UNIVERSITI PUTRA MALAYSIA

SYNERGISM OF SELECTED FLAVONOIDS IN INFLAMMATION

OMAR ABDUL HAFIZ

FPSK(m) 2012 32
SYNERGISM OF SELECTED FLAVONOIDS IN INFLAMMATION

By

OMAR ABDUL HAFIZ HARASSTANI

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

April 2012
To
~My Beloved Family~
SYNERGISM OF SELECTED FLAVONOIDS IN INFLAMMATION

By

OMAR ABDUL HAFIZ

April 2012

Chairman: Professor Daud Ahmad Israf Ali, PhD
Faculty: Medicine and Health Sciences

Inflammation is characterized by the release of various pro-inflammatory mediators, including the free radical nitric oxide (NO), prostaglandin E₂ (PGE₂) 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-αsecreted 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

April 2012

Pengerusi: Profesor Daud Ahmad Israf Ali, PhD

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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, PGE2 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 IC50, flavanoid-flavanoid yang menunjukkan kesan perencatan yang berkesan, sekurang-kurangnya dua daripada tiga perantara, telah digabungkan dalam satu siri nisbah IC50 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 mentic 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. Kesian-kesian 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, PGE₂ dan TNF-α yang dirembeskan daripada sel RAW 264.7 aruhan LPS dengan nilai IC₅₀ 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.
ACKNOWLEDGEMENTS

First and above all, praises to almighty ALLAH for granting me the passion and opportunity to successfully pursue my dreams and studies, I could have never done this without the faith I have in you.

This thesis appears in its form due to the assistance and guidance of several people. I hereby would like to offer my heartfelt thanks to all.

My sincere thanks and gratitude to my supervisor Prof. Dr. Daud Ahmad bin Israf Ali and the supervisory committee; Prof. Dr. Mohamad Roslan Sulaiman and Associate Prof. Dr. Saidi Moin.

A warm appreciation also goes to the lab staff; Zulkhairi Zainol, Abdul Rahman Hassan and Nora Asyikin Mohd Salim.

Many thanks to my colleagues and dearest friends from cell signaling, physiology and medical genetics labs who have contributed to various aspects of this story over the years; Tham Chau Ling, Liew Choi Yi, Norazren Ismail, Revathee Rajajendram, Nadine Nograles, Nuzul Jambari, Nur Hayati Samsul Baharil, Jacklin Suloon, Syamimi Khalid, Nur Izzati Ismail, Ong Hui Ming, Azyyati Mohd Padzil, Azra Elia Zamri and Sally Lai Chin Ping.

And thanks to all those who came in the right time and place and helped me but may not find their names in my narration.

Last but not least, I am indebted to my parents, sisters and brothers for their unconditional love, support and inspiration.

Thank you.
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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Date: 12 JUL 2012
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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<td>°C</td>
<td>celsius</td>
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<tr>
<td>μg</td>
<td>micro gram</td>
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<tr>
<td>μl</td>
<td>micro liter</td>
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<tr>
<td>μM</td>
<td>micro molar</td>
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<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cells</td>
</tr>
<tr>
<td>AST</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>CFUs</td>
<td>colony forming units</td>
</tr>
<tr>
<td>Ch</td>
<td>Chrysin</td>
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<td>CLP</td>
<td>cecal ligation and puncture</td>
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<td>COX</td>
<td>cyclooxygenase</td>
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<td>DAN</td>
<td>2,3-diaminonaphthalene</td>
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<tr>
<td>Dexamethasone</td>
<td>dexamethasone</td>
</tr>
<tr>
<td>D-GalN</td>
<td>D-galactosamine</td>
</tr>
<tr>
<td>dH₂O</td>
<td>deionized water</td>
</tr>
<tr>
<td>DMEM</td>
<td>dulbecco’s modified eagle media</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGCG</td>
<td>epigallocatechin gallate</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
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<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GD</td>
<td>gastroduodenal</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>inhibitory concentration 50</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
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<td>iNOS</td>
<td>inducible NO-synthase</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>IPF</td>
<td>intraperitoneal fluid</td>
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<tr>
<td>Ka</td>
<td>Kaempferol</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>L-NAME</td>
<td>N°- nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>M</td>
<td>molar (mol/liter)</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>millimole</td>
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<tr>
<td>MODS</td>
<td>multiple organ dysfunction syndrome</td>
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<td>MPO</td>
<td>myeloperoxidase</td>
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<td>MTT</td>
<td>3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide</td>
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<td>NF-κB</td>
<td>nuclear factor kappa- B</td>
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<td>nitric oxide</td>
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<td>N-[2-(Cyclohexyloxy)-4-nitrophenyl] methane sulfonamide</td>
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<td>NSAIDs</td>
<td>non-steroidal anti-inflammatory drugs</td>
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<td>OD</td>
<td>optical density</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>prostaglandin E$_2$</td>
</tr>
<tr>
<td>PMNs</td>
<td>polymorphonuclear leucocytes</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
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<tr>
<td>SIRS</td>
<td>systemic inflammatory response syndrome</td>
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<tr>
<td>TLR-4</td>
<td>toll-like receptor-4</td>
</tr>
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<td>TNF-α</td>
<td>tumour necrosis factor – α</td>
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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 intum upregulates the inflammatory process, contributing to the pathogen elimination 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 flavonoids.

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-kB (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\textsubscript{50} of each flavonoid were determined through measurement of selected pro-inflammatory mediators secretion in \textit{in vitro} system. The \textit{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 \textit{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 \textit{in vitro} and \textit{in vivo} via screening of the selected flavonoids for the inhibition of major proinflammatory mediators namely NO, PGE$_2$ and TNF-$\alpha$. \textit{In vitro}, then detecting synergism among \textit{in vitro} flavonoid combinations, followed by testing of the most potent combination for its protective abilities in an \textit{in vivo} model, and finally evaluation of the related synergistic mechanisms \textit{in vivo}. 
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