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SYNERGISM OF SELECTED FLAVONOIDS IN INFLAMMATION

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Masters of Science

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To

~My Beloved Family~

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Master of Science

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By

OMAR ABDUL HAFIZ

April 2012

Chairman: Professor Daud Ahmad Israf Ali, PhD

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Inflammation is characterized by the release of various pro-inflammatory mediators, including the free radical nitric oxide (NO), prostaglandin E_2 (PGE_2) and tumor necrosis factor alpha (TNF-α) from stimulated macrophage cells. Sustained release of these mediators can lead to chronic diseases, tissue injury and multiple organ dysfunction syndrome (MODS). Thus, suppression of these mediators is a useful strategy for the treatment of chronic inflammatory diseases. The use of naturally abundant flavonoid compounds is widely reported to have anti-inflammatory, anti-cancer, anti-oxidant and estrogenic effects. In particular, chrysin, kaempferol, morin, silibinin, quercetin, diosmin and hesperidin are known to alleviate the generation of key pro-inflammatory mediators.

Several of the above mentioned flavonoids have shown biological benefits on models of inflammation. Nevertheless, the combinatorial effects of these flavonoids have not been reported. In this study, the synergistic effects of several flavonoid

combinations on secretion of major pro-inflammatory mediator from lipopolysaccharide (LPS)-stimulated RAW264.7 cells as a cellular model of inflammation, were investigated. To further assess the therapeutic efficacy of flavonoid combination during the progression of sepsis, survival studies against polymicrobial sepsis in ICR mice were done as an animal model of inflammation.

Prior to *in vivo* experiments, the effects of all compounds on NO, PGE_2 and TNF-α secretion from LPS-stimulated RAW 264.7 cells were determined by ELISA and Griess assay; as well as cellular viability by MTT assay. After assessing and obtaining the IC_{50} values, flavonoids that expressed inhibitory effects, in at least two out of the three mediators, were combined in a series of fixed IC_{50} ratios and reassessed to generate dose response curves. Flavonoid combination that exhibited highest synergistic potency as detected by isobolographic analyses, were employed to further investigate its effects in an animal model of sepsis by cecal ligation and puncture (CLP)-induced septic shock in ICR mice. Key inflammatory mediators secreted from septic mice were measured through ELISA and fluorometric determinations; and pharmacological effects upon vital organs were investigated.

Chrysin, kaempferol, morin and silibinin were found to have an adequate potency to produce dose–response effects upon at least two out of the three mediators assayed. Significant synergistic effects have been observed among combinations of the flavonoids mentioned above. In particular, the chrysin / kaempferol combination significantly synergized to increase the potency of inhibiting the mediators NO, PGE_2 and TNF-α secreted from LPS-stimulated RAW 264.7 cells with IC_{50} =

2.27μM, 2.28μM and 20.53μM respectively, as well as a 29% significant increase in survival rate in CLP-induced septic shock in ICR mice.

Conclusively, this study demonstrated that chrysin / kaempferol combination significantly synergized to increase the anti-inflammatory activity through inhibition of several mediators which contributed to improved survival rate. These findings suggest that chrysin / kaempferol combination has reasonable potential as a natural approach in treating inflammation. Further studies are required to investigate the underlying mechanisms involved during inflammation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai Memenuhi keperluan untuk ijazah Master Sains

SINERGISME DARIPADA FLAVONOID TERPILIH DALAM KERADANGAN

Oleh

OMAR ABDUL HAFIZ

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Pengerusi: Profesor Daud Ahmad Israf Ali, PhD

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Keradangan adalah dicirikan dengan pembebasan pelbagai jenis perantara pro-inflamasi termasuk radikal bebas nitrik oksida (NO), prostaglandin E_2 (PGE_2) dan faktor nekrosis tumor alfa (TNF- α) daripada sel makrofaj yang dirangsang. Pembebasan perantara-perantara ini secara berterusan akan mengakibatkan penyakit kronik, kecederaaan pada tisu dan sindrom disfungsi pelbagai organ (MODS). Oleh itu, perencatan penghasilan perantara-perantara ini adalah strategi yang berguna bagi rawatan penyakit keradangan yang kronik. Penggunaan kompaun flavonoid semulajadi dengan banyak telah dilaporkan mempunyai kesan anti-inflamasi, anti-kanser, anti-oksida dan kesan ke atas estrogen. Secara khususnya, krisin, kaempferol, morin, silibinin, kuersetin, diosmin dan hesperidin telah diketahui boleh mengurangkan penghasilan perantara-perantara pro-inflamasi yang utama.

Beberapa flavanoid yang dinyatakan di atas telah menunjukkan kepentingan secara biologi ke atas model keradangan. Walau bagaimanapun, kesan gabungan antara flavanoid-flavanoid masih belum dilaporkan. Dalam kajian ini, kesan sinergi dalam gabungan beberapa flavonoid terhadap penghasilan perantara pro-inflamasi daripada sel RAW 264.7 aruhan lipopolisakarida (LPS) sebagai model sel keradangan telah dikaji. Bagi meneruskan penilaian kesan terapeutik terhadap gabungan flavanoid semasa proses sepsis, ujian kemandirian terhadap sepsis polimikrob telah dijalankan ke atas mencit ICR sebagai model keradangan.

Sebelum eksperimen *in vivo* ini, kesan bagi kesemua kompaun ke atas penghasilan NO, PGE_2 dan TNF-α daripada sel RAW 264.7 aruhan LPS telah ditentukan dengan menjalani ujian asai imunoserap terangkai enzim (ELISA) dan Greiss, ujian kebolehhidupan sel juga telah ditentukan dengan menjalani asai MTT. Selepas menilai dan mendapatkan nilai IC_{50}, flavanoid-flavanoid yang menunjukkan kesan perencatan yang berkesan, sekurang-kurangnya dua daripada tiga perantara, telah digabungkan dalam satu siri nisbah IC_{50} yang telah ditetapkan dan dinilai semula untuk menghasilkan lengkung dos-gerak balas. Kombinasi flavanoid yang mempamerkan potensi sinergistik yang paling tinggi sepertimana yang dikesan daripada analisis isobolografik telah digunakan secara lanjut untuk mengkaji kesannya terhadap model sepsis haiwan dengan menjalani kejutan sepsis aruhan ligasi dan tusukan sekal ke atas mencit ICR. Perantara keradangan yang utama yang dihasilkan daripada mencit septik telah dinilai dengan menggunakan ELISA dan penentuan fluorometrik; dan kesan farmakologikal terhadap organ-organ penting telah dikaji.

Krisin, kaempferol, morin dan silibinin telah ditemui memiliki potensi yang secukupnya untuk menghasilkan kesan dos-gerak balas terhadap sekurang-kurangnya dua daripada tiga perantara yang telah dikaji. Kesan-kesan sinergistik yang ketara telah diperhatikan antara kombinasi flavanoid yang telah dinyatakan di atas. Secara spesifik, kombinasi krisin / kaempferol dilihat sangat sinergik dalam meningkatkan potensi perencatan perantara NO, PGE_2 dan TNF-α yang dirembeskan daripada sel RAW 264.7 aruhan LPS dengan nilai IC_{50} masing-masing 2.27μM, 2.28μM dan 20.53μM, dan juga 29% peningkatan yang ketara dalam kadar kemandirian yang dilakukan dalam kejutan sepsis aruhan ligasi dan tusukan sekal ke atas mencit ICR.

Kesimpulannya, kajian ini menunjukkan gabungan krisin / kaempferol menghasilkan kesan sinergistik yang ketara bagi meningkatkan aktiviti anti-inflamasi dengan merencat beberapa perantara dan ini telah meningkatkan kadar kemandirian. Penemuan ini mencadangkan bahawa kombinasi krisin / kaempferol memiliki potensi yang meyakinkan sebagai rawatan semulajadi untuk merawat keradangan. Kajian yang selanjutnya perlu dijalankan bagi menyelidik mekanisma yang terlibat semasa keradangan.

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I certify that a Thesis Examination Committee has met on 9 April 2012 to conduct the final examination of Omar Al Harasstani on his thesis entitled "Synergism of Selected Flavonoids in Inflammation" in accordance with the Universities and University 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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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or other institutions.

OMAR HARASSTANI

Date: April 2012

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LIST OF ABBREVIATIONS

℃	celsius
µg	micro gram
µl	micro liter
µM	micro molar
AA	arachidonic acid
APC	antigen presenting cells
AST	aspartate aminotransferase
CFUs	colony forming units
Ch	Chrysin
CLP	cecal ligation and puncture
COX	cyclooxygenase
DAN	2,3-diaminonaphthalene
Dexa	dexamethasone
D-GalN	D-galactosamine
dH_2O	deionized water
DMEM	dulbecco's modified eagle media
DMSO	dimethyl sulphoxide
EDTA	Ethylenediaminetetraacetic acid
EGCG	epigallocatechin gallate
ELISA	enzyme-linked immunosorbent assay
FBS	foetal bovine serum
g	gram
GABA	gamma-aminobutyric acid
GD	gastroduodenal
IC_{50}	inhibitory concentration 50
IKK	IκB kinase
iNOS	inducible NO-synthase
i.p.	intraperitoneal
IPF	intraperitoneal fluid
Ka	Kaempferol
LDL	low-density lipoprotein

L-NAME	N^{ω} – nitro-L-arginine methyl ester
LPS	lipopolysaccharide
M	molar (mol/liter)
MAPK	mitogen-activated protein kinase
mg	milligram
ml	milliliter
mM	millimole
MODS	multiple organ dysfunction syndrome
MPO	myeloperoxidase
MTT	3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide
NF-κB	nuclear factor kappa- B
NO	nitric oxide
NS-398	N-[2-(Cyclohexyloxy)-4-nitrophenyl] methane sulfonamide
NSAIDs	non-steroidal anti-inflammatory drugs
OD	optical density
PBS	phosphate buffered saline
PGE_2	prostaglandin E_2
PMNs	polymorphonuclear leucocytes
RT	room temperature
SEM	standard error of mean
SIRS	systemic inflammatory response syndrome
TLR-4	toll-like receptor-4
TNF-α	tumour necrosis factor – α
U	unit

CHAPTER ONE

INTRODUCTION

Inflammatory-related diseases pertain a worldwide significant public health burden. Likewise, sepsis remains the second leading cause of death without any effective remedy in intensive care units (ICUs) in the Malaysian Ministry of Health (MOH) hospitals in 2008 (Ministry of Health Malaysia, 2009). During the progression of sepsis and inflammation, macrophages upon their stimulation with toxins from invading pathogens produce a wide range of inflammatory mediators including prostaglandins (PGs), tumour necrosis factor – α (TNF-α), myeloperoxidase (MPO), nitric oxide (NO) and many others. These mediators were reported to be regulated mainly by two of the most common pathways, mitogen-activated protein kinase (MAPK) and nuclear factor kappa-B (NF-κB) (Kundu and Surh, 2008). The production and release of these mediators inturn upregulates the inflammatory process, contributing to the pathogen elemination and tissue healing. However, if not resolved, imbalance of cytokine regulation is critically involved in the pathogenesis of human autoimmune diseases (Moore and Barton, 2003); the excess production of these mediators will lead to increased leukocyte infiltration, tissue damage, organ failure and eventually death.

A class of drugs known as the non-steroidal anti-inflammatory drugs (NSAIDs) are well adopted for their analgesic, antipyretic and anti-inflammatory properties (Álvarez-Soria *et al.*, 2008). Although they offer inflammatory therapeutic relief, they were found to be associated with several adverse drug reactions. These undesirable side effects including; gastroduodenal (GD) mucosal toxicity, ulceration,

hepatic and nephrotoxic activities which attributed to non-selective inhibition of both cyclooxygenase (COX) isoforms, specifically COX-1 enzyme, which is important for several normal housekeeping activities in the human body (Heeyeong *et al.*, 2004). Moreover, the new generation of selective COX-2-inhibitor NSAIDs such as coxibs, provided the relief from pain and inflammation while avoiding the gastrointestinal side effects associated with non-selective NSAIDs. However, selective COX-2-inhibitor NSAIDs were reported to raise serious concerns about heart toxicities (Grosser *et al.*, 2006) These undesirable or detrimental side effects of the current management of inflammation or sepsis have led to pursuit for alternative therapeutic agents. In the recent past, researchers have investigated inhibitory food compounds and new agents to address the continuing demand for more potent and selective anti-inflammatory agents with minimal adverse side effects. An attractive approach for inflammation treatment is the use of natural compounds, such as flavanoids.

Flavonoids are naturally occurring polyphenolic compounds found in fruits and vegetables, they have been reported to exhibit a wide range of pharmacological properties, including anti-inflammatory, anti-oxidant, anti carcinogenic, anti bacterial and chelating properties (Pereira *et al.*, 2009). Flavonoids were reported to exhibit their drug-like effects and inhibit the production of major pro-inflammatory mediators by disruption of several essential biosynthetic and signal transduction pathways, including MAPK and NF-κB (Jie Wan *et al.*, 2009). While there is a vast research done on flavonoids, there are little or no published data that describes the synergistic effects of combinatorial treatment approach of flavonoids on cellular or animal models of inflammation.

In this study, seven flavonoids, namely chrysin, kaempferol, morin, silibinin, hisperidin, diosmin, and quercetin were selected based on their reported anti-inflammatory effects. The IC_{50} of each flavonoid were determined through measurement of selected pro-inflammatory mediators secretion in *in vitro* system. The *in vitro* system used is LPS-induced RAW 264.7 macrophages. Afterwhich, combinations were constructed and tested for synergy. The combination that exerted synergism against selected pro-inflammatory mediators tested, may serve as an approach for inflammation treatment *in vivo*. Since sepsis is a systemic disorder, the optimal combination were tested in a CLP-induced septic mice. Thus, investigation in the animal model provides more insights into therapeutic potential of the flavonoid combination in the treatment of inflammation and to improve the outcome of sepsis. This novel combinatorial approach aims to induce a response upon multiple targets involved during the inflammatory process yielding a synergistic improvement over inflammation and lethal sepsis.

Objectives of the study:

The advancement of this research is intended to lead to the identification of potent nutritional flavonoid combination/s that synergises to retain substantial therapeutic anti-inflammatory capacity *in vitro* and *in vivo* via screening of the selected flavonoids for the inhibition of major proinflammatory mediators namely NO, PGE_2 and TNF-α *In vitro,* then detecting synergism among *in vitro* flavonoid combinations, followed by testing of the most potent combination for its protective abilities in an *in vivo* model, and finally evaluation of the related synergistic mechanisms *in vivo*.

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