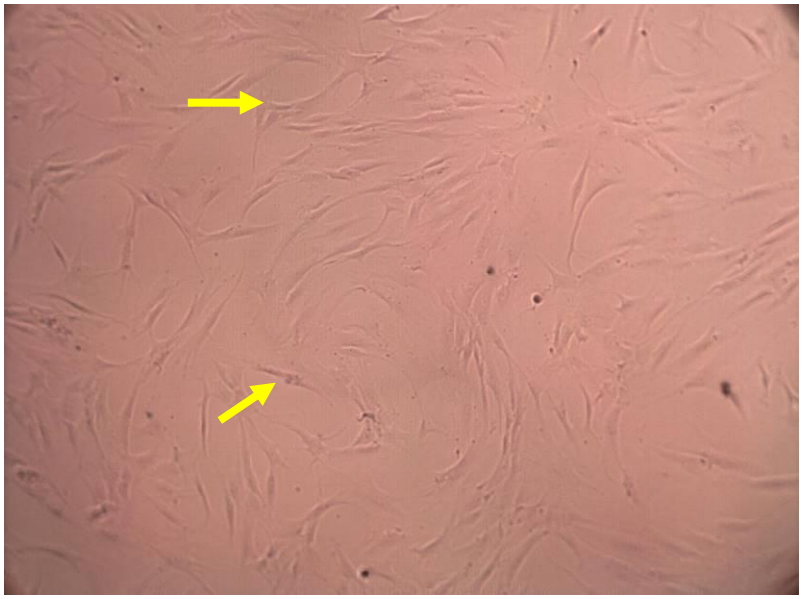

Figure 2.7: Human gingival fibroblast cell line cell line under 100x magnification. Arrow indicates human gingival fibroblast cell line

2.9 MTS assay

There are many assays developed to overcome the disadvantage of MTT assay. Those assays produced insoluble formazan crystals. However, the principles for these methods are similar to 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay where the Malate dehydrogenase (MDH) enzyme in the mitochondria of metabolically viable cells reduces tetrazolium salts to formazan. (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium) (MTS) one of the tetrazolium salt compounds which fulfill this category (Barltrop *et al.*, 1991; Cory *et al.*, 1991; Tominaga *et al.*, 1999). This colorimetric assay has its own advantages such as easy