UNIVERSITI PUTRA MALAYSIA

ANTI-INFLAMMATORY EFFECTS OF ASIATICOSIDE ON TUMOR NECROSIS FACTOR-α AND INTERFERON-γ INDUCED RAW 264.7 MACROPHAGE

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By

NURFARAHDILLA BINTI ZAINUDIN

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Chair : Enoch Kumar Perimal, PhD
Faculty : Medicine and Health Sciences

Centella asiatica is one of the most commonly used medicinal herbs in traditional medicine. Its beneficial effects have been attributed to its major isolated triterpene, Asiaticoside. It has been described to exhibit anti-inflammatory activities in several models. Inflammatory cells produce large amount of nitric oxide, prostaglandin E2 and cyclooxygenase. These activated macrophages functions as effectors molecules and are involved in physiological and pathological responses in neural, vascular and immune system. In this study, tumor necrosis factor-α and interferon-γ-induced inflammatory response was introduced in RAW 264.7macrophages cells to evaluate the potential effects of Asiaticoside (1.25 μM-50 μM) in these cells. Quantification of nitric oxide was determined through the Griess reaction by a microplate assay method. Prostaglandin E2 and cyclooxygenase 2 releases were quantified by EIA. To detect whether the effect of Asiaticoside on tumor necrosis factor-α and interferon-γ-stimulated RAW 264.7 macrophages cells occurred via the mitogen-activated protein kinase signal transduction pathways, western blot was performed. The results showed that Asiaticoside significantly inhibited tumor necrosis factor-α and interferon-γ -induced nitric oxide, prostaglandin E2 and cyclooxygenase-2 productions which is mediated by the suppression of the p38 mitogen-activated protein kinase signaling pathway. Based on our current findings, we concluded that Asiaticoside has anti-inflammatory effects and may be used as a therapeutic agent for the treatment of inflammation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk Ijazah Master Sains

KESAN ANTI-RADANG OLEH ASIATICOSIDE TERHADAP TUMOR NEKROSIS FAKTOR-α DAN INTERFERON-γ YANG DIRANSANG PADA MAKROFAJ RAW 264.7

Oleh

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Pengerusi : Enoch Kumar perimal, PhD
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Centella asiatica adalah salah satu herba perubatan yang paling biasa digunakan dalam perubatan tradisional. Keberkesananya telah dikaitkan dengan triterpenenya yang utama iaitu, Asiaticoside. Ianya telah dihuraikan untuk mempamerkan aktiviti anti-radang dalam beberapa model. Sel-sel radang itu telah menghasilkan sejumlah besar oksida nitrik, prostaglandin E2 dan cyclooxygenase. Sel-sel makrofaj yang telah diaktifkan ini berfungsi sebagai molekul-molekul effektor dan terlibat di dalam tindak balas fisiologi dan patologi pada saraf, vessel dan juga sistem imun. Dalam kajian ini, tumor necrosis factor-α dan interferon-γ- yang meyebabkan tindak balas radang telah diperkenalkan pada sel makrofaj RAW 264.7 untuk menilai keberkesanan Asiaticoside (1.25 μM-50 μM) pada sel ini. Kuantifikasi oksida nitrik ditentukan melalui tindak balas Griess dengan kaedah microplate. Penghasilan prostaglandin E2 dan cyclooxygenase-2 dikuantifikasi oleh kit-kit EIA. Untuk mengesan samada kesan Asiaticoside pada sel-sel makrofaj RAW 264.7 adalah melalui mitogen diaktifkan protein kinase, western blot telah dilakukan. Kesimpulannya, keputusan menunjukkan bahawa Asiaticoside ketara telah menghalang sel yang telah diaktifkan oleh tumor necrosis factor-α dan interferon-γ yang menyebabkan pengeluaran oksida nitrik, prostaglandin E2 dan cyclooxygenase-2, melalui isyarat laluan p38 mitogen diaktifkan protein kinase. Berdasarkan penemuan ini, kami merumuskan bahawa Asiaticoside mempunyai kesan anti-radang. Dengan ini, membenarkan yang sebatian ini untuk menjadi agen terapeutik untuk merawat keradangan.

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I certify that a Thesis Examination Committee has met on 30 November 2016 to conduct the final examination of Nurfarahdilla Zainudin on her thesis entitle "Anti-Inflammatory Effects of Asiaticoside on Tumor Necrosis Factor-α and Interferon-γ Induced RAW 264.7 Macrophage" in accordance with the Universities and Universiti College Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U. (A) 106] 15 March 1998. The committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the Degree of Master of Science. The members of the Supervisory Committee were as follows:

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This is to confirm that:

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Name of Member of Supervisory Committee: Dr. Yong Yoke Keong

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

μM	Micromolar
AA	Arachidonic Acid
AC	Asiaticoside
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
COX	Cyclooxygenase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminenetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
G	Gram
IL	Interleukin
INF-γ	Interferon-gamma
iNOS	inducible Nitric Oxide Synthase
MAPK	Mitogen Activated Protein Kinase
MCP-2	Monocyte Chemoattractant Protein
MIP-2	Murine Macrophages Inflammatory Protein
mM	Milimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium Bromide
NaOH	Sodium Hydroxide
NF-κB	Nuclear Factor κB
ng/mL	Nanogram per mililitre
NO	Nitric Oxide
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
PBS	Phosphate Buffered Saline
PGE_2	Prostaglandin E_2
PMNs	Polymorphonuclear leukocytes
RIPA	Radioimmunoprecipitation Assay
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium dodecyl Sulfate Polyacrylamide Gel
TBS	Tris-Buffered Saline
TNF-α	Tumor Necrosis Factor-alpha

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Inflammation is a localized protective response of living mammalian tissues elicited by the bacterial invasion or injury of tissues (Tracey, 2002). It is a biological process which acts as a body protective reaction to eliminate or limit the spread of injurious agents (Wassung, 2012). This phenomenon is characterized in the acute form by the classical symptoms of pain, heat, redness, swelling, and loss of function while in the chronic form, it is characterized by the infiltration of macrophages, lymphocytes, and plasma cells; tissue destruction; and repair involving fibrosis and new vessel proliferation (Cotran et al., 1999; Lemont et al., 2003).

When inflammation occurs, activated resident macrophages secrete chemokines which functions to attract neutrophils, T cells, and additional macrophages to perpetuate the inflammatory responses. When the injurious stimuli are eliminated, the inflammatory process usually ceases. However, inflammation can sometimes persist and is associated with a number of diseases.

Nowadays, the use of herb is increasingly captured public interest and concern to treat many diseases. *Centella asiatica* (family umbelliferae) or locally known by the Malays as 'Pegaga' is one of the medicinal herbs which widely distributed in tropical countries. Pegaga has been used for centuries in Traditional Medicine including Malay, Chinese, Japan, African and Ayurvedic. It has been used to alleviate symptoms of wound, ulcer and arthritis (Brinkhaus et al., 2000). Many literatures were established regarding its ability to possess the anti-inflammatory, anti-nociceptive, anti-oxidant, anti-tumor, anti-arthritis and neuroprotective effects in several models (Gohil et al., 2010; Somchit et al., 2004; Veerendra Kumar et al., 2003). The leaves of *C. asiatica* contain asiaticoside (AC) which is one of its primary active constituents and the most investigated constituent (Singh et al., 1969). Some studies have reported that AC has effects that help to heal wound, ulcer and arthritis and has capabilities to exhibit anti-oxidant, anti-tumor, anti-arthritis and anti-inflammatory activities (Veerendra Kumar et al., 2003).

In this research, we determined whether Mitogen Activated Protein Kinase (MAPK) signaling can be activated by Tumor Necrosis Factor (TNF)-α and Interferon (IFN)-γ and whether these pathways are responsible for the expression NO, PGE_2 and COX-2. MAPKs regulate key proinflammatory pathways following stimulation with cytokines (Kong et al., 2015); therefore, we examined the anti-inflammatory function via the MAPK signal transduction pathway.

1.2 Problem statement

Inflammation is established when there is an attempt by the body for self-protection against harmful stimuli resulting in the release of bioactive substances (cytokines) by immune cells. These immunocytokines (peptides – ex: TNF-α and IFN-γ) sometimes act on target cells in the periphery to evoke inflammatory interactions to contribute in the pathogenesis of numerous diseases. Current therapies for many diseases have several adverse effects. Traditional plant remedies may provide leads of new treatment. Are AC; a major triterpenoid saponin component of C. *asiatica* could inhibit inflammation by reducing the production of NO, PGE_2 and COX-2 through MAPK pathway which it is important to minimize deleterious consequences of inflammation and reduced the number of dependent on NSAIDs.

1.3 Justification of Study

There have been several claims concerning the underlying mechanisms that involved in the biological actions of AC. But, more scientific data are needed to justify its ever increasing use. The RAW 264.7 macrophage cells model employed for this study will allow us to explore some of the underlying mechanism of AC's anti-inflammatory activity. Here, in this study, the AC's inflammatory properties were investigated by measuring the production of NO, PGE_2 and COX-2 in TNF-α/IFN-γ-induced RAW 264.7 macrophages. Because MAPKs regulate key proinflammatory pathways following stimulation with cytokines; therefore, we examined its anti-inflammatory function via the MAPK signal transduction pathway, particularly, p38 MAPK.

1.4 Research Objectives

1.4.1 General Objectives

To evaluate the effects of AC on TNF-α/IFN-γ-induced murine RAW 264.7 macrophages and its possible mechanism of action.

1.4.2 Specific Objectives

I. To determine the viability of RAW 264.7 macrophages towards AC.
II. To determine the effect of AC on nitric oxide productions, COX-2 activity and PGE_2 release in TNF-α/IFN-γ-induced RAW 264.7 macrophages.
III. To determine the effect of p38 mitogen activated protein kinases (MAPK) by AC in RAW 264.7 macrophages.

1.5 Hypothesis

AC will not affect cells viability and may reduce the COX-2 activity and PGE_2 release and it also will aid in deactivation of p38 mitogen activated protein kinases.

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