



UNIVERSITI PUTRA MALAYSIA

**EFFECTS OF SELECTED PHYTOCHEMICALS FROM MALAYSIAN
PLANTS ON CELLULAR MODELS OF INFLAMMATION**

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By

SYAHIDA BINTI AHMAD

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
Fulfilment of the Requirement for the Degree of Doctor of Philosophy**

May 2006



DEDICATION

To my parents, sisters and brother.

To everyone who believed in my abilities,
and supported me in my intention
to make some of my dreams come true.



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairman: Associate Professor Daud Ahmad Israf Ali, PhD

Institute: Bioscience

Inflammatory diseases of unknown causes are difficult to treat, although non-steroidal anti-inflammatory drugs (NSAIDs) are anticipated to be useful drugs. It was believed that bioactive compounds isolated from plants might have potential anti-inflammatory properties without any side effects. Therefore, the main objectives of this study were to evaluate the effects of selected phytochemicals from Malaysian plants on the production of main inflammatory mediators and to study their mechanism of action in cellular models of inflammation.

Through the preliminary study, four out of the 32 phytochemicals tested were selected. They were atrovirinone, cardamonin, flavokawain B and zerumbone, which were isolated from *Garcinia atroviridis*, *Alpinia rafflesiana*, *Alpinia zerumbet* and *Zingiber zerumbet* respectively. All four compounds inhibited the release of interferon- γ (IFN- γ)/lipopolysaccharide (LPS)-induced nitric oxide (NO) in macrophages cell line (RAW 264.7) without any cytotoxicity effect, which was measured using the Griess assay. In order to determine the mechanism of action of these compounds, further study on the



production of main inflammatory mediators were conducted using several bioassays, namely oxidative stress assay, prostaglandin E₂ (PGE₂) and thromboxane B₂ (TxB₂) radioimmunoassay, and cytokines (tumour necrosis factor- α ; TNF- α , interleukin (IL)-1 β , IL-6 and IL-10) immunoassays. For the regulation and mechanism studies, the four compounds were tested on the main signaling pathways, which were the extracellular signal-regulated kinases (ERKs) and p38 MAPK pathways.

The results showed that atrovirinone, cardamonin, flavokawin B and zerumbone significantly suppressed IFN- γ /LPS-induced NO production dose-dependently with IC₅₀ value of 4.62 μ M, 11.40 μ M, 16.33 μ M and 7.70 μ M respectively and inhibited the reactive oxygen species (ROS) generation with IC₅₀ value of 9.48 μ M, 6.48 μ M, 28.16 μ M and 12.86 μ M respectively. These attenuations were believed to be the result of the perturbation of the inducible nitric oxide synthase (iNOS) enzyme expression in RAW264.7 cells. The data also suggested that all four compounds exhibited anti-inflammatory properties by selectively attenuating COX-2 activation, thus inhibiting the production of prostaglandin E₂ (PGE₂) with IC₅₀ value of 10.31 μ M, 26.18 μ M, 10.32 μ M and 36.20 μ M respectively in human whole blood cells. On the other hand, the ratio of IC₅₀ of COX-2/COX-1, which are associated with the production of thromboxane B₂ (TxB₂) in caprine whole blood cells, demonstrated that flavokawain B (ratio; 0.24 \pm 0.08) was the most selective towards the COX-2 enzyme, followed by atrovirinone (ratio; 0.32 \pm 0.09), cardamonin (ratio; 0.42 \pm 0.07) and zerumbone (ratio; 0.72 \pm 0.17). Interestingly, three of the compounds, namely atrovirinone, cardamonin and zerumbone, have a dual COX/LOX inhibitory activity. In addition, cardamonin,

flavokawain B and zerumbone showed significant inhibition on the synthesis of TNF- α , IL-1 β , IL-6 and IL-10 in RAW 264.7 cells in a dose-dependent manner. In contrast, atrovirinone significantly reduced the synthesis of pro-inflammatory cytokines, namely TNF- α , IL-1 β and IL-6 while it increased the secretion of the anti-inflammatory cytokine, IL-10. It is interesting to note that all four compounds suppressed the phosphorylation of the mitogen-activated protein kinases (MAPKs), namely extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and p38 MAPK.

Furthermore, it was hypothesized that the unique chemical structures of these phytochemicals are the main cause for their varying degrees in anti-inflammatory activities. In this study, two chalcone derivatives, namely cardamonin and flavokawain B were tested. The results demonstrated that substitution on the B ring was not essential for the anti-inflammatory activity of these chalcones. Besides, the presence of one or more hydroxylated and methoxylated benzoyl ring in the chalcones might enhance their scavenging and lipophilicity activities respectively. Similarly, it was suggested that the prenylated benzoquinone skeleton in atrovirinone might be important for its anti-inflammatory effect, which is due to the fact that compounds with prenylated chemical entities as part of their backbone structure are usually more hydrophobic than the non-prenylated compounds for easy penetration into the cell membrane. While in zerumbone, the α,β -unsaturated carbonyl group moiety might be important for its anti-inflammatory and antioxidant effect, which could induce the detoxification enzymes and neutralize the lipid peroxidation.



In conclusion, these findings have shown that the inhibition of main inflammatory mediators production in the cellular models of inflammation might be due to the attenuation of the ERK1/2 and p38 signaling pathways. Thus, this study has demonstrated that atrovirinone, cardamonin, flavokawain B, and zerumbone are potential lead compounds for the discovery of a new generation of drugs for controlling various acute and chronic inflammatory diseases.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN SEBATIAN-SEBATIAN KIMIA TERPILIH DARIPADA TUMBUH-TUMBUHAN MALAYSIA TERHADAP MODEL INFLAMASI DALAM SEL

Oleh

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Penyakit keradangan yang berpunca daripada sesuatu yang tidak diketahui adalah sangat sukar dirawat, meskipun ubat anti-keradangan tanpa steroid telah diperkenalkan bagi merawat penyakit berkenaan. Dipercayai bahawa sebatian kimia yang berasal daripada tumbuh-tumbuhan mempunyai kesan anti-keradangan dan tidak menyebabkan sebarang kesan sampingan. Oleh itu, kajian ini bertujuan untuk menguji kesan sebatian kimia yang berasal daripada tumbuhan terpilih terhadap bahan perantara keradangan yang utama serta kesannya terhadap mekanisma keradangan.

Melalui kajian penyaringan, empat daripada 32 sebatian kimia yang diuji telah dipilih. Empat sebatian kimia tersebut ialah atrovirinone, cardamonin, flavokawain B dan zerumbone yang telah dipencilkan daripada tumbuhan *Garcinia atroviridis*, *Alpinia rafflesiana*, *Alpinia zerumbet* dan *Zingiber zerumbet* masing-masing. Keempat-empat sebatian kimia tersebut merencat pengeluaran nitrik oksida (NO) yang di ransang oleh IFN- γ /LPS daripada sel makrofaj (RAW 264.7) tanpa sebarang kesan toksik melalui

ujian Griess asai. Bagi mengenalpasti mekanisma tindakan sebatian-sebatian kimia ini, kajian lanjut ke atas pengeluaran bahan perantara keradangan telah dijalankan, menggunakan beberapa bioasai iaitu asai tekanan oksidatif (ROS), prostaglandin E₂ (PGE₂), thromboxane B₂ (TxB₂), dan cytokine (TNF- α , IL-1 β , IL-6 dan IL-10). Di samping itu, sebatian-sebatian kimia ini juga diuji dari segi kesannya terhadap tapak jalan berisyarat utama yang terdiri daripada ERK dan p38.

Daripada kajian yang telah dijalankan, didapati atrovirone, cardamonin, flavokawain B dan zerumbone merencat pengeluaran NO dengan nilai perencatan pada kadar 50% (IC₅₀) seperti berikut, iaitu 4.62 μ M, 11.40 μ M, 16.33 μ M dan 7.70 μ M masing-masing. Keempat-empat sebatian kimia ini juga berjaya menurunkan kadar oksigen radikal bebas (ROS) dengan nilai IC₅₀ iaitu 9.48 μ M, 6.48 μ M, 28.16 μ M dan 12.86 μ M masing-masing. Dijangkakan bahawa perencatan ini adalah disebabkan oleh kesan sebatian-sebatian kimia ini terhadap enzim iNOS. Kajian ini juga telah membuktikan bahawa keempat-empat sebatian kimia ini telah menurunkan kadar pengeluaran PGE₂ dengan nilai IC₅₀ iaitu 10.31 μ M, 26.18 μ M, 10.32 μ M dan 36.20 μ M masing-masing. Manakala, melalui nilai nisbah IC₅₀ COX-2/COX-1 yang terhasil daripada perencatan pengeluaran TxB₂, mendapati bahawa flavokawain B (nisbah; 0.24 \pm 0.08) adalah lebih cenderung merencat enzim COX-2 berbanding COX-1, diikuti dengan atrovirone (nisbah; 0.32 \pm 0.09), cardamonin (nisbah; 0.42 \pm 0.07) dan zerumbone (nisbah; 0.72 \pm 0.17). Apa yang mengagumkan daripada kajian ini ialah tiga daripada sebatian kimia ini, iaitu atrovirone, cardamonin dan zerumbone berjaya merencat kedua-dua enzim COX-2 dan LOX. Selain itu, cardamonin, flavokawain B dan zerumbone telah menunjukkan kesan perencatan ke atas pengeluaran TNF- α , IL-1 β , IL-6 dan IL-10.

Sebaliknya, atrovirone telah menurunkan pengeluaran cytokine pro-keradangan (TNF- α , IL-1 β , IL-6) dan pada masa yang sama telah meningkatkan pengeluaran cytokine anti-keradangan iaitu IL-10. Lebih menarik lagi, keempat-empat sebatian kimia ini didapati merencat tindak balas fosforilasi protein MAPK iaitu ERK 1/2 dan p38.

Tambahan pula, dijangkakan bahawa perbezaan kesan anti-keradangan di antara sebatian-sebatian kimia ini adalah disebabkan oleh bentuk struktur kimianya yang unik. Dalam kajian ini, dua sebatian kimia daripada kumpulan chalcone telah diuji, iaitu cardamonin dan flavokawain B. Keputusan kajian telah membuktikan bahawa ketiadaan unsur kimia (substitution) pada gelang B kumpulan ini tidak menjejaskan aktiviti anti-keradangannya. Selain itu, kehadiran satu atau lebih hidroksi dan metoksi pada gelang benzene berkemungkinan memberi kesan kepada aktiviti kumpulan chalcone in untuk memerangkap radikal bebas (scavenging) dan tindakbalas dengan lipid (lipophilicity). Begitu juga dengan atrovirone, dijangkakan struktur benzoquinone yang berprenyl adalah sangat penting bagi meningkatkan kesan anti-keradangannya. Ini disahkan dengan fakta yang menyatakan bahawa sebatian kimia yang mempunyai struktur sedemikian, pada kebiasaannya adalah lebih hidrofobik bagi memudahkan penyerapannya melalui membran sel. Manakala, zerumbone yang mempunyai struktur kumpulan α,β -karbonil tak tepu berkemungkinan sangat penting bagi meningkatkan kesan anti-keradangan dan anti-penuaan, di mana kumpulan struktur ini dapat menyahtoksikkan enzim dan meneutralkan tindakbalas pengoksidaan lipid.

Kesimpulannya, kajian ini telah menunjukkan bahawa perencatan sintesis bahan perantara yang utama dalam model sel keradangan adalah mungkin disebabkan oleh perencatan tapak jalan ERK 1/2 dan p38. Oleh itu, kajian ini telah membuktikan bahawa atrovirinone, cardamonin, flavokawain B dan zerumbone mempunyai potensi sebagai bahan sebatian kimia utama bagi penemuan dadah generasi baru yang boleh digunakan untuk mengawal penyakit keradangan yang akut dan kronik.



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I certify that an Examination Committee met on **25 May 2006** to conduct the final examination of Syahida Binti Ahmad on her PhD thesis entitled “The effects of selected phytochemicals from Malaysian plants in cellular models of inflammation” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

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

SYAHIDA BINTI AHMAD

Date:

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4.6 Microscopy examination above show RAW 264.7 macrophage cells were adherent to plastic and were randomly distributed in culture media at >90% confluency (320X digital magnification).

A) Uninduced RAW 264.7 cells. The resting macrophages were predominantly spherical or irregular. The cytoplasmic membranes were intact and the cytoplasm homogeneous presenting small vacuoles. According to Asfora *et al.* (2005), a healthy macrophage is characterized with a kidney-form centralized nucleous, with chromatin loosely distributed and intact nuclear membrane.

B) RAW 264.7 cells after induced by IFN- γ /LPS with 0.1% DMSO

C) RAW 264.7 cells after induced by IFN- γ /LPS without 0.1% DMSO D) RAW 264.7 cells after treatment with 250 μ M of NOS inhibitor, L-NAME. As shown in the pictures above, both induced and treated RAW 264.7 cells had undergo a remarkable morphological transformation from macrophage-like cells into dendritic-like cells. Dendritic morphology is characterized by multiple prominent cytoplasmic processes, larger nuclei, prominent nucleoli and relatively prominent cytoplasm with increased granularity (Raxena *et al.*, 2003). IFN- γ /LPS-treated RAW 264.7 cells also dramatically increased in size

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4.6 Microscopy examination of RAW 264.7 macrophage cells (320X digital magnification). E) The MTT formazan produced by live cells appear as black, fuzzy crystals. F) The dead macrophages appeared deformed and agglomerated. The cytoplasmic membranes were disrupted in practically all the cells and the cytoplasm of each macrophage was dense with big vacuoles in some areas. According to Asfora *et al.* (2005), the nucleus of dead macrophages was more peripheral and the disruption of the nuclear membrane was conspicuous. The chromatin was dense and no extracellular granulation was observed

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4.7 Structure of chalcone

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