GENE EXPRESSION OF INTERLEUKIN-4 (IL-4) CYTOKINE IN *Clinacanthus nutans*- TREATED MACROPHAGES

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EKSPRESI GEN INTERLEUKIN 4 (IL-4) SITOKIN DALAM MAKROFAJ YANG DIRAWAT *Clinacanthus nutans*

ABSTRAK

Clinacanthus nutans (Burm f.) Lindau (C.nutans) adalah ubat tradisional yang terkenal di Asia Tenggara. Ia didakwa mengandungi beberapa ciri-ciri fitokimia seperti aktiviti anti-radang, antioksida dan anti-herpes di kalangan pengamal perubatan tradisional. Walaupun kajian sebelum ini pada *C.nutans*, banyak yang melibatkan ciri-ciri yang berkaitan dengan menghalang tindak balas keradangan, memahami mekanisma yang terlibat masih perlu dijelaskan. Kajian ini bertujuan untuk menilai ungkapan interleukin-4 (IL-4) dalam makrofaj yang dirawat. Pengenalan sel makrofaj dan pencirian dinilai berdasarkan pemerhatian morfologi di bawah mikroskop terbalik-fasa dan juga ekspresi F4 / 80 dengan menggunakan sitometri aliran (FACS). Kesan sitotoksiti *C.nutans* ekstrak daripada empat pelarut yang berlainan kekutuban iaitu petroleum eter (PE), kloroform (CHL), etanol (ETOH) dan akues (AQ) telah diperiksa menggunakan reagen PrestoBlue ™. Peratus pengurangan menunjukkan perencatan makrofaj RAW264.7 oleh PE, CHL, ETOH, dan AQ-C.nutans ekstrak. Berdasarkan peratusan pengurangan, ETOH dan AQ-*C.nutans* ekstrak menunjukkan bahawa mereka kurang sitotoksik kepada makrofaj RAW264.7 berbanding dengan ekstrak PE dan CHL-*C.nutans* itu. Dari segi ungkapan IL-4 gen, dengan penemuan terhad ini, kami membuat kesimpulan bahawa tidak ada ungkapan IL-4 gen dalam sel RAW264.7 makrofaj yang dirawat dengan ekstrak AQ-*C.nutans*. Walau bagaimanapun, kajian lanjut diperlukan untuk mendapatkan gambaran lengkap ungkapan anti-radang IL-4 dalam RAW264.7 makrofaj yang dirawat dengan AQ-*C.nutans*

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ABSTRACT

Clinacanthus nutans (Burm f.) Lindau (*C.nutans*) is a traditionally well-known medicine in South East Asia. It was claimed to consist several phytomedicinal properties such as anti-inflammatory activities, antioxidants and anti-herpes amongst the traditional healers. Despite previous studies on *C.nutans*, many of which involve properties associated with inhibiting the inflammatory response, understanding the mechanisms involved remain to be elucidated. This study aims to evaluate the expression of an anti-inflammatory cytokine, interleukin-4 (IL-4) cytokine in treated macrophages. Macrophage identification and characterization was assessed based on the observation of morphologies under inverted-phase microscope and F4/80 expression by using flow cytometry (FACS). The cytotoxicity of *C.nutans* extracts using four different polarity of solvents which are petroleum ether (PE), chloroform (CHL), ethanol (ETOH) and aqueous (AQ) were examined using PrestoBlue™ reagent. Percentage of reduction shows inhibition of the RAW264.7 macrophages by the PE, CHL, ETOH, and AQ-C.nutans extracts. Based on the percentage of reduction, ETOH and AQ-*C.nutans* extracts show that they are less cytotoxic to the RAW264.7 macrophages compared to the PE and CHL-*C.nutans* extract. In terms of IL-4 expression, with this limited findings, we concluded that there is no expression of IL-4 in RAW264.7 cells treated with AQ-*C.nutans* extract. However, further studies are needed to get complete overview of the anti-inflammatory cytokine expression in *C.nutans* treated RAW264.7 macrophages.

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LIST OF SYMBOLS AND ABBREVIATIONS

$^{\circ}C$	=	degree Celcius
%	=	percentage
µg/Ml	=	Microgram per milliliter
ng/µL	=	Nanogram per microliter
h	=	hour
g	=	gram
min	=	minute
ml	=	milliliter
Mm	=	miliMolar
nm	=	nanometer
g	=	Gravity force
cm^2	=	Centimeter squared
v/v	=	volume per volume
PE	=	Petroleum Ether

CHL	=	Chloroform
ETOH	=	Ethanol
AQ	=	Aquoeus
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
AAM	=	Alternative Activation of Macrophage
CAM	=	Clasiccal Activation of Macophages
C.nutans	=	*Clinacanthus nutans*
DNA	=	Deoxyribonucleic acid
RNA	=	Ribonucleic acid
DMSO	=	Dimethylsulfoxide
PBS	=	Phosphate Buffer Saline
FBS	=	Fetal bovine serum
FSS	=	Forward scatter
SS	=	Side scatter

CHAPTER 1

INTRODUCTION

1.1 Research Background

Nowadays, almost 80% of the world populations rely on traditional practitioners and their armamentarium of medical plants and herbs in order to meet health care needs (WHO, 2002). Malaysia is one of the developing countries that have more than 2000 plant species that have healing qualities and highly potential to be commercialized (MARDI, 2010). Most of them are used as remedies to treat illness such as fever, diarrhoea, sore throats, sinus problems, respiratory problems and skin condition (Sujaidi, 2009). Due to the increasing interest and research effort made, many herb properties have been well documented.

Clinacanthus nutans (Burm f.) Lindau (C. nutans) is one of the well-known medical plants in South East Asia, including Malaysia, Thailand, Indonesia as well as China. Various studies on its phytomedicinal properties have been done since 1970s and still propagating until now. Despite previous studies on *C. nutans*, many of which involve properties associated with inhibiting the inflammatory response, understanding the mechanisms involved remain to be elucidated. With the increasing concerns about various diseases and disorders in human, medicinal plants and herbs have been interestingly screened for their bioactivity and highly targeted by the scientists who involved in screening of alternative sources for western medicines. Thus, many studies on its anti-inflammatory properties were done to resolve the inflammatory process (Pimentel et al., 2013) that was addressed as a key contributor to the development of diseases.

Inflammation acts as the body's first line defence system against infection and injury. It also involved in healing the damaged tissues. Inflammation process consists of several stages which are initiation, maintenance, and resolution of inflammation (Dalmas et al., 2012). Inflammation diseases may occur due to several factors. For rheumatoid arthritis, the inflammation is induced by the activation of intracellular signalling pathways that finally lead to the production of several proinflammatory cytokines (Piecyk & Anderson, 2001). Thus, the inhibition of the proinflammatory cytokines and increment the secretion of anti-inflammatory cytokines become an effective therapy for inflammation disease such as in rheumatoid arthritis disease (Wax et al., 2003).

Macrophages are involved throughout the inflammation process and act as central role in inflammatory diseases (Cho et al., 2013). They play functions in antigen presentation, phagocytosis, and immunomodulation of the immune system by the secretion of cytokines and growth factors (Dalmas et al., 2012) . They also able to increase the expression of many pro-inflammatory cytokines or protein in macrophages (Oyungerel et al., 2014). For example, in host defence, macrophages play an important role in destroying the dysregulated polymorphonuclear neutrophils (PMNs). PMNs play an important contribution to the propagation and maintenance of acute and chronic inflammation. Malfunction of neutrophils leads to the liberation of granule products, release of toxic oxygen metabolites and production of various inflammatory mediators that finally cause tissue damage associated with inflammatory disorder. Cytokines and other pro-inflammatory agents will induce the apoptosis process of neutrophils so that it can be engulfed by macrophages (Walker et al., 2005).

Macrophage can be classified based on their activities in the host. The three major group of macrophages based on their function in homeostasis are host defence, wound healing and immune regulation (Mosser & Edwards, 2008). Interestingly, macrophages can respond and change their physiology correspond to various cellular signal and stimuli that were confronted by them. The cellular signal may come from innate or adaptive immune responses. These responses can activate macrophages to become more susceptible to various infections by pathogens. At the same time, the production of cytokine by macrophages could also be activated in order to enhance the immune response. Despite their major function in inflammation process, macrophages are also able to induce inflammatory reaction, initiate and maintain certain immune response in the host by releasing different type of cytokines (Poltorak et al., 1998).

Basically, the secretion of cytokines by activated macrophages play role in regulating the cellular interaction but there are some bad effects if it is overproduced. For example, the over expression of inflammatory cytokines may lead to several acute or chronic inflammatory responses (Laskin & Pendino, 1995). Chronic and uncontrolled inflammations may cause the pathogenesis of a variety of diseases, such as arthritis, asthma, multiple sclerosis, inflammatory bowel disease, and atherosclerosis (Kim et al., 2010). In order to understand what is right for the system and what the system actually is, it is very crucial to understand the balance between pro-inflammatory and anti-inflammatory responses of cytokine networks and the networks' roles in inflammatory disease (Seymour & Henderson, 2001).

Previous study showed that *C. nutans* extract able to activate cell-mediated immune-response (CMIR) activity once treated on peripheral blood mononuclear cells

(Yoosook et al., 1999) by the increased the secretion of IL-4 cytokine. Hence, this study has taken a further step in exploring the effect of *C. nutans* extract on gene expression of anti-inflammatory IL-4 cytokine. IL-4 cytokine is a significant cytokine in immune system that produced upon alternative activation of macrophage in order to resolve the inflammation and promote wound healing. Therefore, this work is to further characterize the *C. nutans* effects on macrophages activation by assessing the gene expression of IL-4 in RAW264.7 macrophages. The assessment of IL-4 gene expression will be done in RAW264.7-derived macrophages treated with various extracts of *C. nutans. C. nutans* was extracted from various polarities of solvents [petroleum ether (PE), chloroform (CHL), ethanol (ETOH) and aqueous (AQ)].

1.2 Research Objectives

The main objective of this study was to assess gene expression of IL-4 cytokine in RAW264.7-derived macrophages treated with selected extracts of *C. nutans.* The specific objectives are as follow:

1. To extract the *C. nutans* leaves with different solvent polarities by using successive extraction technique
2. To evaluate the cytotoxicity effect of different *C. nutans* extracts on RAW264.7 derived macrophages
3. To assess the gene expression of IL-4 induced by selected *C. nutans* extracts

CHAPTER 2

LITERATURE REVIEW

Clinacanthus nutans (Burm f.) Lindau (C. nutans) is a traditionally well-known folkfore medicine in South East Asia, included in Malaysia, Thailand, Indonesia as well as China. *C. nutans* which is known as Belalai Gajah or Sabah snake grass in Malaysia is a tall, erect and sometimes rambling shrubs herb plant belongs to the family of Acanthaceae (Yong et al., 2013) .The stems of *C. nutans* are cylindric, yellow when dry, densely striate and subglabrous. *C. nutans* is also characterized by narrowly oblong or lanceolate leaves (2.5-13 cm long; 0.5-1.5 cm wide). They consists of red flowers with dense cymes that usually can be found at the top of the branches for the mature one (Pieroni & Vandebroek, 2007).

Figure 2.1: The structure of *C. nutans* leaves. *C. nutans* leaves are lanceolate in shape. The picture was taken from page: http:// thaiherbinfo.com [Accessed 12 March 2015]

Figure 2.2: The image of mature *C. nutans* plant. The flowers of *C. nutans* be found at the top of the branches. The image was taken from page http://toptropicals.com/ [Accessed 12 March 2015]

C. nutans was claimed to consist several properties such as anti-snake venom, anti-cell lysis, anti-inflammatory activities (Wanikiat et al., 2008), anti-hepatitis, antioxidants and anti-herpes (Wirotesangthong et al., 2009) amongst the traditional healers. The extracts of *C. nutans* leaves have been used in Thailand as anti-inflammatory agents to treat insect bites and several allergic problems. There are also some clinical trials have been reported the successful use of *C. nutans* as a cream or lotion preparation to treat minor skin inflammation and insect bites. But in China, the whole plant of *C. nutans* was used in many purposes. The Chinese practitioners believe that *C. nutans* plant would be able to treat various inflammatory conditions such as rheumatism and sprain of injuries (Arullappan et al., 2014). It also was used among the Chinese healer to regulate menstrual function and relieve the pain in injuries. Nevertheless, the activity of this plant extract on immune system was assessed.

C. nutans consist of several important constituents which are flavonoids, stigmasterol, sitosterol, lupeol, betulin, C-glycosyl flavones, vitexin, isovitexin, shaftoside, isomollupentin, 7-O-gluco pyranoside, orientin, isoorientin, cerebrosides monoacylmonogalactosylglycerol and sulfur-containing glucosides (Teshima et al., 1998; Tuntiwachwuttikul et al., 2004). But, only two glycoglycerolipids have been shown to exhibit antiviral properties. However, in 2008, it was also discovered that there are antiviral chlorophyll a and chlorophyll b related compounds from *C. nutans,* an important Thai medicinal plant used for herpes infections in primary health care. This finding suggested the compounds to be used as the marker in the study of the antiviral activities. Different types of bioactive compounds of *C. nutans* were extracted out by using different polarities of solvents in extraction process. Previously in a study, *C. nutans* were extracted by using nonpolar solvents such as petroleum ether (PE) and chloroform (CHL) to extract out compounds such as alkaloids, terpenoids, coumarins (Nwabueze & Okocha, 2008).

Previous study showed that *C. nutans* extract was able to increase lymphocyte proliferation significantly and at the same time, slow down the activity of natural killer cells (NK cells) (Yoosook et al., 1999). These findings also proposed that *C. nutans* extract able to suppress the secretion of IL-2 while IL-4 secretion was increased. This study shows that the cell-mediated immune-response (CMIR) activity of *C. nutans* extract was partially due to the release of IL-4 from the peripheral blood mononuclear cells.In addition, the extracts of the leaves of *C. nutans* also had been found to possess a strong anti-inflammatory activity corroborating further its extensive used as an anti-inflammatory medicine (Wanikiat et al., 2008). This effect was partly due to its ability

to inhibit the neutrophil responsiveness as evidenced by the significant inhibition of myeloperoxidase (MPO) activity.

In a *in vivo* research on *Barleria lupulina* and *C. nutans* extracts, it has been proposed that methanolic extracts of *C. nutans* possessed significant anti-inflammatory properties in both of the rat paw oedema model induced by injection of carrageenan and the ethyl phenylpropiolate (EPP)-induced rat ear oedema model (Wanikiat et al., 2008). The mechanism of the activity is related to neutrophil migration with the reduction of neutrophil marker enzyme that was known as myeloperoxidase (MPO). The activity of the enzyme was shown in EPP-induced rat ear oedema model.

Furthermore, *C. nutans* extracts also demonstrated inhibitory effects on neutrophils responsiveness *in vitro* without possess any significant cytotoxic effect. The leaves of *C. nutans* also had been used by traditional healers to treat herpes infection. Jayavasu *et al.(*2013) stated that *C. nutans* leaves posses the ability to inactivate herpes simplex virus type-2 (HSV-2) against of acyclovir (Jayavasu et al., 2013). It was proven when they found that the extract of the leaves of *C. nutans* was able to inhibit plaques formation of HSV-2 in baby hamster kidney cell line, even, the previous study done by Yoosook et al. on the anti HSV-2 strain against organic solvent extracts of *C. nutans* did not show any anti-HSV-2 viral activity (Yoosook et al., 1999). This contradictory result may be attributed to differing laboratory factors and plant material as clinical trial done on the extract of *C. nutans* did show significant antiviral properties. The mechanism of action of this plant is believed to be attributed to its anti-cell lysis property rather than as an anti-neuromuscular transmission blocker.

With the recent outbreak of Influenza A (H1N1) globally, the activities of *Clinacanthus siamensis* leaf extract on influenza virus infection was studied (Wirotesangthong et al., 2009). They studied its effects against various mouse-adapted influenza viruses in the NA inhibition and *in vitro* antiviral assay. They found that the extract was superior in its ability to protect the mouse against influenza virus infection as compared to oseltamivir. In 1995, a study was done on the efficacy of topical formulation of *C. nutans* extract. In this study, 51 patients with varicella-zoster virus infection were used as subjects. The results showed that lesion crusting occurs within 3 days of application and healing within 7 days. The medication was applied 5 times per day for 7-14 days until lesion healed. Pain scores were also reduced significantly. No side effects were observed during the course of treatment (Sangkitporn et al., 1995).

The role of free radicals in the pathophysiology of diseases has been very well established. Today many researchers are looking into plants as a source of antioxidants. Amongst the subject of study is *C. nutans.* The antioxidant properties of ethanolic extract of the leaves of *C. nutans* also was discovered (Pannangpetch et al., 2007). They found that this extract had an antioxidant activity and protective effect against free radical-induced haemolysis. This is the evident by the fact that it could scavenge DPPH with a maximum scavenging activity of 67.65±6.59% and IC_{10} of 110.4±6.59 µg/ml; the FRAP value was 17mg ascorbate equivalent to one gram of the extract.

The extract demonstrated a significant inhibition of peroxide production in rat macrophages stimulated by phorbol myristate acetate (PMA) and protected red blood cell against AAPH-induced haemolysis with an IC_{50} of 359.38 ±14.02 mg/ml. Besides, there were only few studies, mostly from Thailand that reported the immunomodulatory

effect of *C. nutans* extracts. For instance, the studies basically focused on investigating the effect of *C. nutans* ethanolic extract on peripheral blood mononuclear cells (PBMC) lymphocytes proliferation, function of natural killer (NK) cells, as well as the production of Interleukin-2 (IL-2) and interleukin-4 (IL-4) (Sriwantha et.al, 1996).

The ability to defend ourselves against disease and infection is dependent upon a highly coordinated effort performed by hematopoietic cells that comprise the immune system. Within this system, extracellular signaling proteins serve as pleiotropic chemical messengers that are critical for regulating cell growth, differentiation and immune homeostasis. Many of these extracellular signaling molecules bind to a highly conserved family of hematopoietic receptors that are void of intrinsic intracellular catalytic domains and share several conserved elements within their extracellular domains.

Macrophages are tissue-resident professional phagocytes as well as antigen presenting cells (APC). Monocytes that were originated from the hematopoietic stem cells (HSC) in bone marrow will be differentiated into either become interstitial dendritic cells, macrophages or microglial cells depending on the location of tissue that they enter (Gordon & Taylor, 2005). Basically, monocytes are short-lived rather than macrophages. They undergo apoptosis spontaneously. But, monocytes are able to escape from fast apoptosis and have longer life span by differentiating into macrophages aided by differentiation factors (Parihar et al., 2010).

Monocytes are actively circulating in the peripheral blood and they circulate for several days before entering the tissue (Gordon & Taylor, 2005). The differentiation of

monocytes occurred upon there were stimulations by different cytokines and other factor cocktail(Le Douce et al., 2010). The signal from the activation of the cytokines and chemokines encourage the monocytes to leave the circulation system and migrate to the site of infection as well as start the differentiation process into macrophages(Davis et al., 2004).

Generally, monocyte and macrophages are the essential cells that involved in the innate immune system along with dendritic cells, neutrophils and mast cells (Parihar et al., 2010). Monocytes and macrophages are categorized into professional phagocytes according to how effective they are in phagocytosis. Both of them express a multitude of receptors on their surface that enabling them to detect signals that are not normally can be found in healthy tissue (Murray & Wynn, 2011). That is the mechanism used by macrophage once they detect the pathogen entering the host and later on, macrophages are inducing specialized activation program to defence the host.

There are two types of macrophages activation; the classically activated macrophage (CAM) and the alternatively activated macrophage (AAM) (Mosser, 2003) as shown in Figure 2.3. These two cross regulated metabolic processes may reflect opposite functional states of macrophages. It is well known that macrophages can be either classically activated or alternatively activated, and are therefore termed as inflammatory or anti-inflammatory cells (Porcheray et al., 2005). A given macrophage may switch from one activated state to another upon a specific signal (Porcheray et al., 2005). Inflammatory mediators and cytokines induce classical activation involving iNOS expression while anti-inflammatory cytokines induce alternative activation with upregulated arginase activity (Yamamoto et al., 1998).

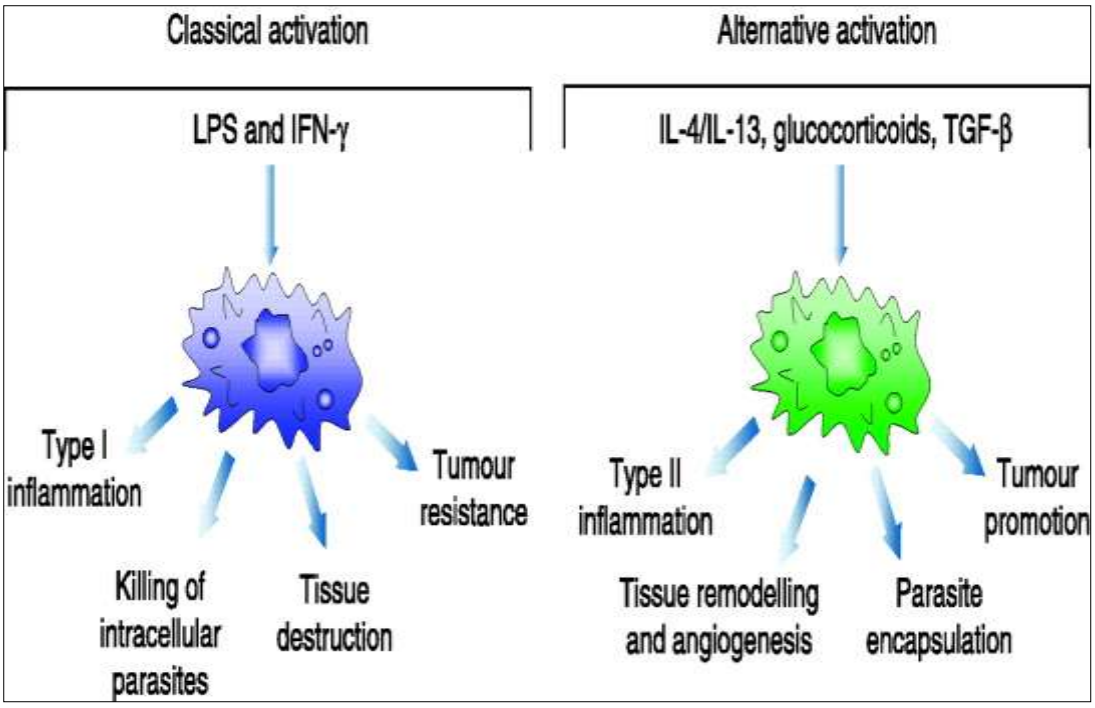

Figure 2.3: Classical and alternative activation of macrophages (Rickard & Young, 2009)

In the CAM activity, a Th1-like phenotype is exhibited and promotes inflammation to tissue destruction (Martinez et al., 2009). This situation will lead to the development of tumour type 1 autoimmune disease and glomerulonephiritis (R&D System Inc., 2013). Therefore, in order to encounter the attack from the invading pathogens, the body is needed to trigger a harsh pro-inflammatory response. Fortunately, the CAM also able to secrete another substance like tumour necrosis factor (TNF) and nitric oxide (NO) that will directly involved in tumour cell killing.

Differently, in AAM activity, the Th2-like phenotype was displayed and facilitates to resolve inflammation as well as promote wound healing (Mosser, 2003). There are two prominent cytokines like IL-4 and IL-13 are produced during this activation. Both of these cytokines are the Th2 molecules that will trigger responses

particularly in allergic, cellular and humoral responses to parasitic and extracellular pathogen (Gordon, 2003).

Cytokines are the most important mediators of the inflammatory response. Cytokines are soluble proteins released by most of the cells in the body. But most of them are primarily produced by the cells of the immune system, which act non-enzymatically with specific receptors to regulate immune responses (Clemens, 1991; Nicola 1994). The major interest in studying cytokines is their role in disease. The secretion of cytokine also can be stimulated by other factor such as pathogen-associated molecular patterns (PAMPs). PAMPS also be able to stimulate the release of pro-inflammatory cytokines, which are crucial to sent signal toward both the innate and adaptive immune response in order to enhance the protection in the host (Hornef et al., 2002).

Cytokines can display autocrine and paracrine functionality (Caldwell et al., 2014). An autocrine action is where a cell produces molecules that affect that cell while a paracrine action is where a cell produces molecules that affect other cells. Cytokines, hormones and hematopoietic growth factors transduce biological signals across the cell membrane via a highly conserved family of single membrane-spanning receptor. The intracellular signal transducing machinery responsible for mediating these responses has remained largely unknown (Gordon, 2003).

The ability to defend ourselves against disease and infection is dependent upon a highly coordinated effort performed by hematopoietic cells that comprise the immune system. Within this system, extracellular signaling proteins serve as pleiotropic

chemical messengers that are critical for regulating cell growth, differentiation and immune homeostasis. At antibiotic age, it is important to understand the network of interacting components between pathogens and their hosts. Major players in these networks are cytokines. Cytokine networks are incredibly complex (Seymour & Henderson, 2001). If too few or too many cytokines are produced the host dies. If the correct amount and combination of cytokines are produced the host overcomes infection.

IL-4 cytokine is a multifunctional peptides that structurally similar to IL-13. Both of them can affect multiple cell types. IL-4 cytokine is a well-characterized regulator of proliferation and immunoglobulin class switching in B cells (Ito et al., 2009). It exerts its actions on immune cells by involving two types of receptors; type I and type II. Type I heterodimer consists of the IL-4R-chain in association with the common cytokine receptor-chain where as type II receptor is comprised of the IL-4R in association with IL-13R (Gessner & Röllinghoff, 2000; Nelms et al., 1999).

Interleukin 4 (IL-4) is one of the most significant cytokine in the immune system. It was firstly discovered in 1982 by Maureen Howard and Bill Paul in the Laboratory of Immunology at the National Institute of Allergy and Infectious Diseases (NIAID). At that time, it was described as a B cell stimulatory factor exist in the supernatant of the phorbol ester-stimulated T cell tumor line EL-4. With the updated findings and researches on IL-4, it is now known that this cytokine give function in many process of immune system regulation such as in Ig isotype switching class II MHC expression by B cells, and the differentiation fate of certain T cell subsets.

In this study, RAW264.7 cell line was used as in the *in vitro* cell model. Mouse RAW264.7 cell line was extensively used previously for many purposes including for monocyte/macrophages differentiation. The RAW264.7 cell line was first discovered by WC Raschke in 1978. It was derived from a adult male mouse with Abelson murine leukaemia virus-induced tumor. RAW264.7 cells have been extensively used as a model cell line for studies of macrophage biology.

RAW264 cells had macrophage-like morphology with deeply stained nuclei and finely granulated cytoplasm. Cells were generally elongated with two short cytoplasmic processes. They found that RAW264.7 cells express high levels of the macrophage and myeloid cell surface markers F4/80 and CD11b. In order to assess the effect of different polarity of *C. nutans* extract to the macrophages, RAW264.7 was used as a model. RAW264 is the most commonly used mouse macrophage cell line in medical research (Hartley et al., 2008). Until 2008, over 1500 publication from Pubmed retrieval list have used the RAW264.7 cell line in the research were reported (Hartley et al., 2008).

They are also often used *in vitro* for the evaluating study on the inhibitory effects of food material and probiotics on inflammation (Kuda et al., 2012). Thus, the anti-inflammatory effects of various probiotics have been well defined in RAW264.7. Due to their properties in ease of cell propagation, efficiency in DNA transfection, sensitive to RNA interference and possess of several receptors for relevant ligands, they also have been chosen as the primary experimental model in studying about signalling pathways especially in the cells (Shin et al., 2006). Previously, RAW264.7 also have been used in anti-inflammatory studies for several plant extracts such as *Angelica*

dahurica ethanolic extract (Lee et al., 2011) and *Magnolia sieboldii* extract (Oyungerel et al., 2014).

CHAPTER 3

METHODS & MATERIALS

3.1 Materials

All glasswares used in this experiment were sterilised and autoclaved at 121°C for 30 min at a pressure of 100 kPA prior to use. The plastic wares used are readily sterilised.

3.2 Methods

3.2.1 Collection of *C. nutans*

C. nutans was harvested from Manjung, Perak, Malaysia. The botanical identification of the *C. nutans* was determined by the Herbarium Unit, School of Biological Sciences, Universiti Sains Malaysia, Penang (Voucher no.:USM Herbarium11465). The leaves were separated from the stem, cleansed and oven dried at 40 to 45ºC for 5 to 7 days.

3.2.2 Extraction of *C. nutans*

The readily dried *C. nutans* leaves were pulverized into fine powder and weighed at 100 g for ultrasonic-assisted maceration by using ultra centrifuge mill (Retsh, ZM200 Germany). *C. nutans* powder was extracted sequentially using petroleum ether (PE) (QRec, Thailand), chloroform (CHL) (QRec, Thailand), ethanol (ETOH) (QRec, Thailand) and aqueous at room temperature. The ratio of powder weight to solvent volume was 1:10. Approximately 100 g of powdered leaves were filled into a conical flask with 1L of PE. The mixture was shaken by using an ultrasonic cleaner bath (Wise Clean WUS ATOH, Korea) for 30 minutes. The macerated leaves were centrifuged at 1250 X g. The supernatant obtained was filtered using a vacuum filtrator (Vacuubrand, MZ2 CNT, Germany) and evaporated off using a rotary evaporator (Eyela, N1100,

USA) at 60 ºC. The percentage yield of the extracts obtained was calculated with the following calculation:

Percentage yields = [Weight of extract (g) ÷ Weight of leaves (g)] x 100%

The PE and CHL extracts were dissolved in dimethylsulfoxide (Sigma Aldrich, USA) to give 1% (v/v) concentration where as for AQ and ETOH extracts, they were dissolved in Dulbecco's Modified Eagle Medium (DMEM) by Gibco, USA to get the appropriate concentrations (0, 0.25, 0.50, 1, 2, 4, 8 mg/mL).

3.2.3 Morphology of RAW264.6 cell line

RAW264.7 cells were obtained from American Type Culture Collection (ATCC). The cells were cultured in complete medium of DMEM containing 10% heat-inactivated fetal bovine serum (v/v), 1% of 100 units/mL penicillin-steptomycin (v/v) and 1% of HEPES (v/v). The cells were cultured in an incubator at 37° C with a humidified atmosphere of 5% CO_2. The morphology of the cells was observed under an inverted-phase contrast microscope (Olympus, USA).

3.2.4 Characterisation of RAW264.7 cell line

Assessment of mouse macrophage (RAW264.7) cell lines was performed using anti-mouse F4/80/EMR1-Phycoerythrin antibody (R&D System, US). Non-specific binding was assessed by using an isotype antibody, rat IgG2A Phycoerythrin-conjugated antibody (R&D System, US). The stained cells were then analysed using BD FACS Canto II flow cytometer (Becton-Dickinson, USA). Briefly, the cells were counted by using a haemacytometer and about $1x10^6$ cells were added into each FACS tube. Each tube was labelled appropriately. Phosphate buffer saline (PBS) was added into each

FACS tube to give a final volume of 200 µl of cells suspension. 2-3µg of fluorescently labelled antibodies (isotype IgG2A PE-Conjugated antibody and anti-mouse F4/80/EMR1-PE) was added into each tube except in the unstained tube. All of the tubes were incubated for 45 minutes on ice, in dark. After that, the tubes were centrifuged at 1200-1500 rpm at 4 °C for 5 minutes. The supernatant was discarded and the pellet was washed three times with 500 µl PBS. After the final washing, the pellet was re-suspended in 400 µl PBS and ready to be acquired. Each of samples was prepared in triplicate. The results obtained were analyzed by using Flow Jo software (Tree Star, Inc., USA).

3.2.5 *In vitro* cytotoxicity assay extracts on macrophages using PrestoBlue™

The plating density of RAW264.7 cell line was determined before performing the PrestoBlue™ reagent assay. Cytotoxicity assay was measured using the PrestoBlue™ (Molecular Probes, California) cell proliferation reagent. Briefly, 10000 of RAW264.7 cells were seeded into each of the 96-wells flat bottom plate (BD Falcon, Canada). The cells were incubated in an incubator at 37 ^{o}C with a humidified atmosphere of 5% CO_2 to get 70-80% confluent of cells. The confluent of the cells were observed under inverted-phase contrast microscope. The next day, filtered-*C. nutans* extracts (PE, CHL, ETOH and AQ) with extract concentrations: 0.25, 0.5, 1, 2, 4, and 8 mg/ml were added into the corresponding wells. The PE and CHL extracts were dissolved in dimethylsulfoxide (Sigma Aldrich, USA) to give 1% (v/v) concentration where as for AQ and ETOH extracts, they were dissolved in Dulbecco's Modified Eagle Medium (DMEM) by Gibco, USA to get the appropriate concentrations (0, 0.25, 0.50, 1, 2, 4, 8 mg/mL). After incubation for 24 hours, the culture medium was removed and cells were washed three times by using 1X PBS to remove excess *C. nutans* extracts. Then,

PrestoBlue reagent, which have been diluted in DMEM were added to each wells and incubated in incubator at 37 ^{o}C with a humidified atmosphere of 5% CO_2. At the same time, PrestoBlue™ reagents diluted in DMEM were also added to blank wells without cells as control. After 20 minutes incubation, the absorbance value at 570nm (normalization wavelength) and 600nm (reference wavelength) was read by an ELISA plate reader (Thermo Scientific Multiskan Spectrum, USA). The percentage reduction of PrestoBlue reagent of each sample was calculated according to the following formula provided in the manufacturer's protocol. Percentage reduction of PrestoBlue Reagent = [(117216×A1) – (80586×A2)] / [(155677×N2) –(14652×N1)] ×100, where A1 is absorbance of test wells at 570 nm, A2 is absorbance of test wells at 600 nm, N1 is absorbance of media only wells at 570 nm, and N2 is absorbance of media only wells at 600 nm. Cells without treatment by *C. nutans* extracts were used as control and corresponding cell viability was set as 100%. Cell viability treated with a specific concentration of *C. nutans* extracts = (% reduction of PrestoBlue reagent at this concentration) / (% reduction of PrestoBlue reagent of cells not treated by *C. nutans* extracts). All tests were conducted in triplicate.

3.2.6 Treatment of macrophages with different concentration of *C. nutans* extracts

Based on the results in cytotoxicity assay, the less cytotoxic effect of *C. nutans* extracts were chosen to be treated on RAW264.7 cells for further experiments. Approximately 1 X 10^{6} of RAW264.7 cells were seeded into T25 cm flasks. The flasks then were incubated overnight in incubator at 37^{o}C with a humidified atmosphere of 5% CO_2. . The next day, the old media was removed and the cells were washed with sterile PBS. The *C.nutans* extracts obtained from PE, CHL, ETOH and AQ were dissolved into

DMEM complete medium then subjected to 2 minutes sonication to make sure the extracts was completely dissolved. The extracts were filtered using 0.2 micron filtration to filter off any contaminants including bacteria. Dilution was carried out to obtain the desired extract concentrations. The appropriate extract concentrations were added into the designated wells of the plate containing the readily washed cells. The plate was incubated in incubator at 37^{o}C with a humidified atmosphere of 5% CO_2 for 24 hours. The cells culture without any addition of extract served as negative control.

3.2.7 RNA Isolation

After 24 h incubation, the medium was discarded. The cells were washed with PBS and the cells were scrapped by using cell scrappers. The cell pellet then was collected by using centrifugation technique. Total Ribonucleic acid (RNA) was isolated by using Qiagen RNA Extraction kit (Qiagen, USA) according to the manufacturer's protocol. Before the RNA was used in RT-PCR, the quality of the RNA was inspected by using Nanodrop Spectrophotometer C2000 (Thermo Fisher, USA) and electrophoresis using 1% gel.

3.2.8 Reverse Transcription-Polymerase Chain Reaction

The reverse primer of interleukin-4 (IL4) and GAPDH were used as gene specific primer in cDNA synthesis by using Superscript ll Reverse Transcriptase kit (Invitrogen, California). Each reaction tube contain corresponding total RNA samples (1 ug) and were reverse-transcribed using SuperScript™ II RT from Invitrogen. First-strand copy of DNA was synthesised according to the manufacturer's protocol.

3.2.9 Assessment of IL-4 gene expression in *C. nutans*-treated macrophages

Polymerase chain reaction (PCR) was performed by using the cDNA that was produced in Section 3.2.8 as template. The primers were designated against human IL-4 gene (Genebank accession number NM_021283.2) and GAPDH gene (Genebank accession number NM_008084.2). The sequences of the primers were shown in Table 3.1. The PCR protocol for IL-4 and GAPDH gene amplification as follows: initial denaturation at 94°C for 2 min, followed by 25-35 amplification cycles at 94°C for 15 seconds (denaturation), 50-70°C for 30 seconds (primer annealing) and 68° C for 1 minute (extension). The PCR mixture details are shown in Table 3.2. The PCR reaction was maintained at 4°C after cycling. PCR products were separated on 1.8 % agarose gel-electrophoresis and visualise with ethidium bromide. Appropriate bands were scanned and quantitated by using the Vilber Lourmat (UV) and the bands were analysed by using Infinity software.

Table 3.1 : The sequences of the primers

Primer	Sequence
IL-4 gene (423 base pairs)	Reverse: 5'-GTACTACGAGTAATCCATTTGCAT GAT-3'
	Forward: 5'-GGTCTCAACCCC CAGCTAGTT-3'
GAPDH gene (171 base pairs)	Reverse: 5'-CACATTGGGGGTAGGAACAC-3'
	Forward: 5'-ACCCAGAAGACTGTGGATGG-3'

Table 3.2 : The preparation of master mix of components for multiple reactions

Component	**Volume**	**Final Concentration**
10x Pfx Amplification buffer	5 µl-10 µl	1X-2X
10mM dNTP mixture	1.5 µl	0.3 mM
50mM MgSO4	1 µl	1 mM
Primer Mix (10 µM each)	1.5 µl	0.3 µM
Template DNA (10 pg-200ng)	>1 µl	1 µg
Platinum Pfx DNA Polymerase	0.4 µl	1 unit
Autoclave, distilled water	To 50 µl	

3.2.10 Statistical Analysis

The statistical analysis was carried out using IBM SPSS Statistics Version 22.0. Values were presented as the mean ± SD from triplicates. The comparisons between control and treated were tested for significance using one-way ANOVA with post hoc Tukey's test. The differences between the two mean data with probability value less than 0.05 ($p < 0.05$) were considered as statistically significant.