REGULATION OF MACROPHAGE CELLULAR RESPONSE BY *Clinacanthus nutans* EXTRACTS IN J774.2 MACROPHAGE CELL LINE

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

AQ	Aqueous
ANOVA	Analysis of variance
cm^2	centimeter squared
C. nutans	*Clinacanthus nutans*
˚C	degree Celcius
EtOH	Ethanol
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FSS	Forward scatter
g	gram
g	Gravity force
h	hour
µg/mL	microgram per millilitre
mL	millilitre
min	minute
ng/µL	nanogram per microlitre

nm	nanometre
%	percentage
PBS	Phosphate-buffered saline
PE	Phycoerythrin
SD	Standard deviation
SS	Side scatter
v/v	volume per volume
w/v	weight per volume

PENGAWALATURAN TINDAK BALAS SELULAR MAKROFAJ OLEH EKSTRAK *Clinacanthus nutans* DALAM SEL MAKROFAJ J774.2

ABSTRAK

Clinacanthus nutans merupakan sejenis tumbuhan perubatan herba dari keluarga 'Acanthaceae', dikenali sebagai Belalai gajah dan 'Sabah Snake Grass' di Malaysia. Ekstrak *C. nutans* dilaporkan mempunyai sifat anti-radang, analgesik, antioksidan, anti-kanser, anti-virus dan anti-bakteria. Namun demikian, kesan ekstrak *C. nutans* terhadap sistem imun dan keupayaan imunomodulasinya masih belum diterokai sepenuhnya sehingga kini. Pengaktifan makrofaj diperlukan untuk membangkitkan sesuatu tindak balas imun. Namun dalam sesetengah difisiensi imun berkait makrofaj, pengaktifan ini tidak mencukupi manakala dalam sesetengah penyakit radang, ia berlaku secara berterusan. Justeru, peranan imunomodulasi ekstrak *C. nutans* dalam mengawalatur tindak balas sel makrofaj dalam makrofaj tikus J774.2 telah dikaji dalam kajian penyelidikan ini kerana hal ini berpotensi untuk memberi manfaat kepada pesakit penyakit radang berkait makrofaj serta pesakit difisiensi imun berkait makrofaj. Sel makrofaj telah dicirikan melalui pemerhatian morfologi dengan menggunakan mikroskop fasa-berbalik dan ekspresi beberapa penanda makrofaj (CD11b, F4/80, CD80 dan CD86) dengan menggunakan sitometri aliran (FACS). Kesan kesitotoksikan ekstrak daun *C. nutans* etanol (EtOH), etanol-akueus (EtOH-AQ) dan akueus (AQ) terhadap makrofaj J774.2 telah ditentukan dengan menggunakan reagen PrestoBlue. Rembesan beberapa jenis sitokin pro- dan anti-radang oleh makrofaj J774.2 yang dirawat 48 jam dengan ekstrak *C. nutans* telah dinilai dengan menggunakan ujian multipleks sitokin berasaskan manik. Aktiviti fagositik makrofaj J774.2 yang dirawat 48 jam dengan

ekstrak *C. nutans* telah dinilai dengan menggunakan biopartikel *E. coli* yang ditag pHrodo hijau dengan menggunakan kaedah mikroskop fluoresens dan sitometri aliran. Akhirnya, ekspresi penanda pengaktifan makrofaj M1 (CD80 dan CD86) dan M2 (CD71 dan CD206) oleh makrofaj J774.2 yang dirawat 48 jam dengan ekstrak *C. nutans* telah dinilai melalui kaedah sitometri aliran. Hasil kajian menunjukkan bahawa makrofaj J774.2 menunjukkan ekspresi CD11b, F4/80 dan CD86 yang tinggi dan kesemua ekstrak yang telah dikaji tidak mempamerkan sebarang kesan sitotoksik yang ketara terhadap makrofaj J774.2 dalam lingkungan kepekatan ekstrak yang diuji. Kesemua ekstrak telah mempamerkan sifat anti-radang dengan mengurangkan penghasilan sitokin pro-radang yang didorong oleh LPS tanpa mengganggu keupayaan fagositik makrofaj J774.2. Ekstrak EtOH-AQ paling menonjol dalam mengekang rembesan sitokin pro-radang dan mengurangkan expresi CD86 yang didorong LPS sekaligus merangsang fungsi fagositik makrofaj J774.2. Namun demikian, kesan ekstrak *C. nutans* terhadap polarisasi makrofaj J774.2 tidak dapat dikenalpasti melalui hasil kajian yang terhad ini. Kajian lanjut perlu dilakukan untuk meneroka dengan lebih mendalam dan mengesahkan peranan ekstrak *C. nutans* dalam pemodulasian pengaktifan dan fungsi makrofaj.

REGULATION OF MACROPHAGE CELLULAR RESPONSE BY *Clinacanthus nutans* EXTRACTS IN J774.2 MACROPHAGE CELL LINE

ABSTRACT

Clinacanthus nutans, which is a medicinal plant from Acanthaceae family, is well-known as 'Belalai gajah' and Sabah Snake Grass in Malaysia. *C. nutans* extracts have been reported to exhibit anti-inflammatory, analgesic, anti-oxidant, anti-cancer, anti-viral and anti-bacterial properties. However, the effects of *C. nutans* extract towards the immune system and its immunomodulatory capabilities have not been well explored until today. Macrophage activation, which is necessary to elicit an immune response, is lacking in certain macrophage related immunodeficiency cases whereas macrophage activation occurs constitutively in certain inflammatory diseases. Thus, the immunomodulatory roles of *C. nutans* extracts in regulating macrophage cellular response in J774.2 mouse macrophages were investigated in this study as it can potentially provide benefits to patients with macrophage related inflammatory diseases as well as to patients with severe macrophage related immunodeficiencies. The macrophage cell line was characterised through morphological observation under inverted phase contrast microscope and expression of a few novel macrophage markers (CD11b, F4/80, CD80 and CD86) using flow cytometric analysis (FACS). The cytotoxicity effect of *C. nutans* leaves ethanol (EtOH), ethanol-aqueous (EtOH-AQ) and aqueous (AQ) extracts on J774.2 macrophages was determined using PrestoBlue assay. The secretion of a mixture of pro- and anti-inflammatory cytokines by the macrophages after 48 hours of extracts incubation was assessed using multiplexed cytokine bead-based assay. The phagocytic activity of 48-hours extracts-treated J774.2 macrophages

was assessed using Green pHrodo-conjugated *E. coli* Bioparticles by both fluorescence microscopy and flow cytometry approaches. Finally, the M1 (CD80 and CD86) and M2 (CD71 and CD206) activation markers expression on the macrophages after 48 hours of extract incubation was assessed using flow cytometry technique. The results showed that J774.2 macrophages have high expression of CD11b, F4/80 and CD86 and all three extracts tested did not exhibit any significant cytotoxic effect towards J774.2 macrophages within the extract concentration range tested. All three extracts displayed their anti-inflammatory properties by reducing LPS-induced inflammatory cytokines secretion without disintegrating the phagocytic ability of the macrophages. EtOH-AQ extract has the highest potential in downregulating LPS-induced inflammatory cytokines production and CD86 expression level as well as stimulating the phagocytic function of J774.2 macrophages. However, the effect of the extracts on J774.2 macrophage polarisation could not be determined with these limited findings. Further studies should be carried out to further explore and confirm the role of *C. nutans* extracts in modulating macrophage activation and function.

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Traditional medicine practitioners around the world use medicinal herbs as a primary healthcare medicine to treat various illnesses. The formulations of herbal plants based traditional medicine are preserved over many generations which have resulted in herbal plant-based drug discovery. The biological properties such as the anti-inflammatory, anti-cancer and anti-microbial activities possessed by medicinal plants have been investigated extensively to explore their therapeutic potential in treating various illnesses including cancer, immune disorders and infectious diseases (Zulkipli et al., 2017).

Plants species from Acanthaceae family possess various potential medicinal properties (Khan et al., 2017). Thus, Acanthaceae family serves as a repertoire of medicinal herbs which would be essential to be implemented as traditional medicines for various health complications (Alam et al., 2016). *Clinacanthus nutans*, a type of herbal plant which has been studied extensively for its medicinal use, is a novel species of Acanthaceae family. *C. nutans* is well-known as 'Belalai gajah' and also as Sabah Snake Grass in Malaysia (Aslam et al., 2015). Herbal tea made of *C. nutans* leaves is famous among Malaysians while fresh drinks are prepared by mixing *C. nutans* leaves with sugarcane, apple juice or green tea in Thailand. Traditional medicine practitioners in Thailand use *C. nutans* to treat snake and scorpion bites (Alam et al., 2016).

The anti-inflammatory (Mai et al., 2016, Le et al., 2017, Khoo et al., 2018), anti-viral (Pongmuangmul et al., 2016, Sakdarat et al., 2017, Haetrakul et al., 2018), anti-bacterial

(Arullappan et al., 2014, Sekar and Rashid, 2016, Nyawai et al., 2017), anti-oxidant (Yong et al., 2013, Lee et al., 2014, Akowuah et al., 2018) and anti-proliferative (Kong and Abdullah Sani, 2017, Quah et al., 2017, Teoh et al., 2017, Zakaria et al., 2017, Roslan et al., 2018) properties of extracts made from this plant parts were studied previously.

However, there are limited reports on *C. nutans* activity towards the immune system and its immunomodulatory capabilities have not been explored well until today. Sriwanthana et al. (1996) stated that lower doses of *C. nutans* ethanol extract significantly increased lymphocyte proliferation and induced IL-4 expression at higher extract concentrations. While another study reported that methanolic crude extract of *C. nutans* is able to reduce neutrophil migration (thus, impede neutrophil responsiveness) and as such possessed a significant anti-inflammatory property. (Wanikiat et al., 2008). Meanwhile, *C. nutans* aqueous extract displayed great anti-inflammatory property by suppressing nitric oxide (NO) production by LPS and IFN-γ-stimulated RAW 264.7 macrophages (Khoo et al., 2018). Besides that, polar and non-polar leaf and stem extracts of *C. nutans* inhibited LPS-challenged pro-inflammatory cytokines secretion, thus revealing the immunomodulatory capabilities possessed by *C. nutans* extracts (Mai et al., 2016b). In addition, results from previous studies performed in our lab showed that *C. nutans* polar leaf extracts were capable of modulating IL-4 cytokine production in U937 monocytes and RAW 264.7 macrophages (unpublished data). Findings from these studies suggested that the potential immunoregulatory properties of *C. nutans* extracts should be further investigated.

Cells of the innate immune system such as macrophages, dendritic cells, mast cells and neutrophils become activated upon encountering invading foreign substances and tissue damage. These activated effector cells clear the infectious particles and host debris by phagocytosis and also triggers inflammatory cytokines secretion to initiate T-cell-mediated adaptive immune response. Thus, an inflammatory response is crucial to fight against infections and to heal tissue injury (Newton and Dixit, 2012). However, unregulated hyperinflammatory response causes over-production of inflammatory mediators that leads to chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease (IBS), Crohn's disease, cancer and others.

1.2 Problem statement

Macrophage activation which is necessary to elicit an immune response is lacking in certain macrophage related immunodeficiency cases whereas macrophage activation occurs constitutively in certain inflammatory diseases. Thus, macrophage activation should be regulated in a proper manner to fight against pathogenic invaders and concurrently to prevent chronic inflammation that leads to inflammatory diseases. Taking into account the increasingly popular use of *C. nutans* and its potential benefits, this study was carried out to focus on investigating the immunomodulatory role of *C. nutans* extracts in macrophages and their effect in regulating macrophage function. Such study is crucial in providing scientific evidence of its potential and benefits particularly to patients with macrophage related inflammatory diseases and those with severe macrophage related immunodeficiencies.

1.3 Objectives of the research

Main objective:

To determine the immunomodulatory role of *C. nutans* extracts in regulating macrophage cellular response in J774.2 mouse macrophages.

Specific objectives:

i) To determine the cytotoxicity effect of the extracts on J774.2 cells.

ii) To assess the secretion of cytokines in the extracts-treated J774.2 cells.

iii) To assess the phagocytic function of the extracts-treated J774.2 cells.

iv) To assess the expression of macrophage activation markers in extracts-treated J774.2 cells.

Hypothesis:

C. nutans extract should display some immunomodulatory role in regulating macrophage activation and function of J774.2 macrophages without disintegrating the phagocytic function of the macrophages.

CHAPTER 2

LITERATURE REVIEW

2.1 *Clinacanthus nutans*

2.1.1 Origin, nomenclature & structural features of *C. nutans*

C. nutans is a type of medicinal plant with various therapeutic potential which have not been fully explored and elucidated yet (Aslam et al., 2015). It is a small shrub which belongs to the Acanthaceae family and can be found in China and Southeast Asia, especially in Malaysia and Thailand (Tuntiwachwuttikul et al., 2004). This plant is taxonomically classified and nomenclature as follows (Yahaya et al., 2015):

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Lamiales

Family: Acanthaceae

Genus: *Clinacanthus*

Species: *nutans*- Lindau

Scientific name: *Clinacanthus nutans* (Burm. f.) Lindau

Figure 2.1: The *C. nutans* plant.

Figure 2.2: The lanceolated leaves of *C. nutans* plant.

This plant is able to grow until about 1 to 3-meter-tall in height with matured branches (Figure 2.1). The leaves of this plant are lanceolate, long, thin and arranged oppositely, whereas the stems are cylindrical, smooth and striated (Figure 2.2) (Zulkipli et al., 2017). This plant is also known as Daun Belalai Gajah (elephant's trunk leaf) in Malay as it has slightly curved stems which bear resemblance to an elephant's trunk (Shim et al., 2013). Besides that, this plant is also called "Sabah snake grass" in Malaysia as it can be found easily in Sabah, which is located in East Malaysia (Yahaya et al., 2015). This plant is known as Dandang Gendis and Ki tajam (Sunda) in Indonesia, while it is known as Phaya yo and Phaya plongtong in Thailand and as twist of flowers, alligator flower and e zuihia in China (Alam et al., 2016).

2.1.2 Phytochemicals extracted from *C. nutans*

A few novel bioactive metabolites that have been extracted from different plant parts of *C. nutans* are summarised in Table 2.1. A polysaccharide-peptide complex (CNP-1-2), which possesses potential anti-proliferative activity against SGC-7901 human gastric cancer cells, was isolated from *C. nutans* leaves (Huang et al., 2016). Janwitayanuchit et al. (2003) reported that 1,2-O-dilinolenoyl-3-O-b-D-glucopyranosyl-sn-glycerol exhibited the highest anti-viral activity towards HSV-1 and HSV-2 among 19 monoglycosyl diglycerides synthesised and investigated by them. Moreover, glycoglycerolipids and chlorophyll a and chlorophyll b related compounds, which were 13^2-hydroxy-(13^2-R)-pheophytin B, 13^2-hydroxy-(13^2-S)-pheophytin a and 13^2-hydroxy-(13^2-R)-pheophytin A, also possess some anti-viral property against HSV (Satakhun, 2001, Sakdarat et al., 2009). Meanwhile, Khoo et al. (2018) suggested that sulfur-containing compounds (clinamide A, B and C), sulfur-containing glucosides,

Table 2.1: Metabolites isolated from different parts of *C. nutans* plant.

Plant part	Metabolites	References
Leaf	Cerebrosides, monoacylmonogalactosylglycerol	(Tuntiwachwuttikul et al., 2004)
	13^2-hydroxy-(13^2-S)-chlorophyll b, 13^2-hydroxy-(13^2-R)-chlorophyll b, 13^2-hydroxy (13^2-S)-phaeophytin b, 13^2 -hydroxy-(13^2-R)-phaeophytin b, 13^2-hydroxy-(13^2-S)-phaeophytin a, 13^2-hydroxy-(13^2-R)-phaeophytin a, purpurin 18 phytyl ester, phaeophorbide-a	(Sakdarat et al., 2006, Sakdarat et al., 2009)
	Saponins, phenolics, flavonoids, diterpenes, phytosterols	(Yong et al., 2013)
	C-glycosidic flavones (vitexin, isovitexin, shaftoside, orientin and isoorientin)	(Chelyn et al., 2014, Latiff et al., 2017)
	Polysaccharide–peptide complex (CNP-1-2)	(Huang et al., 2016)
	Sulfur-containing compounds (clinamide A, B and C), sulfur-containing glucosides, phytosterols, triterpenoids, flavones, organic and amino acids	(Khoo et al., 2018)
	19-Oxo-all-trans-retinoic acid	(Roslan et al., 2018)
Aerial	Clinamides A-C, 2-*cis*-entadamide A and its geometric isomer (entadamide A)	(Tu et al., 2014)
	Flavonoids (shaftoside, apigenin 6,8-C-a-	

	L-pyranarabinoside, orientin, isoorientin, vitexin and isovitexin)	(Huang et al., 2015)
Stem	Lupeol, β-sitosterol Stigmasterol, phenolic acids, terpenoids, inositol, cyclitol, sulfur-containing glycosides, fatty acids and organic acids	(Dampawan et al., 1977) (Alam et al., 2017)
Stem and leaf	Sulfur-containing glucosides (clinacoside A and B)	(Teshima et al., 1998)
Callus and cell suspension cultures	Quercetin, catechin, luteolin	(Phua et al., 2018)

phytosterols, triterpenoids, flavones and some organic and amino acids present in *C. nutans* leaves aqueous extract contributed to the potential anti-inflammatory property of the extract. 19-Oxo-all-trans-retinoic acid from *C. nutans* leaves chloroform extract was reported to be efficient in inhibiting the proliferation of human cervical cancer, HeLa cells and hence, would be useful in cervical cancer treatment (Roslan et al., 2018).

2.1.3 Traditional uses of *C. nutans*

Plant parts of *C. nutans* have been used as traditional medicines in several countries including Malaysia. *C. nutans* serves as anti-venom, anti-inflammatory, analgesic, anti-diabetic, anti-rheumatism, anti-viral and anti-oxidant for traditional medicine practitioners in these countries (Arullappan et al., 2014). The fresh leaves of this plant are used in the preparation of herbal tea in our country while people in Thailand and Indonesia consume *C. nutans* by boiling the fresh leaves with water to treat dysuria, dysentery and diabetes (Alam et al., 2016). Meanwhile, scientists in Thailand discovered

that *C. nutans* can also be used to treat dysentery and fever. The anti-cell lysis property possessed by this plant had contributed to its use as an anti-venom for snake and scorpion bites and the removal of nettle rashes. While, Chinese traditional medicine practitioners use *C. nutans* to regulate normal menstruation, alleviate pain, anaemia, repairing bone cracks and jaundice (Arullappan et al., 2014).

Moreover, this plant is used to treat inflammatory conditions such as haematoma, eye bruises, anxieties, injuries and rheumatism due to its anti-inflammatory property (Arullappan et al., 2014). In Thailand, alcoholic extract made from fresh leaves of *C. nutans* is used externally to heal skin rashes, snake and insect bite, herpes simplex virus (HSV), and varicella-zoster virus (VZV) lesions (Sookmai et al., 2011). This plant is also widely used in our country to treat kidney problems, liver cancer, nasal cavity cancer, uterine fibroid, gout and urinary neuropathies (Arullappan et al., 2014). *C. nutans* had been approved to be used in the treatment of herpes simplex, herpes zoster and skin psoriasis in Primary Health Care Programme in Thailand (Wanikiat et al., 2008). Besides that, various types of commercial products such as concentrated liquid drinks, soap, tea, balm, cream, massage ointments and capsules made from this popularity gaining plant are available in the market (Zulkipli et al., 2017).

2.1.4 Properties of *C. nutans*

2.1.4 (a) Anti-proliferative properties

The anti-proliferative properties of extracts from *C. nutans* were also studied previously. Sulaiman et al. (2015) reported that *C. nutans* leaves ethanol and ethyl acetate extracts caused a significant decrease in proliferation of MCF-7 human breast

cancer estrogen positive cells with IC_{50} values of 24.04 ± 1.7 μg/mL and 28.90 ± 2.1 μg/mL, respectively. In addition, 35 and 30 μg/mL *C. nutans* root methanol and ethyl acetate extracts, respectively, inhibited 50% proliferation of MCF-7 cells. However, these extracts only inhibited about 40% cell viability of human cervical cancer, HeLa cells at maximum extract concentration (50 μg/mL) tested (Teoh et al., 2017). On the other hand, *C. nutans* leaves aqueous extract significantly suppressed the growth of HeLa cells with IC_{50} 13±0.82 μg/mL but methanol leaves extract failed to inhibit HeLa cells even at the maximum extract concentration 50 μg/mL (Zakaria et al., 2017). Moreover, 19-Oxo-all-trans-retinoic acid, which is isolated from *C. nutans* leaves chloroform extract, exerted potential anti-proliferative activity on HeLa cells with IC_{50} 27 ± 2.6 μg/mL (Roslan et al., 2018). This shows that different active compounds present in different extracts might have exerted different effects on the survival of different types of cancer cells.

Besides, *C. nutans* hexane and chloroform extracts showed significant inhibition on the proliferation of lung cancer (A549), nasopharyngeal cancer (CNE1) and liver cancer (HepG2) cells with IC_{50} values ranging from 25 to 200 μg/mL (Kong and Abdullah Sani, 2017). 138.82 ± 0.60 μg/mL *C. nutans* aqueous extract significantly inhibited 50% growth of A549 lung cancer cells (Fazil et al., 2016) while 43.9367 μg/mL *C. nutans* methanol extract suppressed 50% viability of HepG2 cells (Hamid and Yahaya, 2016). Meanwhile, HepG2 and breast cancer oestrogen negative (MDA-MB-231) cells were inhibited by *C. nutans* methanol leaves extract with IC_{50} values 13.33 μg/mL and 18.67 μg/mL, respectively (Quah et al., 2017). A crude methanol extract of *C. nutans* also displayed strong cytotoxicity effect towards D24 melanoma cells with IC_{50} of 950

μg/mL (Fong et al., 2016). *C. nutans* leaves chloroform extract displayed significant cytotoxic effect towards human erythroleukemia (K-562) and human Burkitt's lymphoma (Raji) cell lines with IC_{50} values 47.7 μg/mL and 47.31 μg/mL, respectively. Furthermore, 100 μg/mL of this extract inhibited almost 91.28 ± 0.03% and 88.97 ± 1.07% activity of K-562 and Raji cells, respectively (Yong et al., 2013). *C. nutans* extract only displayed cytotoxicity effect towards cancer cell lines tested and were less toxic towards normal cells used as controls in these studies. The selective inhibition of cancer cells by *C. nutans* extracts reveals its prominent anti-cancer property, thus, signifies the potential of this plant to be used in cancer treatment.

2.1.4 (b) Anti-oxidant properties

The anti-oxidant properties of *C. nutans* extracts were investigated previously. *C. nutans* ethanolic leaves extract (1-300 μg/mL) showed dose-dependent free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity compared to ascorbic acid used as the positive control. The extract scavenged 50% of DPPH at 110.4 ± 6.59 μg/mL, however, the extract only inhibited 67.65% DPPH at the highest extract concentration studied. Moreover, 30, 100 and 300 μg/mL of the extract also significantly suppressed the production of PMA-stimulated free radicals by rat macrophages (Pannangpetch et al., 2007). *C. nutans* methanolic leaves extract was shown to suppress 50% DPPH at 1126.63 μg/mL (Lee et al., 2014). Besides, 4.0 mg/mL *C. nutans* petroleum ether leaves extract and 10.0 mg/mL *C. nutans* methanolic stems extract scavenged 82.0% and 70.0% of DPPH radical, respectively, compared to ascorbic acid and α-tocopherol used as positive controls (Arullappan et al., 2014).

Yong et al. (2013) showed that *C. nutans* leaves chloroform extract was very efficient in scavenging DPPH and galvinoxyl radicals compared to aqueous and methanolic extracts. However, this chloroform extract failed to scavenge NO and hydrogen peroxide (H_2O_2) radicals. Meanwhile, *C. nutans* aqueous and methanolic extracts negated approximately 30% of NO and H_2O_2, respectively, at highest extract concentration (100 µg/mL) tested (Yong et al., 2013). In addition, Akowuah et al. (2018) also demonstrated that *C. nutans* leaves methanolic and aqueous extracts (50-400 µg/mL) exhibited potential dose-dependent NO scavenging activities. These findings suggested that different types of *C. nutans* extracts possess anti-oxidant activity specific for certain species of free radicals probably due to the different types of active constituents present in them.

2.1.4 (c) Anti-inflammatory properties

In 1996, Sriwanthana and his co-workers discovered that lower concentrations (0.5, 2.5 and 5 µg/mL) of *C. nutans* extract significantly induced lymphocyte proliferation while higher extract concentrations (2.5 and 5 mg/mL) significantly suppressed lymphocyte proliferation and natural-killer cells activity in human immunocompetent peripheral blood mononuclear cells (PBMC). The upregulation of IL-4 cytokine secretion at higher extract concentrations (2.5 and 5 mg/mL) employed might have reduced lymphocyte activation and proliferation at such doses. This is because IL-4 could suppress IL-2 induced proliferation of peripheral blood cells. Besides that, the reduced activity of NK cells was also due to the upregulation of IL-4 cytokine since IL-4 exhibit an inhibitory effect on the function of NK cells (Sriwanthana et al., 1996).

Moreover, Le et al. (2017) reported that stigmasterol and β-sitosterol isolated from hexane fractions made from *C. nutans* leaves suppressed proliferation of Concanavalin A (ConA)-induced T cell proliferation in murine splenocytes culture. However, only β-sitosterol significantly inhibited the proliferation of T helper cells ($CD4^+CD25^+$) and cytotoxic T cells ($CD8^+CD25^+$) upon ConA-induced T cell activation and inhibited Th2 (IL-4 and IL-10) cytokines secretion by helper T cells. The binding of these compounds competitively or non-competitively to T cell receptor (TCR) on T cells might have inhibited T cell activation and secretion of cytokines. These compounds might also have interfered antigen presentation by antigen-presenting cells or blocked TCR activation on naive T cells since ConA-induced T cell activation involves antigen-presenting cells (Le et al., 2017).

Besides that, the anti-inflammatory property of *C. nutans* methanolic crude extract on neutrophils functions and migration was studied using EPP-induced ear oedema and carrageenan-induced paw oedema in rat models. Results from this study stated that *C. nutans* extract significantly inhibited fMLP-induced chemotaxis, myeloperoxidase (MPO) activity, superoxide anion generation, and MPO and elastase release, which reflects reduced neutrophil migration and promotion of healing process although neutrophil apoptosis was not affected. This proves the strong anti-inflammatory property of *C. nutans* extract that produced inhibitory effects on neutrophil functions and migration (Wanikiat et al., 2008). This is parallel to findings from Tu et al. (2014) which reported that 10 µg/mL of 80 % ethanol extract made from aerial parts of *C. nutans* strongly inhibited neutrophil elastase release with an inhibition rate of 68.33% when

MeO-Suc-Ala-Ala-Pro-Valp-nitroanilide was used as the elastase substrate in elastase release experiment.

Khoo et al. (2018) demonstrated that *C. nutans* leaves aqueous, 20%, 50%, 70% and 100% ethanol extracts suppressed nitric oxide (NO) production by LPS and IFN-γ-stimulated RAW 264.7 macrophages. However, only aqueous extract strongly inhibited the NO production with half-maximal inhibitory concentration, IC_{50} 190.43 ± 12.26 µg/mL compared to curcumin used as the positive control. This anti-inflammatory activity displayed by the aqueous extract was associated with the active constituents present in the extract, which were phytosterols, flavones, sulfur-containing glucosides, sulfur-containing compounds, triterpenoids, and some organic and amino acids (Khoo et al., 2018).

2.1.4 (d) Anti-bacterial properties

Apart from that, Arullappan et al. (2014) stated that fractions isolated from *C. nutans* ethyl acetate leaves extract showed strongest anti-bacterial activity towards *Bacillus cereus* at minimum inhibitory concentration (MIC) of 1.39 mg/mL and this is associated with the presence of flavonoids in the plant, which have enhanced the anti-bacterial response. This is due to the action of carbonyl groups present in the flavonoids where the functions of some vital enzymes such as xanthine oxidase, aldose reductase, ATPase and phosphodiesterase have been shut down and hence, blocking the surveillance of the microbes (Arullappan et al., 2014). Moreover, *C. nutans* acetone leaves and stems extract was shown to inhibit the growth of *B. cereus* with MIC 12.5 mg/mL (Kong and Abdullah Sani, 2017). On the other hand, Ho et al. (2013) reported that *C. nutans*

methanolic leaves crude extract did not inhibit the activity of *B. cereus* (MIC > 12.5 mg/mL).

C. nutans extracts did not display any significant inhibition at all concentrations tested for anti-bacterial activity against methicillin-resistant *Staphylococcus aureus* (Chomnawang et al., 2009), *Propionibacterium acnes* and *Staphylococcus epidermidis* (MIC > 5 mg/mL) (Chomnawang et al., 2005). However, *C. nutans* methanolic leaves extract significantly inhibited the activity of *S.aureus* and *Escherichia coli* with MIC 12.5 mg/mL, but not *P. acnes* and *S. epidermidis* (MIC > 12.5 mg/mL) (Yong et al., 2013). Meanwhile, Kong and Abdullah Sani (2017) showed that *C. nutans* acetone extract suppressed *S. aureus, S. epidemidis, E. coli, B. subtilis, Salmonella typhimurium, Shigella boydii, Klebsiella pneumonia, Listeria monocytogenes* and *Pseudomonas aureginosa* activities at MIC 12.5 mg/mL. Findings from these studies suggest that different active compounds present in different types of *C. nutans* extracts might possess anti-bacterial activity specific for some bacterial strains. Sekar and his colleague demonstrated that *C. nutans* methanolic leaves extract and ointment formulated from the extract exhibited anti-bacterial activity against *S. aureus, B. cereus, E. coli* and *P. aeruginosa* and thus, suggesting that *C. nutans* extract might be useful in developing ointments for external use (Sekar and Rashid, 2016).

2.1.4 (e) Anti-viral properties

In 1992, Jayavasu and his co-workers discovered that *C. nutans* leaf extract possesses anti-viral activity towards herpes-simplex virus type 2 (HSV-2). The extract showed inhibition of plaque formation by HSV-2 in baby hamster kidney cell line (Jayavasu et

al., 1992). Kunsorn et al. (2013) also reported that n-hexane, dichloromethane and methanol extracts of *C. nutans* inhibited 50% of plaque formation by HSV-1 (KOS) and HSV-2 (Baylor186) viruses at 100 μg/mL in Vero cells. Furthermore, a few *in vitro* studies performed had declared that active metabolites isolated from *C. nutans*, which include monoglycosyl diglycerides, glycoglycerolipids and three pure chlorophyll a and chlorophyll b related compounds ('13^2-hydroxy-(13^2-R)-phaeophytin b', '13^2-hydroxy-(13^2-S)-phaeophytin a' and '13^2-hydroxy-(13^2-R)-phaeophytin a'), were found to exhibit anti-HSV activity (Satakhun, 2001, Janwitayanuchit et al., 2003, Sakdarat et al., 2009). Sakdarat et al. (2009) stated that the virus was suppressed by the three chlorophyll derivatives isolated from *C. nutans* chloroform extract prior to the virus entry into the host cells by interfering with the adsorption or penetration processes of the virus into the host. Meanwhile, monogalactosyl diglyceride and digalactosyl diglyceride isolated from *C. nutans* leaves chloroform extract inhibited HSV-1 and HSV-2 viral replication process post-infection in Vero cells (Pongmuangmul et al., 2016). Based on these findings, *C. nutans* extract might serve as a potential target in drug development against HSV-1 and HSV-2 viruses.

However, Yoosook et al. (1999) reported some contrasting findings, which stated that *C. nutans* methanol extract did not display significant intracellular anti-viral activity against HSV-2 in Vero cells, compared to the acyclovir used as the positive control. Yoosok et al. also demonstrated that *C. nutans* aqueous extract did not exhibit any significant effect on HSV-1 and HSV-2 viral activities in Vero cells (Yoosook et al., 1999, Yoosook et al., 2000). Different extract concentrations and extraction techniques employed that might have yielded different active constituents, may probably

contributed to the differences in the results obtained (Yoosook et al., 1999). Moreover, *C. nutans* extract significantly inactivated Varicella-zoster virus, which is a type of herpes virus that causes chickenpox upon infection and zoster after reactivation (Thawaranantha et al., 1992). This corresponds to an *in vivo* study conducted to investigate the efficacy of *C. nutans* extracts in the treatment of *Herpes genitalis* and *Herpes zoster* based on randomised clinical trials and discovered that *C. nutans* extract might be useful in treating *H.genitalis* with HSV-2 infection. This is because routine use of cream made from *C. nutans* resulted in crust formation on lesion within 3 days and complete healing within 7 days compared to placebo (Kongkaew and Chaiyakunapruk, 2011).

Besides that, *C. nutans* ethanolic extract prepared from aerial parts of the plant was found to exhibit considerable anti-dengue virus activity at IC_{50} 31.04 μg/mL when studied using naïve Huh-7 cells (Tu et al., 2014). Meanwhile, compound 2 (phaeophorbide a), among the four chlorophyll a and chlorophyll b related compounds isolated from *C. nutans* hexane and chloroform leaf crude extracts, was reported to suppress the synthesis of viral RNA and proteins of dengue virus 2 in A549 infected cells (Sakdarat et al., 2017). Furthermore, Sookmai et al. (2011) discovered that compounds extracted from *C. nutans*, which were 136C and 136D, displayed anti-human papillomavirus (HPV16) activity since direct binding of these compounds between viral particles and host cell receptor suppressed the adsorption of virus onto host cells and hence, prevented virus entry into host cells. Therefore, *C. nutans* extract might provide a new platform for efficient treatment of dengue fever and cervical cancer, respectively.

Apart from that, 500 µg/mL *C. nutans* ethanolic crude extract was proven to be effective against fish pathogenic viruses like infectious hematopoietic necrosis virus (IHNV) and Oncorhynchus masou virus (OMV) (Direkbusarakom et al., 1996). *C. nutans* ethanolic leaf extract also inhibited yellow-head rhabdo-like virus (YRV) activity at MIC of 1 µg/mL when tested in black tiger shrimp (Direkbusarakom et al., 1998). In this context, Direkbusarakom et al. (1998) suggested that inhibition of virus by the extract occurs through the reaction between the viral envelope and the extract. This is because *C. nutans* extract was found to be efficient against enveloped viruses such as YRV, OMV, IHNV and herpes simplex virus but less efficient in inactivating non-enveloped virus-like infectious pancreatic necrosis virus (IPNV) (Direkbusarakom et al., 1996, Direkbusarakom et al., 1998). Recently, *C. nutans* crude extract was reported to exhibit efficient anti-viral activity against cyprinid herpesvirus 3 (CyHV-3) or koi herpesvirus (KHV) both pre- and post- infections and thus, would be efficient in treating viral infection in common carp and koi aquaculture (Haetrakul et al., 2018).

C. nutans possesses various potential properties that should be further explored to fully exploit the benefits of this plant to mankind. The immunomodulatory property of extracts made from this novel species is further explored in this study in order to reveal the therapeutical potential of this medicinal plant.

2.2 Macrophages

2.2.1 The role of macrophages in the immune system

The immune system is composed of innate and adaptive immune responses. The innate immunity, which is comprised of epithelial barriers, phagocytes (macrophages,

neutrophils, dendritic cells and natural killer cells) and the proteins of the complement system, provides the first line of defense against invading foreign substances such as microbes, cells and their constituents (Tsirogianni et al., 2006). On the other hand, the adaptive immunity provides the second line of defense, which is more specific and effective, towards the invading foreign molecules. Furthermore, the adaptive immune response is further divided into cellular and humoral immunity where cellular immunity is mediated by T-lymphocytes and their cytokines, while humoral immunity is mediated by B-lymphocytes and antibodies secreted by them (Alberts et al., 2002, Tsirogianni et al., 2006).

Monocytes derived from bone marrow enter circulating blood and later differentiate and give rise to macrophages (Gordon & Taylor, 2005). Macrophages play a key role in both innate and adaptive immune responses (Koppensteiner et al., 2012). Macrophages which stay longer in the same tissue are termed as fixed macrophages while macrophages which travel to different tissues through blood capillaries are termed as the wandering macrophages (Wood, 2006). Macrophages are termed based on their location of origin in the body. For example, macrophages in the liver, lungs and brain (central nervous system) are termed as Kupffer cells, alveolar macrophages and microglia, respectively.

The human's body reacts towards infections through an inflammatory process which increases blood supply to the infected area and increases the permeability of blood capillaries to ease the transfusion of white blood cells such as macrophages, monocytes and neutrophils (Olsson, 2006). Once reaching the site of infection, macrophages act as professional phagocytes that engulf exogenous pathogens and as antigen-presenting cells

which present antigenic peptides to CD4 T cells via MHC class II pathway (Koppensteiner et al., 2012). The phagocytosed exogenous pathogens derived antigens are degraded in lysosome into smaller fragments and transported to the cell surface to be presented by MHC class II molecule to naïve CD4 T cells. Then the activated CD4 T cells secrete cytokines to initiate adaptive immune responses to trigger antibody responses through the proliferation of B-lymphocytes and production of antibodies (Storni and Bachmann, 2004, Wood, 2006). Meanwhile, when a macrophage is infected with viral particles, these endogenous pathogen-derived antigens are degraded in proteasome. The processed antigenic peptides are then transported to the cell surface for presentation to naïve CD8 T cells by MHC class I molecule. The activated CD8 T cells induce the apoptosis of the macrophage to prevent the spread of the viral infection (Wood, 2006, Leone et al., 2013).

2.2.2 Types of macrophage activation

Morphologically, activated macrophages are usually larger in size with irregular plasma membrane, extending pseudopods and increased adherence and spreading ability compared to resting macrophages (Olsson, 2006). There are three different types of macrophage activation, which are the classical activation (M1), alternative activation (M2) and type 2-activation of macrophages, where the biological function of each population of activated macrophages differs from the other (Mosser, 2003). Hence, all three populations of activated macrophages work as a team in preventing diseases. Exposure to IFN-γ and pathogenic products such as LPS results in classical activation of macrophages and secretion of inflammatory cytokines such as TNF, IL-12, IL-1 and IL-6, which act as the effector cells to boost Th1 immune response. On the other hand,

exposure to IL-4 results in alternative activation of macrophages and secretion of anti-inflammatory cytokines such as IL-10, which plays a major role in the regulatory and healing process (Stein et al., 1992, Mosser, 2003). Classically activated macrophages possess increased antigen presenting and pathogen killing capability while alternatively activated macrophages possess enhanced phagocytic ability but decreased pathogen killing ability (Pace and Russell, 1981, Stein et al., 1992, Lee et al., 2013). Meanwhile, Toll-like receptors (TLRs) ligations result in type 2-activated macrophages which secretes IL-10 and involves in type-2 humoral immune response that triggers antibody production by B-lymphocytes towards antigen (Mosser, 2003).

Unlike M1 activated macrophages, M2 activated macrophages are further sub-divided into a few categories namely M2a, M2b, M2c and M2d (Figure 2.3). Exposure to IL-4, IL-13 or helminth and fungal infections induces M2a activated macrophages, exposure to immune complexes and LPS induces M2b activated macrophages while exposure to IL-10, transforming growth factor (TGF)-β and glucocorticoid hormones induce M2c activated macrophages (Mantovani et al., 2004). Hence, the type-2 activated macrophages proposed earlier by Mosser were classified as M2b by Mantovani and his co-workers (Mantovani et al., 2004). Finally, exposure to IL-6 and adenosines induce the fourth group, which is M2d activated macrophages (Wang et al., 2010, Ferrante et al., 2013).

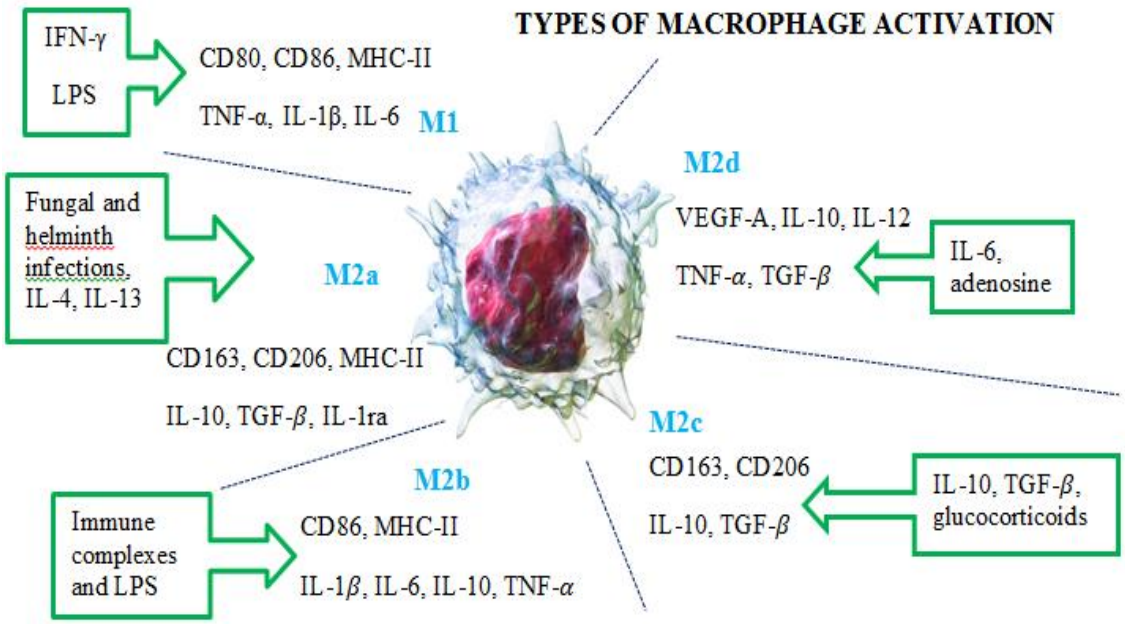

Figure 2.3: The full spectrum of macrophage activation. The figure shows the list of cytokines and markers associated with each macrophage activation phenotype. [Edited from (Rőszer, 2015, Rojas et al., 2015)].

2.2.3 The role of macrophages in pathogenesis of diseases

Over-activation of macrophages causes hyperinflammation which then leads to inflammatory diseases such as macrophage activation syndrome (MAS) that is mostly found in rheumatic diseases of childhood. The excessive pro-inflammatory cytokines secreted by constitutively activated macrophages in patients with MAS results in uncontrollable fatal inflammation since the cytotoxic T cells failed to promote apoptosis of activated macrophages to regulate the homeostasis within the immune system. Moreover, regulatory pathways like IL-10 are suppressed in patients with severe MAS (Schulert and Grom, 2014).

Besides that, the failure of macrophages to abolish cancerous cells causes inflammation which leads to the growth of tumour (Lee et al., 2013). Previous studies had revealed the correlation between macrophage and cancer by elucidating that the rate of development of cancer is affected by the amount of macrophages present within a tumour, production of macrophage-derived angiogenic cytokines, the antigen presentation and phagocytosis ability of apoptotic cells by macrophages (Coussens and Pollard, 2011, Yi et al., 2011).

Apart from that, macrophages also played a crucial role in the pathogenesis of atherosclerosis. Atherosclerosis is the thickening of the arterial wall due to the accumulation of cholesterol (Lee et al., 2013). Atherogenic modified low-density lipoprotein (LDL), which is recognised by macrophages through TLRs and scavenger receptors, are phagocytosed through a different unregulated phagocytosis pathway. This contributes to the accumulation of lipid-laden macrophages (foam cells) that forms atherosclerotic lesions in the arteries (Bobryshev et al., 2016). Since the modified LDL itself is an inflammatory mediator, the uptaken modified LDL enhances the polarisation of macrophages into a pro-inflammatory phenotype that secretes inflammatory cytokines that further reinforce the uptake of modified LDL by macrophages. Hence, increases the cholesterol build up in the arteries (Lee et al., 2013, Bobryshev et al., 2016).

Moreover, prominent changes in the amount and inflammatory features of adipose tissue macrophages (ATM) plays a vital role in the evolution of obesity (Boutens and Stienstra, 2016), which in turn reveals the connection between obesity and macrophages. During the development of obesity, the number of classically activated ATM, which induce aerobic glycolysis increases and contributes to adipose tissue inflammation.

Conversely, alternatively activated macrophages, which promote oxidative metabolism to regulate the homeostasis level in adipose tissue, predominate in lean individuals (Castoldi et al., 2016). Obesity is often related to chronic inflammatory condition in which the inflammatory cytokines secreted by adipose tissue macrophages attracts more macrophages and leads to accumulation of classically activated macrophages in the adipose tissue (Lee et al., 2013). This chronic inflammatory condition in adipose tissue is the major factor that contributes to the development of insulin resistance and type 2 diabetes in obesity (Boutens and Stienstra, 2016).

On the other hand, there are a few primary immunodeficiencies which deteriorate the function of macrophages. Immunodeficiency is a condition where the capability of the immune system to resist or fight against infections is reduced or totally absent. Primary immunodeficiency is inherited and present since birth, while secondary immunodeficiency develops later in life, such as Acquired Immunodeficiency Syndrome (AIDS) which develops upon Human Immunodeficiency Virus (HIV) infection (Wood, 2006). Deficiency in macrophage activation results in failure of the immune system in prompting an immune response against any infections and hence, causes recurrent infections and muted T- and B-cell responses. Inherited IFN-γ receptor deficiency, is an example of these diseases. Patients with this deficiency have complications in macrophage activation and inflammatory cytokines secretion. Besides that, defects in the phagocytosis process of pathogens by macrophages such as deficiency in actin polymerisation, interfere the formation of phagocytic vesicle. Moreover, defects in TLR signalling pathways result in failure to recognise pathogens and hence, causes ‘cold infections’ or infections where inflammation and fever response are impaired. Defects in