ANTI-INFLAMMATORY ACTIVITY

OF *Centella asiatica*

by

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percent
µg/ mL	Microgram per milliliter
µM	Micromolar
CO_2	Carbon dioxide
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FBA	Fetal bovine serum
g	gram
HRP	Horseradish peroxidase
H_2O_2	Hydrogen peroxide
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
mL	milliliter
MTT	3-(4.5-dimethythiazol-2-yl)-2.5-diphenyltetrazolium bromide
NaCl	Sodium chloride / Natrium chloride
NED	N- [1-naphthyl]-ethylenediamine dihydrochloride
NO	Nitric oxide
NaOH	Sodium hydroxide / Natrium hydroxide
NOS	Nitric oxide synthase
ng/mL	Nanogram per milliliter
nm	Nanometer

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
Pen-Strep	Penicillin-streptomycin
SEM	Standard error of mean
SDS	Sodium dodecyl sulphate
U/ μL	Unit per microliter

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ABSTRAK

Centella asiatica (C.asiatica) merupakan ahli kumpulan tumbuhan tropika yang banyak digunakan dalam perubatan tradisional sebagai anti-inflamasi dan untuk penyembuhan luka. Makrofaj memainkan peranan utama dalam tindak balas sistem imun semula jadi. Peningkatan peranan makrofaj akan merangsang dan mempercepatkan tindak balas sistem imun terhadap sebarang rangsangan. Kajian ini dilakukan untuk mengkaji kesan ekstrak methanol daripada daun, petiol dan akar *C.asiatica* pada kepekatan yang berbeza terhadap aktiviti anti-inflamasi sel makrofaj J774A.1. Viabiliti sel makrofaj dikaji dengan menggunakan asai proliferasi sel MTT dan aktiviti anti-inflamasi *C.asiatica* ditentukan dengan mengukur kesannya terhadap penghasilan H_2O_2 dan NO oleh sel tersebut. Keputusan menunjukkan ekstrak methanol daun tumbuhan tersebut meningkatkan proliferasi sel makrofaj J774A.1 pada kepekatan 150 µg/Ml. Data dianalisis dengan menggunakan analisis statistik ANOVA dan ujian Tukey 'post hoc'. Ekstrak daun *C.asiatica* mempunyai kesan anti-inflamasi yang signifikan dari segi proliferasi sel, menghalang penghasilan NO dan H_2O_2 dalam kadar yang bergantung secara langsung kepada kepekatan berbanding kumpulan kawalan.

Kata kunci: *C.asiatica,* sel makrofaj J774A.1, anti-inflamasi, proliferasi, H_2O_2, NO.

ABSTRACT

Centella asiatica (C.asiatica) is a member of a tropical plant family, traditionally used as anti-inflammation and for wound healing. Macrophage plays a primary role in innate response of immune system. Enhancing the role of macrophage would result in promoting and accelerating the response of our immune system towards any form of stimuli. The present study was designed to assess the effects of different concentrations of methanolic extract of leaves, petiole and root of *C.asiatica* (50, 100 and 150 µg/mL) on anti-inflammatory activity of J774A.1 macrophage cell line. The viability of macrophage cells stimulated with the plant extracts was initially investigated by MTT cell proliferation assay and the anti-inflammatory activity of *C.asiatica* was determined by measuring its effect on the H_2O_2 and NO production by the stimulated macrophage cells. The data were analyzed using ANOVA and Tukey's post hoc test. The result showed that the methanolic leaves extract of the plant enhances the highest proliferation of the J774A.1 macrophage cell at 150 µg/mL. It was also found that the extract has a significant anti-inflammatory effect in terms of cell proliferation, suppressing NO and H_2O_2 production in concentration-dependent manner as compared to the control groups.

Keywords: *C.asiatica,* J774A.1 cell line, anti-inflammatory, proliferation, H_2O_2, NO.

CHAPTER I

INTRODUCTION

1.1 Introduction

Nature sources have been used traditionally for medicinal purposes and have been the source of active pharmacology components which are exploited in the traditional medication (Coe and Anderson, 1996). There are various plants that play roles in this traditional medical system. Among others, *Centella asiatica* (*C.asiatica*) also known as *pegaga* in Malaysia, is one of plants which has been exploited in many ways by mankind for its medicinal value and advantages.

C.asiatica from the Apiaceae (Umbelliferae) family is an herbaceous perennial plant and native to India, China, Indonesia, Australia, the South Pacific, Madagascar, and southern and middle Africa. It grows in damp swampy areas, up to 700 meters above sea level. This slender creeping plant has long, prostrate, filiform stems with long internodes, rooting at nodes. The long petioled leaves, 1-5 in number from each node, are orbicular and deeply cordate, 1-7 cm in diameter (refer to Appendix A). The small, purple to white-green flowers, 3-6 in number, are arranged in umbels arising from the axils of the leaves. The fruit is 8 mm long and ovoid, hard with strongly thickened pericarp (Cesarone *et al.*, 1992).

In Ayurvedic medicine, *C.asiatica* is used for various pathological disorders such as healing of wounds, leprosis, chronic diseases and mental disorders. This plant has been listed in the pharmacopoeia and is recommended for the treatment of skin diseases like

leprosy, psoriasis and eczema (Zainol *et al.*, 2008). *C.asiatica* has also been listed officially in the Chinese Pharmacopoeia as one of the Traditional Chinese Medicine (TCM) (Tang *et al.*, 1992).

Inflammation is the first response of the immune system to infection, injury or irritation and is one of the mechanisms of innate immunity. Symptoms of inflammation include redness (rubor), swelling (tumor), heat (calor), pain (dolor) and dysfunction of that particular part of tissues (functio laesa). When tissues are damaged, mast cells release chemicals called histamine. Stimulations of nerve ending by the chemicals result in pain. Histamine increases blood flow to damaged tissue resulting in redness and heat, and causing capillaries to start leaking (Ennis *et al.*, 1980). The accumulation of the exudate results in swelling.

Inflammation may also gives arise to fever, leukocytosis, the presence of acute-phase proteins including C-reactive proteins (CRP), fibrinogen and serum amyloid A protein (SAA), and sepsis. A large variety of proteins is involved in inflammation and susceptible to a genetic mutation which impairs the normal function and expression of that protein. Inflammatory results in both immunological and non-immunological abnormalities. Examples of disorders associated with inflammation include asthma, autoimmune diseases, glomerulonephritis, hypersensitivities, inflammatory bowel diseases, pelvic inflammatory disease, reperfusion injury, rheumatoid arthritis, transplant rejection, and cardiovascular diseases.

Inflammatory mediators are the substances that tend to direct the inflammatory response. These inflammatory mediators come from plasma proteins or cells including mast cells, platelets, neutrophils and monocytes/macrophages after being triggered by bacterial products or host proteins. They bind to specific receptors on target cells and able to increase vascular permeability and neutrophil chemotaxis, stimulate smooth muscle contraction, direct enzymatic activity, induce pain or mediate oxidative damage. Examples of chemical mediators include vasoactive amines, arachadonic acids and cytokines.

These aforementioned chemical mediators involve in cell signaling which then propagates inflammation process. Cell signaling is one of the most important aspects of biological sciences involving cells. Many of the reactions that occur are based on redox reaction which means that there is reduction and oxidation of compounds taking place. Main compound thought to be involved in redox-mediated signal transduction are nitric oxide (NO) and reactive oxygen species (ROS) which includes hydrogen peroxide (H_2O_2) and superoxide anions (O^{2-}) (Hancock, 2009). Reactive nature of ROS makes them seem potentially lethal oxidants. However, signaling is mainly operated by H_2O_2, an oxidant less reactive than other ROS (Toledano *et al.*, 2010). Therefore, in this present study, *C.asiatica* was selected in order to evaluate their anti-inflammatory activity, focused mainly on the production of NO and H_2O_2 by the treated murine macrophage cells.

CHAPTER II

LITERATURE REVIEW

2.1 Literature review

2.1.1 Inflammation

Inflammation is a response of the immune system to infection, injury or irritation. Inflammation acts as a central executor in the pathogenesis of many diseases such as rheumatoid arthritis, arteriosclerosis, myocarditis, infection, cancer, metabolic disorders and many more. This process attracts macrophages and resulting in production of inflammatory mediators which further propagates the process. Macrophages release different cytokines, reactive oxidative species and growth factors that can promote tissue injury, inflammation, and fibrosis. These includes H_2O_2, nitric oxide synthase (NOS), fibronectin, and colony-stimulating factors that can act directly on target tissues and cells or may indirectly activate infiltrated leukocytes, thus amplifying the inflammatory response. Nonspecific, humoral and cellular immune function is depressed in the tissue injured by burn or wound, primarily due to impaired leukocyte chemotactic function.

Activation of a cascade of immune cells is the main event of inflammation. Among these cells, macrophages play an important role in production of various inflammatory mediators. Macrophages migrate to an injured area to kill invading organisms, produce cytokines that recruit other inflammatory cells and are responsible for the diverse effects of inflammation (Cho *et al.*, 2003). Macrophages are extraordinarily versatile cells, they detect pathogenic substances through pattern-recognition receptors and subsequently

initiate and regulate inflammatory response using a wide range of soluble pro-inflammatory mediators (Medzhitov *et al.*, 1997).

Laskin and Pendino (1995) demonstrated the role of macrophage and the inflammatory mediators in tissue injury, tissue cytotoxity, hepatotoxicity, and pulmonary toxicity. In this study it is stated that induced tissue injury is a complex process that involves a variety of cell types and mediators. Reactive mediators produced by inflammatory macrophages may act as primary mediators of tissue injury, or may participate in the inflammatory response by initiating a cascade of additional immunologic reactions that result in tissue damage.

According to another study by Raison *et al.* (2006) patients with major depression who are medically healthy have been repeatedly observed to have activated inflammatory pathways, with increased pro-inflammatory cytokines, increased acute phase proteins and increased expression of chemokines and adhesion molecules. The data in the research also suggested that inflammatory processes contribute to the therapeutic effects of the currently available anti-depressants and could provide targets for novel pharmacological and non-pharmacological treatment strategies. (Raison *et al.*, 2006)

2.1.2 *Centella asiatica*

C.asiatica has been used traditionally all over the world for various purposes. It has also been scientifically proven that it has healing capability (Cho *et al.*, 2003). Natural plant

compounds which are able to suppress the production of inflammatory mediators from activated macrophages can act as potential anti-inflammatory agents. It is being used as antipyretic and diuretic, and in the treatment of icterus, heatstroke, diarrhoea, ulcerations, eczema, and traumatic diseases (Tang *et al.*, 1992).

In Thailand, *C.asiatica* is normally consumed as tea or juice (Punturee *et al.*, 2004) while Japanese and Chinese use in their dishes as one of the ingredients. The leaves of the plant are also used for treating headache, dizziness and shoulder stiffness. In alternative medicine, *C.asiatica* has been used in treatment like mental disorders, inflammation, rheumatism, circulatory problems, asthma and bronchitis, and immune system deficiencies (Farnsworth and Bunyapraphatsara, 1992). It has been used for wound healing in folk medicine of Malay Peninsula, Java and other islands, Madagascar, and Kenya (Tang *et al.*, 1992).

C.asiatica is highly effective against factors responsible for vascular and neuronal degeneration associated with dementia and Alzheimer disease (Matshushima and Morimoto, 2009). Chen *et al.* (1999) also showed that the *C.asiatica* extracts are able to reduce acute radiation reactions by anti-inflammatory activity. In experimental studies, the plant showed evidence of wound healing (Suguna *et al.*, 1996), anti-tumor activity and cognition enhancement in rats (Kumar and Gupta, 2002).

Punturee *et al.* (2004) has demonstrated that *C.asiatica* modulates NO and tumor necrosis factor-α in J774.2 mouse macrophage cells. In his study, the production of NO from the lipopolysaccharide (LPS) stimulated macrophage increased upon treatment with aqueous extract from *C.asiatica*. However, the ethanol extract of the plant suppressed the production of NO from the stimulated cells. Yun *et al.* (2007) has shown that asiatic acid, a phytochemical compound from *C.asiatica* had a stronger inhibitory effect on LPS-induced NO and PGE_2 production compared to another compound, asiaticoside of the same plant (Yun *et al.*, 2007). This has been found to be supported by Kobuchi *et al.* (1997) which stated *C.asiatica* reduces the level of NO in LPS and interferon-gamma activated macrophages.

Topical and oral administration of alcoholic extract of *C.asiatica* on rat showed an increased DNA, protein and collagen content of granulation tissues which indicates a significant increase of cellular proliferation and collagen synthesis at the wound site (Suguna *et al.*, 1996). Quicker and better maturation and cross-linking of collagen was observed in the extract treated rats. The extract treated wounds were found to epithelialise faster and the rate of wound contraction was higher, as compared to control wounds (Suguna *et al.*, 1996).

In another study, the intraperitoneal administration of *C.asiatica* extract significantly reduced the PGE_2 induced paw edema in *Sprague Dawley* rats (Somchit *et al.*, 2004). Jayathirtha and Mishra (2004) showed that *C.asiatica* significantly increased the

phagocytic activity and total WBC count. The study showed that the plant has a positive effect on proliferation and viability of macrophage cells. In a nutshell, the study indicated preliminarily that *C.asiatica* holds promise as immunomodulatory candidate. According to Kobuchi *et al.* (1997), *C.asiatica* also reduces the level of NO in macrophages activated in vitro with lipopolysaccharides and interferon-gamma.

An in vitro study by Punturee *et al.* (2005) revealed preliminary effects of this plant extract on non-specific cellular immune response. The water extract exerted immune stimulating effect on mitogen stimulated proliferation of human PBMCs in a dose-dependent relationship whereas ethanol extract of the plant exerted a strong immune suppressive activity which suggested different active components exist in water and ethanol extract. The exact mechanism of the effect is not known, however the possible explanation mentioned in the study was the interaction between active components of extract and cell surface molecules or growth factor. It is also stated that immune parameters such as macrophage activity including cell signaling and cytokine production should be studied further to evaluate the effect of this plant extract.

C.asiatica contains diverse phytochemicals especially triterpenoids namely, asiaticoside, asiatic acid, madecassoside, and madecassic acid. These bioactive compounds are also quoted in the protective effect of anti-inflammatory (Channarong *et al.*, 2007). Numerous studies are being conducted using these phytochemicals to evaluate the medicinal property of the compounds.

Triterpenoids, a phytochemical compound from *C.asiatica*, have shown promising effects when applied as anti-inflammatory agents (Rao *et al.*, 2000). Triterpenic fraction of *C.asiatica* is used for treatment of venous hypertensive microangiopathy (Cesarone *et al.*, 2001). Compounds released from *C.asiatica* inhibit the formation of reactive oxygen species (Perez *et al.*, 2003) and possess platelet activating factor (PAF) antagonist property (Smith *et al.*, 1996). Moreover, it also attenuates oxidative stress in macrophages and endothelial cell. Punturee *et al.* (2004) stated that clinical study and data on sedative, analgesic, anti-depressive, antimicrobial, antiviral, anti-inflammatory and immunomodulating effects of *C.asiatica* is still lacking. Foreseeing the potential of this plant as an anti-inflammatory agent, it has been chosen as the main interest in present study.

2.1.3 Lipopolysaccaride

Lipopolysaccaride (LPS) is one of the most powerful activators of macrophages. Macrophages induced by LPS are known to be activated through the production of inflammatory mediators including NO, cytokines, such as TNF-α, IL-1β and IL-6 and other free radicals (Yun *et al.*, 2008). NO is produced by the constitutive forms of NOS, namely, endothelial and neuronal NOS, and functions principally as a vasodilator and neurotransmitter (Ignarro, 2002). The third form of NOS, known as iNOS, is generally not present in resting cells, but is induced by various stimuli, which include bacterial LPS, TNF-α, IL-1β, picolinic acid, and INF-γ (Salvemini *et al.*, 2003).

2.1.4 Hydrogen Peroxide (H_2O_2)

There have been studies on role of ROS in inflammatory diseases but little is being concentrated on role of H_2O_2 in certain diseases like hyperalgesia (Keeble *et al.*, 2009). Peripheral inflammation leads to significant production of ROS at the affected site, including superoxide anion O_2^-, peroxynitrite ($ONOO^-$), hydroxyl radical (OH^-) and H_2O_2. The rates of production of superoxide anion and H_2O_2 are interdependent as the enzyme superoxide dismutase (SOD) catalyzes formation of H_2O_2 from superoxide anion.

Toxicity of H_2O_2 is hypothesized to be due to its reaction with transition metals to form hydroxyl radicals which able to further react with nucleic acids, phospholipids, amino acids and sugars and eventually causes cell death (Mann *et al.*, 1997). Keeble *et al.* (2009) demonstrated a notable effect of H_2O_2 in mediating inflammatory hyperalgesia where the carrageenan-induced pain and inflammation is accompanied by a significant increase in H_2O_2 levels in the mouse hind paw. Significantly less mechanical and thermal hyperalgesia observed in paws that received carrageenan co-injected with active superoxide dismutase (SOD) and catalase than the paws injected with denatured SOD and catalase. Thus emphasizing H_2O_2 elimination is a novel therapeutic target for anti-hyperalgesic drugs in clinic (Keeble *et al.*, 2009).

Mann *et al.* (1997) studied the toxic effects of H_2O_2 in CSM 14.1 neural cells. The study showed that H_2O_2 causes dose-dependent toxicity and DNA fragmentation in a ladder

pattern in the cells. However, over expression of catalase which transfected into the cells by a vector containing human cDNA inhibited H_2O_2 induced toxicity. The cells experienced shrinkage and rounding up of cells and appearance of vacuoles early after exposure to H_2O_2 before occurrence of cell death. These studies showed the importance of the role of H_2O_2 in cell apoptosis and necrosis which can be the consequences of uncured inflammation and hence one of the aim for present anti-inflammatory study on *C.asiatica.*

2.1.5 Nitric Oxide (NO)

Inflammatory mediators interact with each other and exert effects in an inflammation process. A study by Noronha-Dutra *et al.* (1993) on reaction of NO with H_2O_2 to produce potentially cytotoxic singlet oxygen as a model for NO mediated killing proved that NO reacts with H_2O_2 and releases large amounts of highly cytotoxic species, singlet oxygen which accounts for the cytotoxic function of NO. It also hypothesized that this mechanism might play a role in ischemia- reperfusion injury. The study clearly stated the relationship between NO and H_2O_2.

NO like other small neutral gases, can diffuse across cell to its site of action. Generation of toxic mediators, especially reactive nitrogen species, regulates activity of macrophage. NO produced from macrophage has important role in antimicrobial, antiviral and antitumor activities. However, in excess NO also can contribute to pathogenesis of inflammation diseases including hypertension, stroke and septic stroke

(Punturee *et al.,* 2004). The major mechanism of termination of biological action of NO is its reaction with O_2 to form NO_2 which in aqueous solution results in the formation of N_2O_3 and then $N_2O_2^-$ which then further oxidized by a variety of endogenous oxidants to NO_3^-, which is relatively innocuous. NO is an important inflammatory mediator which is synthesized from arginine by NOS (Kim *et al.,* 1999).

Nitric Oxide synthases (NOS) is a family of enzyme that catalyzes oxidation of one of the terminal guanidine nitrogens of L-arginine to yield NO and citrulline as mentioned above. NO is then converted to oxygenated solutions and then to nitrite and nitrate. There are at least three types of NOS isotypes; neuronal NOS, endothelial NOS and inducible NOS. Inducible NOS expressed over stimulation by cytokines or endotoxin in macrophages, endothelial cells, vascular smooth muscle cells and cardian myocytes. Preliminary immunohistological studies stated that inducible NOS is present in monocytes in human artherosclerotic lesions (Yang *et al.*, 1993)

There were studies on natural compounds that able to directly inhibit expression of cytokines, iNOS and COX-2 which then reduces inflammation (Hehner *et al.,* 1998; Ma *et al.,* 2003). A study by Heo *et al.* (2010) on brown algae explained anti-inflammatory activity of one of the active compounds from the algae, fucoxanthin, is by suppressing NO production and iNOS expression. The study mentioned that the NO production was correlated with the active compound and it down regulated protein expression of iNOS and thereby inhibited LPS-induced NO production (Heo *et al.*, 2010). Another study by Hseu *et al.* (2005) on anti-inflammatory potential of *Antrodia camphorata*, a Chinese

medicine, shown the plant inhibited LPS-induced NO production in dose-dependent manner. High level of NO produced by iNOS also has been defined as cytotoxic in inflammation (Hseu *et al., 2005*).

Inhibition of LPS-induced NO and PGE_2 production by asiatic acid, an active compound from this *C.asiatica* was studied by Yun *et al.* (2007) in RAW 264.7 macrophages. The compound significantly inhibited LPS-induced NO production and iNOS expression in concentration-dependent manner. The study showed clearly that inhibition of NO production by this compound was due to reduced expression of iNOS protein. All these studies suggest that NO production correlates with inflammation process and thus justifies the purpose of this study on *C.asiatica.*

Hence, based on all these findings from numerous studies, this present study aims to study anti-inflammatory activity of methanolic extract of different parts of *C.asiatica* in J774A.1 murine macrophage cell line.

2.2 Objective

2.2.1 General Objective

To determine the anti-inflammatory activity of methanolic extract of different parts of *C.asiatica* in J774A.1 murine macrophage cell line.

2.2.2 Specific Objective

2.2.2 (a) To determine the viability of murine macrophage cell line J774A.1 treated with methanolic extract of leaves, petiole and root of *C.asiatica*.

2.2.2 (b) To determine the production of hydrogen peroxide by murine macrophage cell line J774A.1 treated with methanolic extract of leaves, petiole and root of *C.asiatica*.

2.2.2 (c) To determine the production of nitric oxide by murine macrophage cell line J774A.1 treated with methanolic extract of leaves, petiole and root of *C.asiatica*.

The overview of the research is visualized in the Figure 2.1.

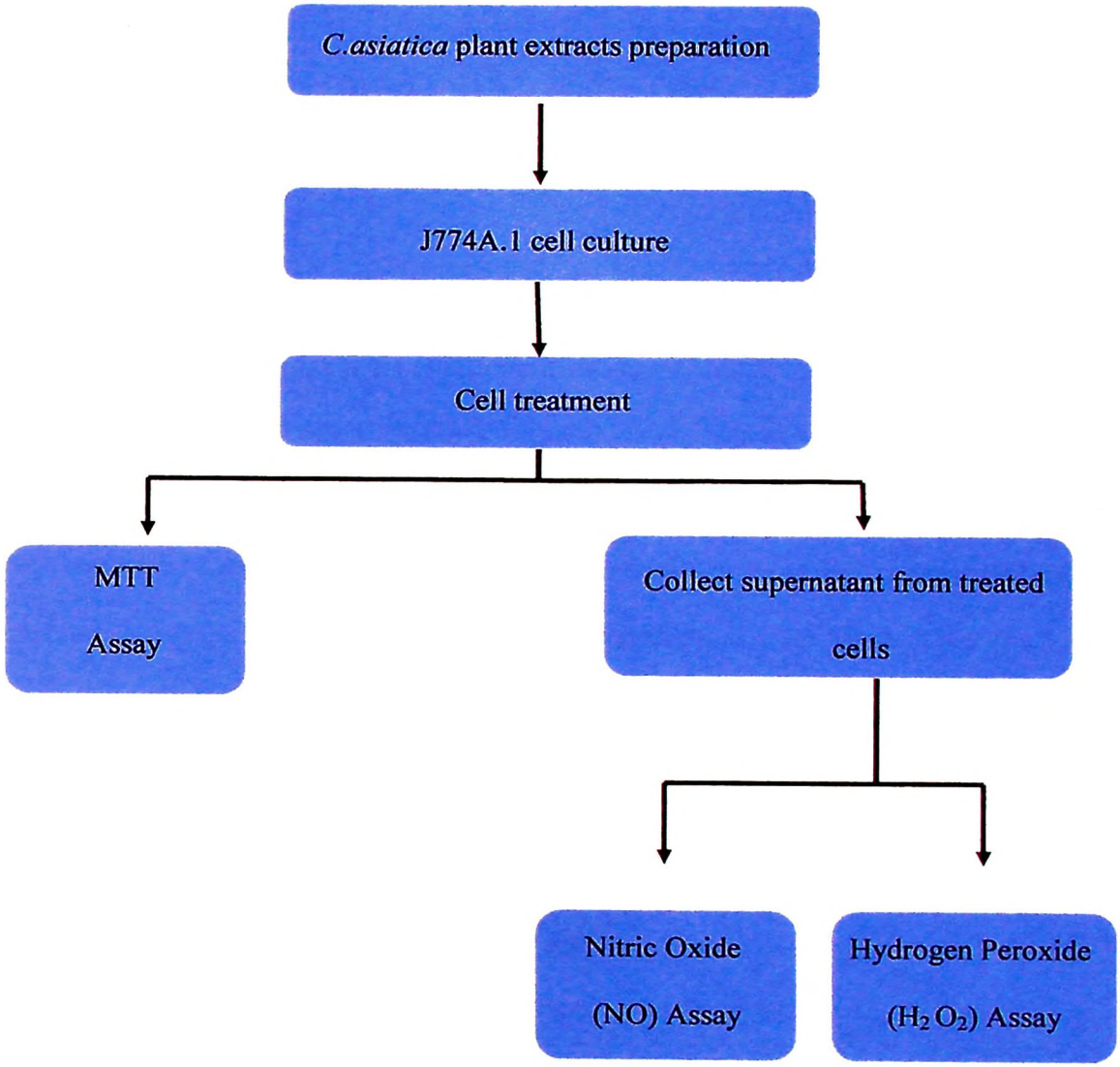

Figure 2.1: Summary of the study

CHAPTER III

MATERIAL & METHOD

3.1 Material

3.1.1 Chemicals and reagents

Locally obtained *C.asiatica* plant after identification by botanist was used for extraction. Dulbecco's modified Eagle's medium (DMEM) was obtained from Sigma Aldrich company where as fetal bovine serum (FBS) and penicillin-streptomycin (PenStrep) were purchased from Life Technologies Inc. (Grand Island, NY, USA). *Escherichia coli* lipolysaccharide (LPS) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

3.1.2 Commercial Kit

Vybrant® MTT Cell Proliferation Assay Kit (V-13154) was purchased from Molecular Probes Inc. Griess reagent Assay Kit for NO Assay was purchased from Promega Corporation.

3.2 Methodology

3.2.1 Plant extraction

The plant was collected from Bachok, Kelantan, Malaysia. The plant was authenticated by USM botanist and the voucher specimen number. The whole plant was separated into leaves, petioles and root prior to cleaning and drying process. The separated parts were

then washed with water, soaked in methanol (MeOH, Analytical Grade, BDH Laboratory supplies) and dried at 60°C for 3 days. The plant parts were grinded and extracted using soxhlet apparatus (500 mL) with methanol separately. Each filtrate was then evaporated under reduced pressure at 55°-60° C. The subsequent residue dissolved to 100 mg/mL with 1 mL of Dimethyl Sulfoxide (DMSO) and 100 µL from this was diluted in 9900 µL of DMEM making it to 1000 µg/mL for cell treatments. From this diluted extract, calculation was made (Appendix B) to aliquot volumes for cell treatment according to the concentrations needed for this study (50 µg/mL, 100 µg/mL and 150 µg/mL).

3.2.2 Cell culture and treatment

Murine macrophage J774A.1 cell line was grown in DMEM medium supplemented with 10% FBS and 100 units/mL Penstrep (penicillin-streptomycin) and incubated at 37°C in a humidified atmosphere of 5% CO_2. The cells were grown to confluent in sterile tissue culture flasks and then collected by gentle detachment using scrapping technique. The cell number and viability were assessed using trypan blue exclusion method on a haemacytometer. The cells were then cultured at a density of $1x10^6$ cells/mL in tissue culture flasks and treated with different concentrations of methanolic extract of leaves, petioles and root of *C.asiatica.*

The treated cultures were then incubated for 24 hours in a humidified 5% CO_2 incubator. LPS (100 µg/mL) activated J774A.1 cells was used as positive control and untreated cells was used as negative control. After 24 hours incubation, the cells were then gently

detached by scrapping and centrifuged at 3000 rpm (Hettich) for 20 minutes. Culture supernatants were transferred into individual microcentrifuge tubes, sealed and stored at -80°C for NO and H_2O_2 concentration determination.

3.2.3 MTT (3-(4.5-dimethythiazol-2-yl)-2.5-diphenyltetrazolium bromide) assay

Cell viability studies were performed in 96-well flat bottom plate. J774A.1 cells were scraped and plated at $1x10^5$ cells/mL in 96-well plate containing 100 µL of DMEM medium with 10% FBS and 100 U/mL PenStrep and incubated overnight for 24 hours in a humidified 5% CO_2 incubator. The prepared plant extract was then added to wells in volume calculated as per the concentrations needed. The treated well-plate was then incubated for 24 hours in a humidified 5% CO_2 incubator. Cell proliferation and viability in the treated macrophage culture were determined using Vybrant® MTT Cell Proliferation Assay Kit (V-13154) according to the protocol provided. This assay involves conversion of water soluble MTT to an insoluble formazan. Briefly, 10 µL of 12 mM MTT stock solution (5 mg/mL MTT in phosphate buffer saline) added to each well of macrophage cultures and incubated for 4 hours at 37°C in humidified chamber. After incubation, 100 µL of SDS-HCL solution added to each wells and mixed thoroughly with pipette and incubated at 37°C for 4 hours in humidified chamber. Then, absorbance (OD) was read at 570 nm using spectrophotometer.

Percentage of cell viability was calculated based on OD reading obtained using following formula:

$$\text{Cell viability (\%)} = \frac{\text{OD (treated J774A.1 cells)}}{\text{OD (untreated pure J774A.1 cells)}} \times 100\%$$

3.2.4 Hydrogen Peroxide (H_2O_2) assay

H_2O_2 in treated cell supernatant will be determined by using ELISA principle and is performed in 96-well flat bottom assay plate. Materials were first prepared in following quantities:

Table 3.1: Reagent preparation for H_2O_2 assay

Reagent	Materials and quantities
1. Blank solution (4N NaOH)	0.32 g sodium hydroxide (NaOH) 3.96 mg phenol red 20 mL of distilled water
2. HRP solution	40.95 mg sodium chloride (NaCl) 4.95 mg glucose 0.99 mg phenol red 5 mL horseradish peroxidase (HRP) 8.71 µL potassium phosphate 4991.29 µL deionised-distilled water
3. Stop solution (1N NaOH)	40 g NaOH 1 L distilled water

Standards were first established by adding 100 µL of prepared blank solution into row A of well-plate and 50 µL of blank DMEM into row B to H in triplicates. Serial dilution was performed from row A until row G and 50 µL discarded from row G to generate H_2O_2 standard reference curve (100, 50, 25, 12.5, 6.25, 3.13 and 1.56 µM). Row H is left with only blank DMEM (0 µM). 50 µL of each supernatants prepared from treated cells

was added to new wells in triplicates. 100 µL of prepared HRP solution was then added to all wells including standards and incubated for 1 hour in humidified CO_2 chamber. After incubation, 10 µL of prepared stop solution added to all wells to stop the reaction and absorbance is read at 620 nm using a spectrophotometer.

3.2.5 Nitric oxide (NO) assay

The nitrite accumulated in culture medium is measured as an indicator of NO production based on Griess reaction as the nitrite is a stable reaction product of NO and oxygen. A nitrite standard reference curve was prepared for each assay for accurate quantification of nitrite levels in experimental samples. Briefly, 100 µM of nitrite solution prepared by adding 1 µL of nitrite reagent into 999 µL of DMEM and 50 µL of this was then added into row A of 96-well plate in triplicate. 50 µL of blank DMEM was added into row B to row H in triplicates. 6 serial twofold dilutions (50 µL/well) performed down the plate from row A to row G and 50 µL from row G was discarded to generate nitrite standard reference curve (100, 50, 25, 12.5, 6.25, 3.13 and 1.56 µM). Row H is left with only blank DMEM as negative control (0 µM). 50 µL of individual supernatants prepared from treated J774A.1 cells were then added to new wells in triplicates. 50 µL sulfanilamide solution was added to all wells including standards and incubated 5-10 minutes at room temperature, protected from light. 50 µL of NED solution (0.1% *N*-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) then added to all wells including standards and incubated at room temperature for 5-10 minutes, protected from light. A purple color formed upon adding NED solution. After incubation, the absorbance was read at 540 nm using spectrophotometer within 30 minutes as the color fades after this time period.

3.2.6 Statistical analysis

All experiments were carried out in triplicate and repeated three times. Data were represented as the arithmetic mean $\pm$ standard error mean (S.E.M). Comparison between treated groups and control groups was analyzed using One Way ANOVA. The accepted level of significance was at $P< 0.05$.

CHAPTER IV

RESULT

4.1 MTT Assay

Result showed that all the cell viabilities for plant extract treated cells have increased in the concentration-dependent manner from 50 µg/mL, 100 µg/mL to 150 µg/mL. However, the cell viability of methanolic extract of leaves part of *C.asiatica* was the highest compared to the petiole, root and both controls. Cell viability after treated with methanolic extract of leaves increased from 113.46%, 117.0% to 121.96% for the three concentrations respectively. However, only cells treated with 100 µg/mL and 150 µg/mL of leaves methanolic extract were significantly different at the level of $P<0.05$ as compared to the negative control. Furthermore, only cells treated with 150 µg/mL of leaves methanolic extract showed significant difference when compared to the positive control. Cells treated with 50 µg/mL of the leaves extract did not show significant difference as compared to both controls.

Petiole methanolic extract treated cells shown viabilities of 98.78%, 109.2% and 109.36% for the three concentrations respectively where as for root methanolic extract treated cells, viabilities of 88.7%, 101.26% and 116.0% were shown after treated with the same concentrations of extract. Viabilities of cells increased in concentration-dependent manner for both petioles and root methanolic extract treated cells. However, both petiole and root methanolic extract treated cells did not show significant difference as compared to both controls. Detailed ANOVA result is attached in Appendix C.

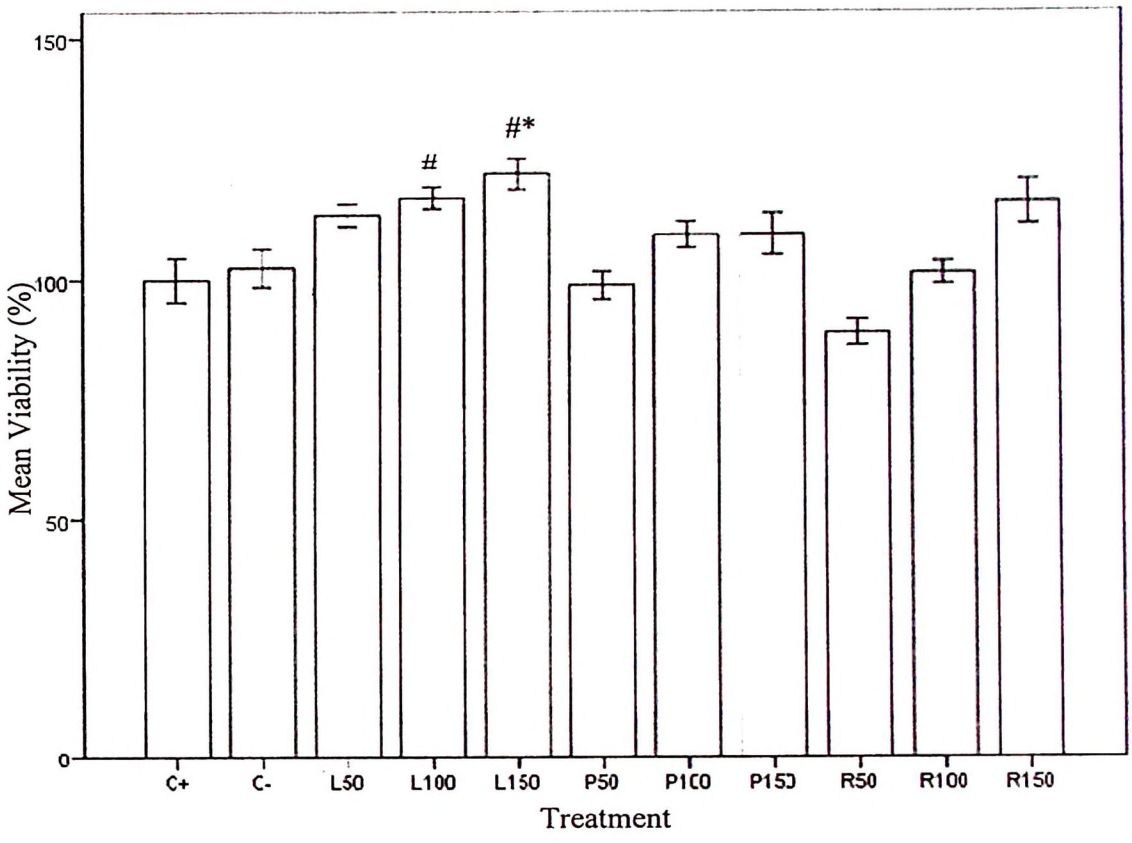

Figure 4.1: Cell viability (%) of treated J774A.1 cells

The data represents the mean ± S.E.M. for viability of J774A.1 cells treated with leaves (L), petiole (P) and root (R) methanol extract of *C.asiatica* at concentrations of 50 µg/mL, 100 µg/mL and 150 µg/mL respectively for each plant parts. LPS (100 ng/mL) treated J774A.1 cell culture was used as a positive control (C+) and untreated cell culture was used as negative control (C-). * Mean value was significantly different from positive control (P<0.05); # Mean value was significantly different from negative control (P<0.05). Tabular data is attached in Appendix C.

4.2 Hydrogen Peroxide (H_2O_2) Assay

Result showed that H_2O_2 production from J774A.1 cells treated with methanolic extract of leaves and petiole part of *C.asiatica* decreased in concentration-dependent manner from 50 µg/mL, 100 µg/mL to 150 µg/mL. In contrast, cells treated with methanolic extract of root part of this plant showed increasing trend in concentration-dependent manner.

H_2O_2 production from the cells after treated with methanolic extract of leaves reduced from 11.12 µg/ml, 10.1 µg/ml and 9.8 µg/ml for the three concentrations respectively. Nevertheless, only cells treated with 100 µg/mL and 150 µg/mL of leaves methanolic extract were significant at the level of $P<0.05$ compared to positive control. However, all three concentrations did not show significant difference compared to negative control.

Petiole methanolic extract treated cells yielded H_2O_2 concentrations of 10.46 µg/mL, 9.68 µg/mL and 9.38 µg/mL for each extract concentration respectively. H_2O_2 concentration was significantly different for all three extract concentrations compared to positive control. However, in comparison to negative control the results for all three concentrations were not significantly different.

Whereas for root methanolic extract treated cells, H_2O_2 concentration increased in concentration-dependent manner from 7.52 µg/mL, 10.22 µg/mL to 11.12 µg/mL for