行政院國家科學委員會專題研究計畫 成果報告

類黃酮對脂泡細胞形成的分子機轉研究
研究成果報告(精簡版)

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行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

類黃酮對脂泡細胞形成的分子機轉研究

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計畫主持人：吳明娟
共同主持人：
計畫參與人員： 連子偉、洪靖婷、涂宜辰等

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

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中　華　民　國　96 年　　1 月　　28 日

I. 研究計畫中文摘要：

本研究已完成黃酮醇(flavonols, 包括 kaempferol, quercetin, morin, fisetin, myricetin)的結構與體外抗氧化活性及在 RAW264.7 的抗發炎活性的探討，並已發表在 Journal of Agricultural and Food Chemsitry (1)。此外，也利用 TBARS, conjugated diene, electrophoresis 探討黃酮醇抑制氧化型 LDL 氧化能力，發現 qucercetin 和 fisetin 在此三種指標的表現皆最佳。進一步以 U937 單核球細胞衍生的巨噬細胞為模式，利用 RT-Q-PCR 分析脂泡形成的相關基因的表現。結果顯示在 15d-PGJ2 誘導 PPARγ活化的情況下發現 morin 及 myricetin 皆可調降 CD36 的 mRNA 表現。進一步以 flow cytometry 分析則發現 fisetin, myricetin 皆可顯著調降 15d-PGJ2 誘導的 CD36 蛋白質的表現。

關鍵詞: 血管粥狀硬化、黃酮醇、CD36、ABCA1、脂泡細胞、抗氧化、抗發炎

II. Abstract

We have completed the investigation regarding to the structure-function relationship of flavonols, including fisetin (F), kaempferol (K), morin (MO), myricetin (MY) and quercetin (Q). Our result has been published in Journal of Agricultural and Food Chemistry. We found that although structural similarity, these five flavonols have distinctive antioxidant and anti-inflammatory effects (1).

The effects of flavonols in copper-induced low density lipoprotein oxidation and the effects on the expression of atherogenic-related genes were further investigated. We found that Q and F have the strongest inhibitory effect in LDL oxidation revealed by TBARS, conjugated dienes and lipid electrophoresis assay. In the presence of PPARγ ligand, 15d-PGJ2, MY can attenuate CD36 mRNA and protein expression in U937-derived macrophages. This may confer MY's ability in inhibition of foam cell formation.

Keywords: atherosclerosis, flavonol, CD36, ABCA1, foam cell, antioxidant, anti-inflammatory

III. Introduction

It has been suggested that flavonols, which have 3-OH, are the strongest antioxidants among flavonoids (*2, 3*). The B-ring OH moiety has been shown to be the most significant determinant factor in the scavenging of ROS (*2, 4*). In addition to OH moieties in the structural arrangements of flavonols, the resonance of electrons between A and B rings may also be important for their antioxidant and biological activities. In this study we examine the antioxidant and anti-inflammatory activities of selected flavonols, including fisetin, kaempferol, morin, myricetin and quercetin, to seek possible structure-function relationship. Current results show that flavonols exhibit distinctive antioxidant and anti-inflammatory potentials and the structures required to strengthen these activities vary with sorts of free radicals and mechanisms (1).

Atherosclerosis is a multifactorial process that leads to the development of a complex, cellular lesion within the arterial intima. One of the characteristic features of this lesion is the presence of lipid laden "foam-cells". Oxidised low-density lipoprotein (ox-LDL) has been suggested, could interfere with macrophage phagocytosis in vivo. Macrophages within atherosclerotic lesions become lipid-laden foam cells through unregulated uptake of ox-LDL via varied scavenger receptors (SRs). SR class B (SR-B), has been identified as the major OxLDL receptor. It is an 88-kDa transmembrane glycoprotein expressed on monocyte-derived macrophages, platelets, adipocytes, and certain endothelial and epithelial cells. CD36 may play a quantitatively significant role in modified LDL binding to macrophages.

The aim of this study was to determine the effects of flavonols on inhibition of LDL oxidation as well as CD36 expression and function in human monocyte derived macrophages.

IV. Materials and Methods

4.1 Chemicals

Kaempferol was from Fluka Chemie (Bushs, Switzerland). Fisetin, morin, myricetin, quercetin, luminol (5-amino-2,3-dihydro-1,4- phthalazinedione), DPPH, xanthine, xanthine oxidase, *f*MLP (N-Formyl-Methionyl-Leucyl- Phenylalanine), Greiss reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid) and other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise indicated.

4.2 1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging effect

The DPPH scavenging effect was measured according to Dinis et al. (*5*).

4.3 Superoxide radical scavenging effect **(1)**

4.4 Hydroxyl radical scavenging effect **(1)**

***4.5 Preparation of human polymorphonuclear Nneutrophils (PMN)*(1)**

***4.6 Measurement of N-Formyl-Methionyl-Leucyl-Phenylalanine (fMLP)-induced ROS Production in Human PMN* (1)**

4.7 RAW264.7 cell Cell Culture for Anti-Inflammatory Analysis (1)

4.8 Nitrite and Prostaglandin E_2 (PGE_2) Measurement (1)

4.9 Analysis of LDL Oxidation.

Peroxidation of LDL was measured by the determination of thiobarbituric acid reactive substances (TBARS) and expressed as malondialdehyde (MDA) equivalents. The quantity of conjugated diene in LDL was assessed by monitoring the change at A234. To measure the electrophoretic mobility, the ox-LDL was concentrated by filtration (Centricon 4) at 450g and 4 °C for 120 min. About 2 μL of each concentrated sample was loaded onto Titan lipoprotein gel (Helena Laboratories, Beaumont, TX) and run at 80 V for 45 min. The electrophoretic mobility of LDL was determined by Fat Red 7B staining according to the manufacturer'sinstructions.

4.10 U937-Derived Macrophage

U937 monocytes were cultured in RPMI 1640 medium containing 25 mM Hepes (pH 7.0) and 10% (v/v) fetal bovine serum. Differentiation into macrophages was achieved by treating the cells in 24-well plates with PMA (80 ng/ml) for 72 h.

4.11 RNA extraction and analysis.

In order to analyze the expression of CD36 mRNA, differentiated U937 cells were incubated at 37^{o}C with the required test agents or control vehicle (0.1% v/v DMSO), for 24 h. Total RNA was then prepared. The expression of each mRNA was analyzed by carrying out TaqMan real time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) amplification on a Prism 7300 sequence detection system (Applied Biosystems, USA). The sequences of the primers and probes used were as follows: CD36: forward primer, 5'-CTGAGGACAACAC AGTCTCTTTCC-3'; reverse primer, 5'-ACTGTGAAGTT GTCAGCCTCTGTTC-3'; and probe, 5'-6FAM-TGGTGCCATCTTCGAACCTTCACTATCAG-TAMRA-3'. Internal control GAPDH (TaqMan GAPDH control reagents) was purchased from Applied Biosystems (Cat No. 4331182 Assay ID Hs99999905_m1).

4.12. Flow cytometry for CD36 protein expression

Cells were washed with PBS and detached with scraper. The cells were incubated with monoclonal anti-CD36-fluorescein isothiocyanate (FITC) in ice for 1 h and then subjected to flow cytometry analysis.

4.13. Statistical Analysis

All experiments were repeated at least three times. The results were analyzed by Student's unpaired t-test and a *p* value of < 0.05 was taken to be significant.

V. Results and Discussion

5.1 The DPPH scavenging ability of flavonols

Table 1. 1,1 diphenyl-2-picrylhydrazyl (DPPH) radical scavenging effects of selected flavonols.

Tested Compounds[1]	DPPH Scavenging Activity[2] IC_{50} (μM)
fisetin	11.84 ± 0.39
kaempferol	25.70 ± 1.02
morin	20.86 ± 0.24
myricetin	12.29 ± 0.59
quercetin	8.05 ± 0.52
α-tocopherol	27.36 ±1.64

5.2 Superoxide radical scavenging effect

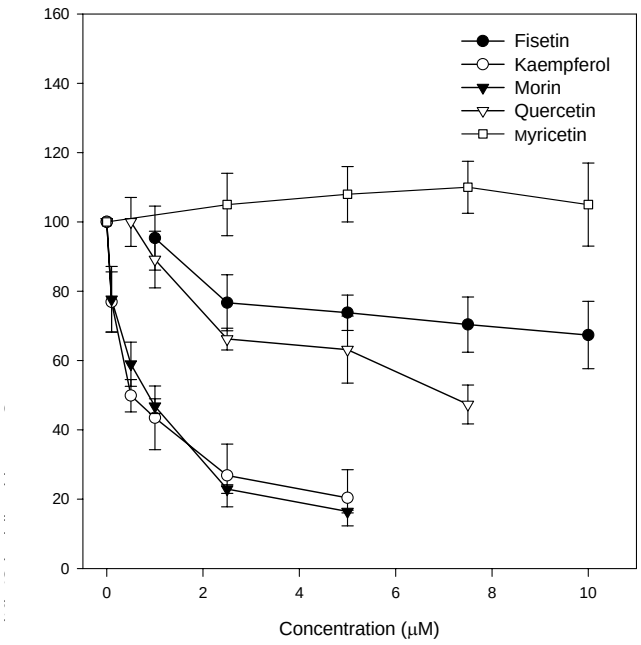

Figure 1. Flavonols inhibit superoxide-elicited chemiluminescence (CL)**.**

5.3 Hydroxyl radical scavenging effect

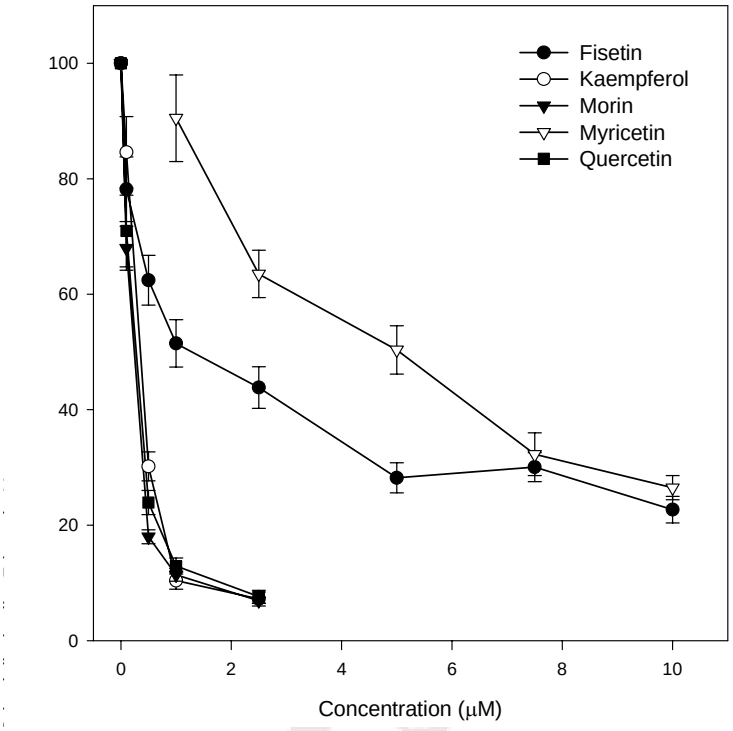

Figure 2. Flavonols inhibit hydroxyl radical-elicited chemiluminescence (CL).

5.4 Measurement of N-Formyl-Methionyl-Leucyl-Phenylalanine (fMLP)-induced ROS Production in Human PMN

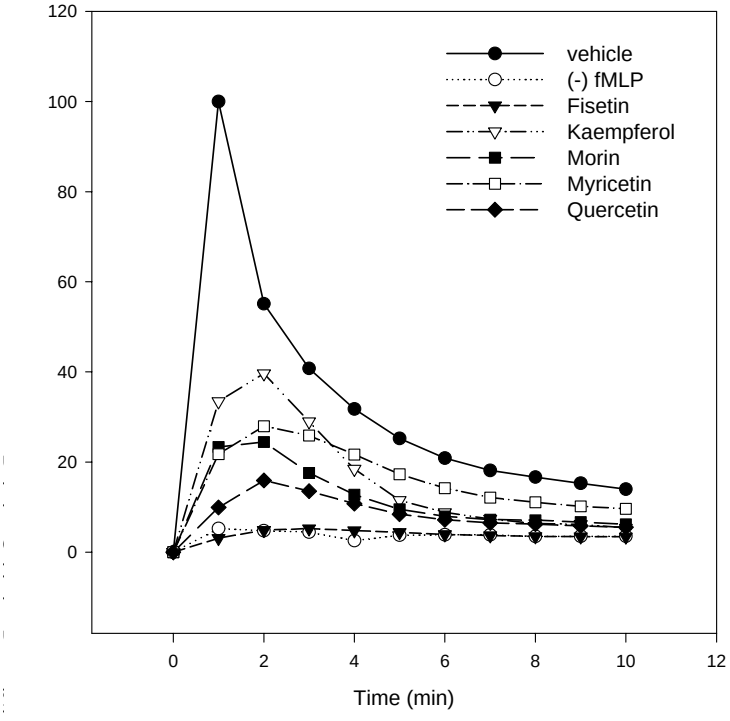

Figure 3. Flavonols inhibit *f*MLP-induced oxidative burst in PMN.

5.5 Inhibition of NO and PGE2 production in LPS-induced RAW264.7 cells by flavonols

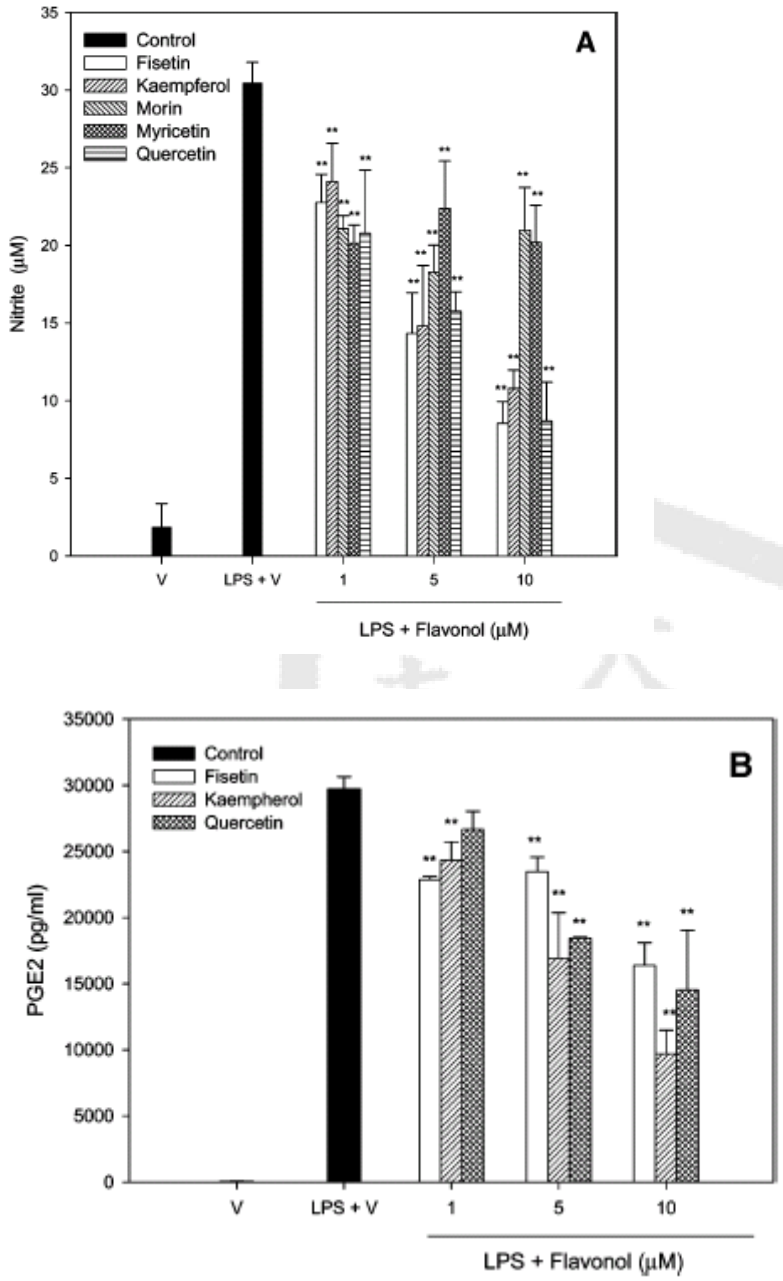

Figure 4. Effects of selected flavonols on nitrite and PGE_2 formation in RAW 264.7 macrophages.

5.6 Inhibition of Cu++-induced LDL oxidation

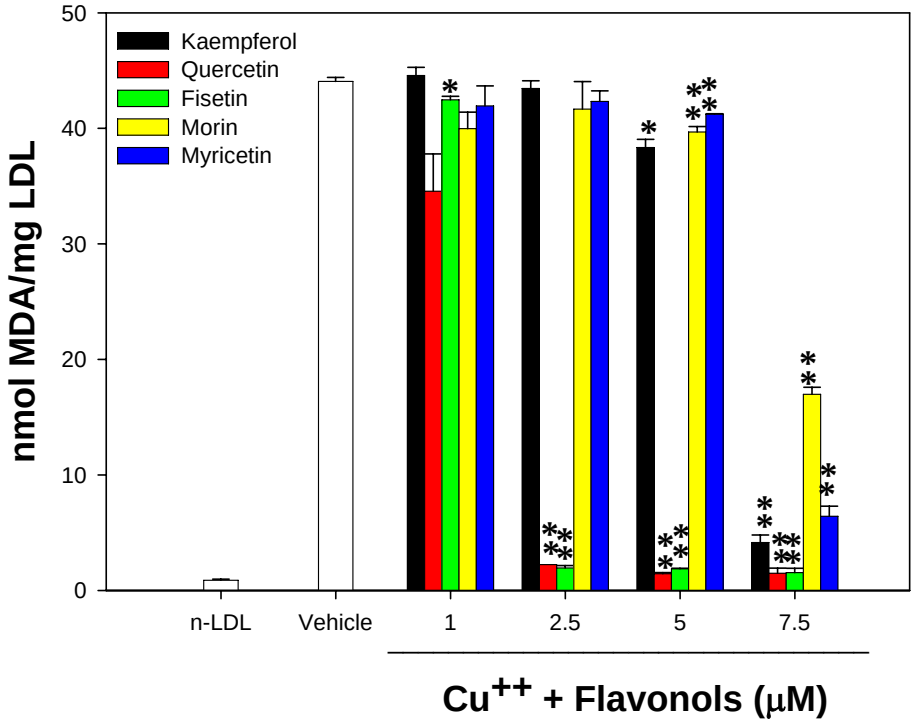

Figure 5. Inhibition of MDA formation by flavonols

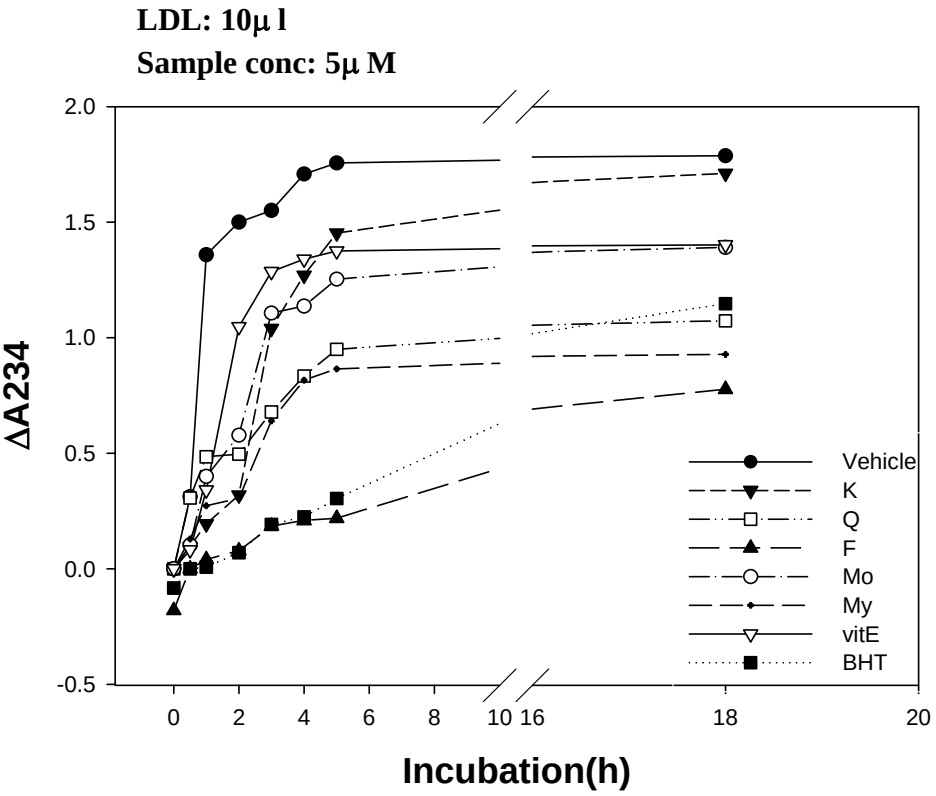

Figure 6. Inhibition of conjugated diene formation by flavonols

5.7 Effect of flavonols on CD36 mRNA and protein expression in 15d-PGJ2- induced U937-derived macrophages

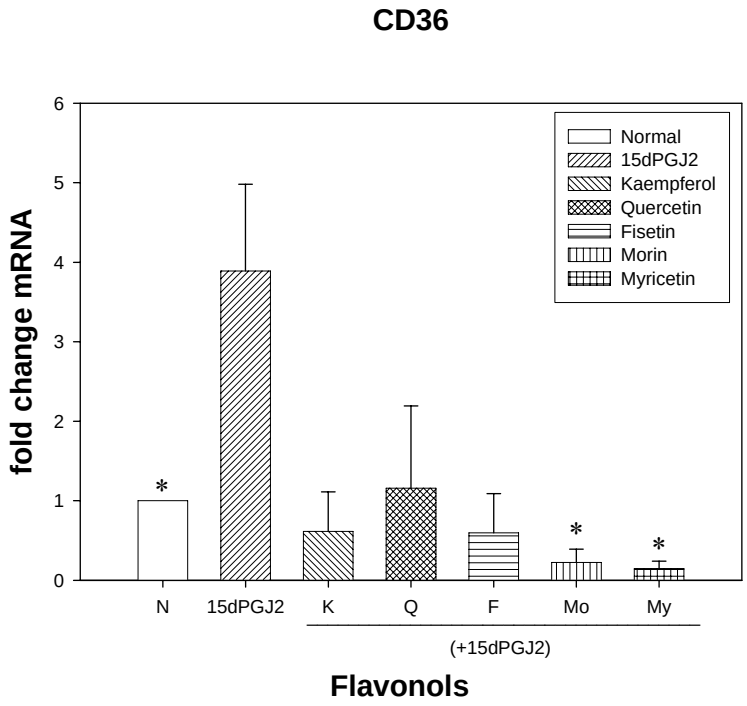

Figure 7. Effect of flavonols (20 μM) on CD36 mRNA expression in in 15d-PGJ2-induced U937-derived macrophages.

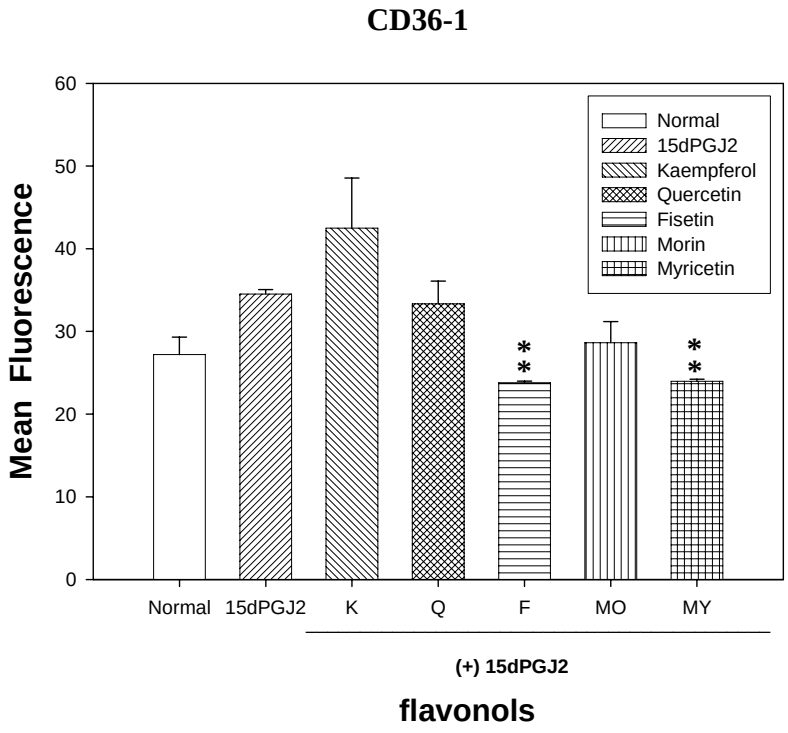

Figure 8. The effect of flavonols (20 μM) on CD36 protein expression 15d-PGJ2-induced U937-derived macrophages.

VI. References

1. Wang, L., Tu, Y.C., Lian, T.W., Hung, J.T., Yen, J.H., Wu, M.J., Distinctive antioxidant and antiinflammatory effects of flavonols. *J Agric Food Chem.* 2006, 54(26):9798-804
2. Burda, S.; Oleszek, W., Antioxidant and antiradical activities of flavonoids. *J Agric Food Chem* 2001, 49, (6), 2774-9.
3. Hollman, P. C.; Katan, M. B., Absorption, metabolism and health effects of dietary flavonoids in man. *Biomed Pharmacother* 1997, 51, (8), 305-10.
4. Sekher Pannala, A.; Chan, T. S.; O'Brien, P. J.; Rice-Evans, C. A., Flavonoid B-ring chemistry and antioxidant activity: fast reaction kinetics. *Biochem Biophys Res Commun* 2001, 282, (5), 1161-8.
5. Dinis, T. C.; Maderia, V. M.; Almeida, L. M., Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch Biochem Biophys* 1994, 315, (1), 161-9.

VII. 計畫自評

本研究已利用試管及細胞實驗探討黃酮醇抑制血管粥狀硬化可能之機轉，並將前半段的研究結果整理，刊登於 *J Agric Food Chem.* 2006, 54(26):9798-804。現正致力將後半部份的 PPARγ-CD36 實驗整理，因此達成計畫書的預期成果。

VIII. 附件：發表之期刊論文

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Distinctive Antioxidant and Antiinflammatory Effects of Flavonols

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The antioxidant and antiinflammatory effects of flavonols have been suggested to be structure-related. Results revealed that selected flavonols, including fisetin (F), kaempferol (K), morin (MO), myricetin (MY), and quercetin (Q), exhibited distinctive free radical scavenging properties against different kinds of free radicals. The H donation (DPPH bleaching) potential was Q > F ≈ MY > MO > K, indicating that the presence of a 3′,4′-catechol moiety in the B ring correlated with high activity. The 4′-OH in the B ring was suggested to be important for reducing xanthing/xanthine oxidase-generated superoxide; while an additional OH moiety on the ortho sites (3′ or 5′) attenuated the effect as the observed inhibitory potency was K ≈ MO > Q > F > MY. The relative inhibitory effect for Fenton-mediated hydroxyl radical was K ≈ MO ≈ Q > F > MY. This result implies the involvement of 4-keto, 5-OH region in Fe^{++} chelating and the negative effect of pyrogallol moiety in the B ring. Similar to the inhibitory activity against a *N*-formyl-methionyl-leucyl-phenylalanine (*f*-MLP)-stimulated oxidative burst in human polymorphonuclear neutrophils (PMN), our result showed that the structural peculiarity of the di-OH in the B ring obviously rendered F, Q, and MO more potent as ROS inhibitors than MY and K, which have tri- and mono-OH in the B ring, respectively. All of the previous data indicated that the structure prerequisite to reinforce the free radical scavenging activity varies with the type of free radical. We further analyzed the effects of flavonols on nitric oxide (NO) production in endotoxin-stimulated murine macrophages, RAW264.7 cells. Results showed that all flavonols (up to 10 μM) inhibited NO production without exerting detectable cytotoxicity. F, K, and Q dose-dependently repressed iNOS mRNA expression and prostaglandin E_2 (PGE_2) production, in part through an attenuating NF-κB signaling pathway. This result indicates that flavonols, despite structural similarity, have different antioxidant and antiinflammatory effects.

KEYWORDS: Antioxidant; anti-inflammatory; kaempferol; quercetin; fisetin; NF-κB

INTRODUCTION

Hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide radicals ($O_2{\bullet}^-$), and hydroxyl radicals (OH•), collectively known as the reactive oxygen species (ROS), are the most reactive species derived from the metabolism of oxygen in aerobic systems (*1*). Activated phagocytic cells produce large amounts of ROS. These cells, when encountering microorganisms or other mediators, have the membrane-bound NADPH oxidase complex generate a superoxide anion, which can either spontaneously or enzymatically dismutate to hydrogen peroxide. The hydroxyl radical is thought to be made by a metal-catalyzed reaction (the well-known Haber−Weiss reaction) between superoxide anion and hydrogen peroxide (*2*).

Nitric oxide (NO) is synthesized from L-arginine by constitutive and inducible nitric oxide synthase (cNOS and iNOS) in numerous mammalian cells and tissues (*3*). Constitutively expressed NO by neuronal NOS (nNOS) and endothelial NOS (eNOS) is a key regulator of homeostasis. However, NO synthesized by iNOS is induced by a variety of stimuli, such as oxidants, lipopolysaccharide (LPS), bacteria, viruses, and proinflammatory cytokines. NO can be directly cytotoxic but can also interact with superoxide anions and result in the formation of peroxynitrite ($ONOO^-$), which is the most reactive RNS. Excess production of ROS, NO, and RNS can damage DNA, lipids, proteins, and carbohydrates, leading to impaired cellular functions and enhanced inflammatory reactions.

It is well-known that the expression of several genes involved in immune and inflammatory responses is regulated at the

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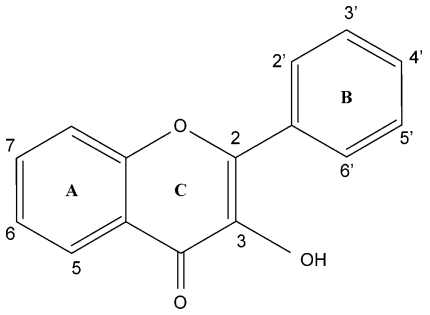

Flavonols	Hydroxyl group subsitituions
fisetin	3, 7, 3', 4'
kaempferol	3, 5, 7, 4'
morin	3, 5, 7, 2', 4'
myricetin	3, 5, 7, 3', 4', 5'
quercetin	3, 5, 7, 3', 4'

Figure 1. Generic structure of flavonols and selected compounds where the hydroxyl groups of rings A and B are shown.

transcriptional level by the nuclear factor-κB (NF-κB) (*4*). NF-κB exists within the cytoplasm in an inactive form associated with regulatory proteins, called inhibitors of κB (IκB). Upon stimulation by various extracellular signals, including LPS, the IκB kinase (IKK) phosphorylates IκB, inducing its ubiquination and subsequent degradation. NF-κB is then free to translocate to the nucleus where it facilitates the transcription of many genes, including proinflammatory cytokines, chemokines, and antiapoptotic factors.

Classically oxidative stress is described as an imbalance between generation and elimination of ROS and RNS. Oxidative stress plays a prominent role in the pathogenesis of many diseases such as respiratory distress syndrome, ischemeia/reperfusion injury, renal failure, rheumatoid arthritis, local or systemic inflammatory disorders, diabetes, atherosclerosis, cancer, and neurodegenerative diseases (*5*, *6*). Dietary intake of naturally occurring antioxidants, which scavenge free radicals, may be effective to prevent such diseases. This is the reason for the current strong interest in natural antioxidants and their roles in human health.

The flavonoids have long been recognized to possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities (*7*). The flavonoids also act as potent metal chelators and free radical scavengers and are powerful chain-breaking antioxidants (*8*). The activities of flavonoids are dependent on their chemical structures. The position and the degree of hydroxylation have been demonstrated to be the most important for their biochemical and pharmacological actions (*9*−*11*).

It has been suggested that flavonols, which have 3-OH, are the strongest antioxidants among flavonoids (*12*, *13*). The B ring OH moiety has been shown to be the most significant determinant factor in the scavenging of ROS (*12*, *14*). In addition to OH moieties in the structural arrangements of flavonols, the resonance of electrons between A and B rings may also be important for their antioxidant and biological activities. In this study, we examine the antioxidant and antiinflammatory activities of selected flavonols, including fisetin, kaempferol, morin, myricetin, and quercetin (**Figure 1**), to seek possible structure−function relationships. Current results show that flavonols exhibit distinctive antioxidant and antiinflammatory potentials and that the structures required to strengthen these activities vary with sorts of free radicals and mechanisms.

MATERIALS AND METHODS

Chemicals. Kaempferol was from Fluka Chemie (Bushs, Switzerland). Fisetin, morin, myricetin, quercetin, luminol (5-amino-2,3-dihydro-1,4- phthalazinedione), DPPH, xanthine, xanthine oxidase, *f*-MLP (*N*-formyl-methionyl-leucyl-phenylalanine), Greiss reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid), and other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise indicated.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Scavenging Effect. The DPPH scavenging effect was measured according to Dinis et al. (*15*). The reaction was performed in 1 mL of solution containing 0.1 mM freshly prepared DPPH in methanol and various concentrations of tested samples (in DMSO). After incubation at 37 °C for 30 min, the absorbance at 517 nm was measured in triplicate, and the scavenging effect was calculated against vehicle control (DMSO).

Superoxide Radical Scavenging Effect. The modified xanthine/luminol/xanthine oxidase assay was used to evaluate the superoxide scavenging effect of selected flavonols (*16*). Briefly, the reaction was carried out in a mixture containing 80 μL of 10 mM luminol (in PBS) and 10 μL of different flavonols (in DMSO). Subsequently, 5 μL of xanthine oxidase (0.02 unit/mL) was added. The reaction was started by the addition of 5 μL of xanthine (0.03 M in 1 N NaOH). The superoxide-induced luminol CL during the first 1 min was measured. The inhibitory efficiency in response to the CL of vehicle control (DMSO) was calculated.

Hydroxyl Radical Scavenging Effect. The hydroxyl radical-quenching activity of selected flavonols was measured by Fe (II)−H_2O_2−luminol CL method modified from literature (*17*). Briefly, the reaction was carried out in a mixture containing 50 μL of 10 mM luminol (in PBS), 20 μL of ferrous (100 μM)−EDTA (500 μM) complex, 20 μL of 5% H_2O_2, and 10 μL of flavonol (in DMSO). The hydroxyl-induced luminol CL during the first 1 min was averaged. The inhibitory efficiency in response to the CL of vehicle control (DMSO) was calculated.

Preparation of Human Polymorphonuclear Neutrophils (PMN). PMN was isolated from heparinized blood donated by healthy volunteers using Ficoll-Paque (Amersham Pharmacia, Upsala, Sweden) density gradient centrifugation according to the manufacturer's instructions. The isolated PMN was resuspended in RPMI-1640 medium containing 2 mM glutamine and 2.5% autologous plasma.

Measurement of *N*-Formyl-methionyl-leucyl-phenylalanine (*f*-MLP)-Induced ROS Production in Human PMN. ROS production was measured using a modified luminol-enhanced CL method (*18*). The CL response of PMN was measured using microtiter plate luminometer within 5 h after blood collection. Each well that contained 3×10^5 PMN, 1 mM luminol, and vehicle (1% DMSO) or flavonol (10 μM, in vehicle) was incubated at 37 °C in 5% CO_2 for 15 min. The activator, 10 μM *f*-MLP, was then added, the light emission, expressed as relative light units (RLU), was monitored every 1 min for 5 s during a 10 min observation period, and the kinetic curve was obtained.

Cell Culture. RAW 264.7 cells were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and cultured in Delbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 2 mM glutamine, 1% nonessential amino acid, 1 mM pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen Life Technologies, Carlsbad, CA). The cells were maintained in a humidified incubator at 37 °C in 5% CO_2.

Nitrite and Prostaglandin E_2 (PGE_2) Measurement. RAW 264.7 cells were cultured in 96-well plates until confluent. Vehicle (DMSO) alone or LPS (1 μg/mL) in combination with vehicle or indicated amount of flavonol was added into well and incubated at 37 °C in 5% CO_2 for 24 h. Nitrite production, an indicator of NO synthesis, was then determined by the Griess reaction. The culture supernatant was mixed with an equal volume of Griess reagent. The optical density at 550 nm (A550) was measured and calculated against a sodium nitrite standard curve. The level of PGE_2 in the supernatant of the culture medium was measured using an ELISA kit (Cayman Chemical, Ann Arbor, MI). Assays were performed according to the manufacturer's instruction.

Table 1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Effects of Selected Flavonols

tested compounds[a]	DPPH scavenging activity[b] IC_{50} (μM)
Gisetin	11.84 ± 0.39
Kaempferol	25.70 ± 1.02
Morin	20.86 ± 0.24
Myricetin	12.29 ± 0.59
Quercetin	8.05 ± 0.52
α-tocopherol	27.36 ± 1.64

[a] All tested samples were dissolved in DMSO. [b] The reaction was performed against 1 mM freshly prepared DPPH. After incubation at 37 °C for 30 min, the absorbance at 517 nm was measured in triplicate, and the scavenging effect was calculated against vehicle control (DMSO).

RT-PCR for iNOS and β-Actin. RAW 264.7 cells were cultured in 6-well plates until confluent. LPS (1 μg/mL) alone or in combination with an indicated amount of flavonol was added and incubated at 37 °C in 5% CO_2 for 15 h. Total cellular RNA was prepared using a RNA miniprep System (Viogene, Taipei, Taiwan). RT-PCR was performed using the Access RT-PCR System (Promega, Madison, WI). This reaction was carried out in a total volume of 50 μL, containing 1 μg of RNA, reaction buffer (1X), 0.2 mM dNTP, 50 pmol of each primer, 1 mM $MgSO_4$, AMV reverse transcriptase (5 U), and T*fi* DNA polymerase (5 U). Forward and reverse primers used for PCR for iNOS were 5′-CCCTTCCGAAGTTTCTGG CAGCAGC-3′ and 5′-GGCTGTCAGAGAGCC TCG TGGCTTTGG-3′, respectively. Forward and reverse primers used for PCR for β-actin were 5′-ATGCCATCCTGCGTCTGGAC CTGG-3′ and 5′-AGCATTTGCGGTGCACGATGGAGGG-3′, respectively. After reverse transcription at 48 °C for 45 min, the PCR was performed as follows: initiation of denaturation at 94 °C for 2 min, 10 cycles of primary amplification (94 °C for 45 s, 65 °C for 45 s, and 72 °C for 2 min), and secondary amplification (94 °C for 45 s, 67 °C for 45 s, and 72 °C for 2 min), followed by an extension at 72 °C for 10 min. RT−-PCR products were separated by 2% agarose gel electrophoresis followed by ethidium bromide staining.

Electrophoreic Mobility Shift Assay (EMSA). RAW264.7 cells were grown in 6-well plates and stimulated with LPS (1 μg/mL) alone or in combination with fisetin, kaempferol, and quercetin (10 μM) for 30 min. Nuclear extracts were prepared by NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce Endogen, Rockford, IL). EMSA experiments were performed using a LightShift Chemiluminescent EMSA Kit (Pierce Endogen). Briefly, 20 μg of nuclear protein was incubated with 50 fmol of 5′-biotinate double-stranded oligonucleotide probes containing a consensus-binding sequence for NF-κB (5′-AGTTGAGGGGACTTT CCCAGGC-3′) for 20 min at room temperature and resolved in an 8% nondenaturing polyacrylamide gel. The protein−DNA−biotin complexes were blotted onto a nylon membrane followed by UV cross-linking. The complexes were revealed with streptavidin−horseradish peroxidase conjugate and LightShift chemiluminescent substrate. The specificity of the DNA−protein complex was confirmed by competition with a 100-fold excess of an unlabeled NF-κB probe.

Statistical Analysis. All experiments were repeated at least 3 times. The results were analyzed by Student's unpaired *t*-test, and a *p* value of <0.05 was taken to be significant.

RESULTS

Effects of Flavonols on Free Radical Scavenging. To evaluate the relative antioxidant activity of flavonols, we started by investigating stable free radical (DPPH), superoxide ($O_2^{\bullet -}$), and hydroxyl radical (OH•) scavenging actions. **Table 1** demonstrates that the relative DPPH scavenging potential was on the order of Q > F ≈ MY > MO > K. Among them, quercetin was the strongest with an IC_{50} value of 8.05 ± 0.52 μM, while kaempferol was the weakest with IC_{50} of 25.70 ± 1.02 μM. The positive control, α-tocopherol, was the weakest DPPH scavenger as compared with the tested flavonols.

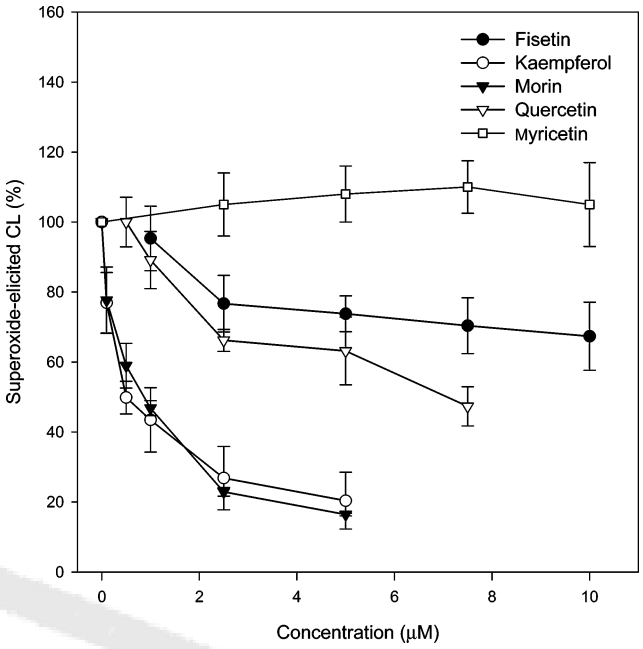

Figure 2. Flavonols inhibit superoxide-elicited chemiluminescence (CL). The reaction was carried out in a mixture containing 80 μL of 10 mM luminol (in PBS) and 10 μL of different samples. Subsequently, 5 μL of xanthine oxidase (0.02 unit/mL) was added. The reaction was started by the addition of 5 μL of xanthine (0.03 M in 1 N NaOH). The superoxide-induced luminol CL during the first 1 min was measured. The inhibitory efficiency in response to the CL of vehicle control (DMSO) was calculated. Data represent the mean ± SEM ($n = 3$) of vehicle control.

To further investigate the ROS scavenging potentials of flavonols, luminol-enhanced CL was employed to evaluate the superoxide and hydroxyl radical scavenging activities. Superoxide produced by xanthine/xanthine oxidase caused an increase in luminol-enhanced CL, and the addition of flavonols markedly inhibited CL in dose-dependent manners as shown in **Figure 2**. It was found that kaempferol and morin were the strongest superoxide scavengers with a compatible IC_{50} about 0.5 μM, followed by quercetin and fisetin in succession. Myricetin did not show detectable scavenging activity for superoxide. For comparison, the IC_{50} of a specific superoxide acceptor, 4,5-dihydroxy-1,3-benzene disulfonic acid (tiron) (*19*), was found to be about 1 mM (data not shown).

Figure 3 showed that kaempferol, morin, and quercetin were the strongest scavengers for Fenton-mediated hydroxyl radical with compatible potency, and the estimated IC_{50} was around 0.5 μM. Similar to those for a superoxide radical, myricetin was the weakest hydroxyl radical scavenger with an IC_{50} about 10-fold higher than those of kaempferol, morin, and quercetin.

Production of ROS by leukocytes is a potent microbicidal mechanism, but unrestrained production of these toxic metabolites has been indicated to mediate tissue damage. To examine the relative efficiency of flavonols for inhibiting ROS production in activated human leukocytes, we set up an in vitro method with luminol-enhanced CL to measure the ROS production induced by *f*-MLP in PMN. Chemoattractive peptide *f*-MLP activates an oxidative burst by its binding to a membrane receptor and activating a signal transduction pathway that leads to an oxidative burst being induced (*20*). **Figure 4** showed the kinetic profile of a *f*-MLP-stimulated oxidative burst in PMN. Flavonols (10 μM) differentially inhibited an oxidative burst. Among them, fisetin had the strongest potency and almost completely abolished *f*-MLP-induced ROS. On the other hand,

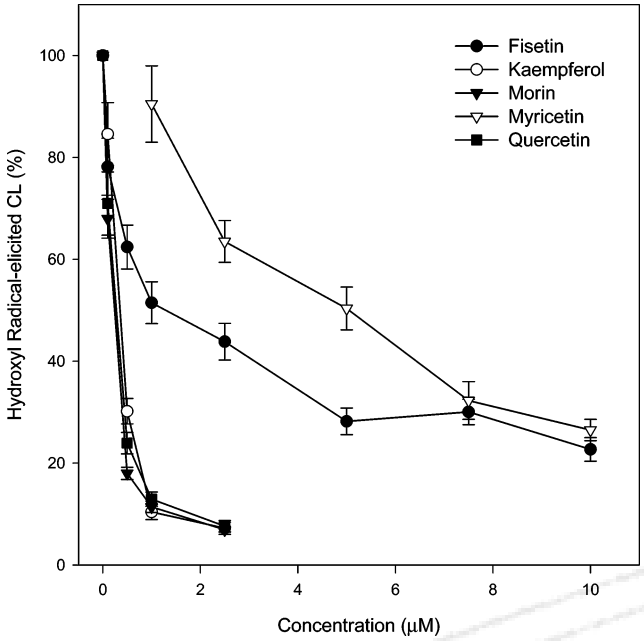

Figure 3. Flavonols inhibit hydroxyl radical-elicited chemiluminescence (CL). The reaction was carried out in a mixture containing 50 μL of 10 mM luminol (in PBS), 20 μL of ferrous (100 μM)–EDTA (500 μM) complex, 20 μL of 5% H_2O_2, and 10 μL of flavonols (in DMSO). The hydroxyl-induced luminol CL during the first 1 min was averaged. The inhibitory efficiency in response to the CL of vehicle control (DMSO) was calculated. Data represent the mean ± SEM (n = 10) of vehicle control.

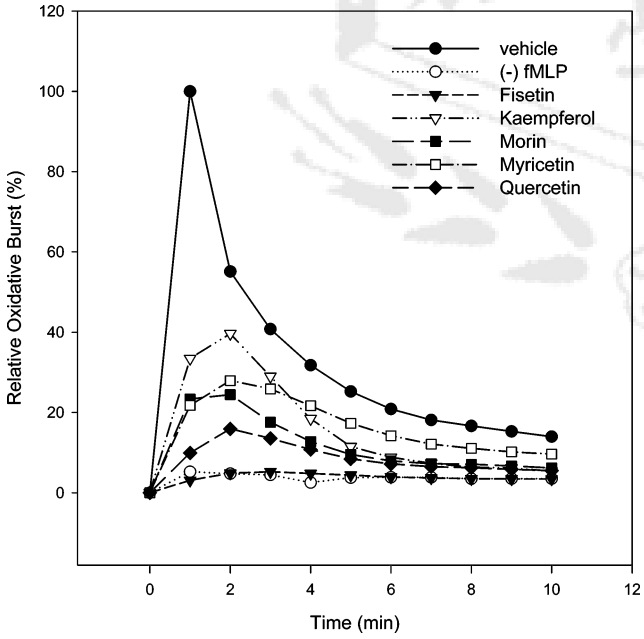

Figure 4. Flavonols inhibit *f*-MLP-induced oxidative burst in PMN. Each well containing 3×10^5 PMN, 1 mM luminol and vehicle, or 10 μM indicated flavonol was incubated at 37 °C in 5% CO_2 for 15 min. The activator, 10 μM *f*-MLP, was then added, and the light emission, expressed as relative light units (RLU), was monitored every 1 min for 5 s during a 10 min observation period. The experiments were repeated 3 times with a representative result shown.

kaempherol showed the weakest inhibitory activity and inhibited only about 60% of oxidative burst at the same concentration.

Effects of Flavonols on Nitrite Release. Nitric oxide (NO) synthesized by activated inflammatory cells regulates the functions of other cells involved in the inflammatory process

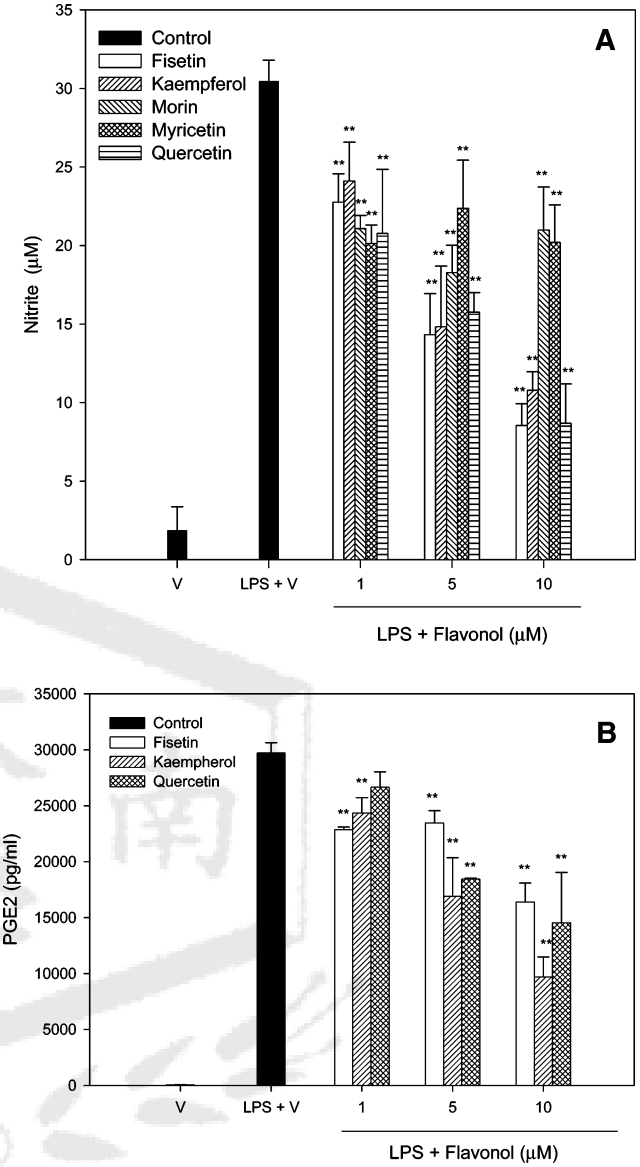

Figure 5. Effects of selected flavonols on nitrite and PGE_2 formation in RAW 264.7 macrophages. RAW 264.7 macrophages were cultured at 37 °C for 24 h in a 96-well plate in the presence of vehicle control (V, DMSO), LPS (1 μg/mL) plus vehicle (V), or LPS (1 μg/mL) in combination with indicated concentrations of flavonol. (**A**) The culture supernatant was mixed with Griess reagent for nitrite analysis. (**B**) The level of PGE_2 in the supernatant was measured using an ELISA kit. Data are expressed as the mean ± SEM of three individual experiments. Statistically significant inhibition (**p < 0.01), as compared with the groups treated with LPS plus vehicle.

and appears to act as a secondary mediator of some actions of proinflammatory cytokines. The effects of flavonols on NO production in LPS-stimulated RAW264.7 macrophages are shown in **Figure 5A**. Stimulation of cells with LPS (1 μg/mL) for 24 h induced a dramatic increase in nitrite production from the basal level (1.86 ± 1.52 μM) to 30.4 ± 1.3 μM. Fisetin, kaempferol, and quercetin evoked a dose-dependent inhibition on nitrite release (n = 3), and the inhibition reached 72, 65, and 71% at 10 μM, respectively. In contrast, morin and myricetin are significantly weaker and with no dose-dependent effect. A MTT test revealed that none of the flavonols (up to 10 μM) caused significant cytotoxicity of RAW264.7 cells (data not shown). This result implies that flavonols inhibited nitrite release without causing cell death.

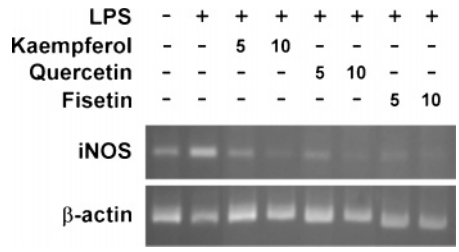

Figure 6. RT-PCR analysis of iNOS mRNA expression. RAW 264.7 cells were cultured with LPS (1 μg/mL) alone or in combination with indicated amounts of flavonol at 37 °C in 5% CO_2 for 15 h. Total RNA was isolated for iNOS and β-actin mRNA analyses. The experiments were replicated 3 times with similar results.

Effects of Fisetin, Kaempferol, and Quercetin on iNOS mRNA Expression. To further assess the effect of fisetin, kaempferol, and quercetin administration on iNOS mRNA levels, RAW264.7 cells were cotreated with LPS (1 μg/mL) and three flavonols (5 and 10 μM) for 15 h. RT-PCR analysis of the extracted RNA revealed that LPS caused an increase in iNOS mRNA expression as compared with the control group (**Figure 6**). Fisetin, kaempferol, and quercetin, in conjunction with the stimuli, blocked this induction dose-dependently. Thus, the action of fisetin, kaempferol, and quercetin on NO release was caused, at least in part, by inhibition of iNOS mRNA expression.

Effects of Fisetin, Kaempferol, and Quercetin on PGE_2 Release. It has been shown that quercetin and kaempherol exerted their antiinflammatory and anticancer effects through inhibition of COX-2 gene expression (*21*, *22*). To the best of our knowledge, literature regarding to the effect of fisetin on PGE_2 production has yet been reported. **Figure 5B** showed that stimulation of RAW 264.7 cells with LPS (1 μg/mL) for 24 h induced a dramatic increase in PGE_2 production from the basal level (69 $\pm$ 6 pg/mL) to 29719 $\pm$ 917 pg/mL. Fisetin, kaempferol, and quercetin evoked a dose-dependent inhibition of LPS-activated PGE_2 release (n = 3), and the inhibition reached 45, 74, and 51%, respectively, when the concentration was 10 μM.

Effects of Fisetin, Kaempferol, and Quercetin on NF-κB Activation. A search for common pathways involved in the regulated induction of diverse inflammatory gene expression has focused on transcriptional control mechanisms and has identified NF-κB as a likely converging point of various immune and inflammatory responses (*23*). It has also been shown that quercetin and kaempferol suppressed NF-κB/IκB signal transduction pathways in LPS-stimulated macrophages cells (*24*, *25*). However, there is no report with regard to the effect of fisetin on NF-κB activation so far. **Figure 7** demonstrated that nuclear extracts from LPS-stimulated macrophages exhibited strong κB-binding activity in electrophoretic mobility shift assays (EMSA) using a biotin-labeled oligonucleotide containing a consensus NF-κB-binding site. The binding was specific since it was inhibited with an excess of unlabeled, identical oligonucleotide and was absent from the nuclear extract of nonstimulated cells (data not shown). Nuclear extract from macrophages stimulated with LPS plus kaempferol or quercetin (10 μM) showed a significantly decreased κB-binding activity, while less effects were found for LPS plus fisetin.

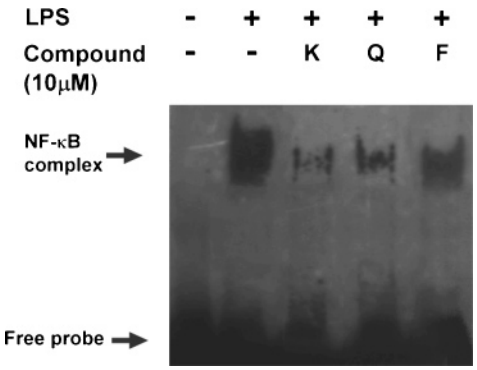

Figure 7. Effect of fistetin (F), kaempferol (K), and quercetin (Q) on LPS-induced κB DNA binding. RAW 264.7 cells were treated with LPS (1 μg/mL) with vehicle or indicated flavonol (10 μM) for 30 min. Nuclear extracts were prepared and analyzed for κB DNA binding using the electrophoreic mobility shift assay (EMSA). Assays were repeated 3 times with a representative result shown.

DISCUSSION

Flavonoids have been suggested to have several potential health benefits due to their antioxidant and antiinflammatory activities, which are attributed to the presence of phenolic hydroxyl (OH) moieties on the structure (*26*). The association of the number of OH moieties with the antioxidant activity of flavonoids has been investigated extensively (*9*−*11*, *27*, *28*). In general, free radical scavenging by flavonoids occurs via electron donation from the free hydroxyls on the flavonoid nucleus with the formation of less reactive flavonoid aroxyl radicals (*29*).

We started our study of the structure−function relationship of flavonols from DPPH free radical bleaching, which has been routinely used to test hydrogen atom donation activity for antioxidants (*15*). We found that quercetin, myricetin, and fistin, which have ortho 3′,4′-di-OH in the B ring (catechol), have a significantly stronger DPPH scavenging activity than kaempherol (4′-OH) or morin (2′,4′-di-OH). This result indicates that the structure prerequisite to reinforce DPPH scavenging is the catechol arrangement. This result is in agreement with published data (*9*), which suggested that the high scavenging activity of 3′,4′-catechol was attributed to their rapid reaction with DPPH to form dimers.

Xanthine oxidase is a source of oxygen free radicals. In the reperfusion phase (i.e., reoxygenation), xanthine oxidase reacts with molecular oxygen, thereby releasing superoxide free radicals. In the present paper, we demonstrated that flavonols inhibited superoxide production generated by xanthine/xanthine oxidase. Among them, morin (2′,4′-di-OH) and kaempherol (4′-OH) have stronger activities than qucercetin or fisetin (3′,4′-di-OH), while myricetin (3′,4′,5′-tri-OH) has no detectable activity. It has been demonstrated that flavonoids inhibited xanthine oxidase activity and/or scavenged superoxide (*30*). As a result, the decrease of superoxide may be due to the combinatory effects of scavenging superoxide and inhibiting xanthine oxidase activity. It has also been reported that kaempferol is a very good superoxide scavenger, even though it has only one hydroxyl group on the B ring (4′-OH) possibly because of the combination of the other characteristics (C2═C3 double bond, 3-OH group, and 4-oxo group on the C ring) (*31*). The structural peculiarities of the C ring obviously are common for the tested flavonols; therefore, the difference in the reduction of superoxide production may be attributed only to the location rather than the number of OH substitutions on the B ring. 4′-OH may be sufficient to render a flavonol as an inhibitor of xanthine oxidase and/or scavenger of superoxide. The additional OH group on the ortho sites (3′- or 5′-OH) attenuated the activity possibly through steric hindrance, as the OH group on the meta site (2′-OH) did not influence the effect.

We found that kaempherol, morin, and qucercetin exerted compatible inhibitory activity against Fenton-generated hydroxyl radicals, while fisetin and myricetin were significantly weaker. It has been well-known that metal-binding properties of flavonoids offer antioxidant action by encapsulation of a prooxidant iron species, which generates hydroxyl radical species through the Fenton reaction (*32*). The structural features contributed to the metal chelating have been suggested to be 4-keto, 5-OH region, 4-keto, 3-OH region, and 3′,4′-di-OH (*33*). Current results suggested that the 4-keto, 5-OH region conferred the activity for chelating Fe^{++}, while the pyrogallol moiety in the B ring attenuated the effects.

From the previous in vitro data, it seems that myricetin, which has six OH moieties, showed the least antioxidant activity against both superoxide and hydroxyl radicals. It has been reported that the level of antioxidant activity increased depending on the numbers of OH groups when OH derivatives up to five but compounds with six OH groups conversely declined (*11*). It has also been suggested that myricetin bearing a pyrogallol moiety has a lower antioxidant potential than a quercetin containing catechol moiety (*34*).

To look for compounds from plants or natural products for the prevention of ROS-associated disorders by inflammatory cells is one of the important strategies for antioxidant therapy in recent research. Current studies demonstrated that ROS production by *f*MLP-elicited PMN was attenuated by flavonols on the order of fisetin > quercetin > morin > myricetin > kaempherol. This result implies that di-OH on the B ring renders the compounds more potent as inhibitors for oxidative bursts. Higher or lower numbers of OH moieties would decrease the activity. Suppression of ROS production by flavonols might be attributed to the combinatory effects, including inhibiting NADPH oxidase activity and scavenging free radicals (*35*).

To further investigate the effect of flavonols on another free radical, nitric oxide (NO), we found that only fisetin, kaempferol, and quercetin dose-dependently inhibited nitrite release in LPS-stimulated RAW 264.7 cells through down-regulating iNOS mRNA expression. Fisetin, kaempferol, and quercetin (up to 10 μM) also dose-dependently inhibited the release of PGE_2, another inflammatory mediator, in activated RAW 264.7. EMSA further revealed that fisetin, kaempferol, and quercetin inhibited κB binding, which is necessary for the expression of iNOS and COX-2, with its binding motif in the promoter of target genes. It has been proposed that in addition to attenuating NF-κB activation, phenolic compounds may exert their antiinflammatory activity by inhibiting ERK1/2 phosphorylation or JAK/STAT-1 activation or by directly interrupting LPS binding to toll-like receptors (*36*). The fact that fisetin was weaker in inhibiting κB binding as compared with kaempferol and quercetin, while it was compatible in inhibiting NO or PGE_2 production, might reflect the possibility that fisetin inhibited either one of the previous pathways as well.

Taken together, current results suggest that fisetin, kaempferol, and quercetin exert antiinflammatory effects at least in part through down-regulating the NF-κB signaling pathway and in turn repressing iNOS and COX-2 gene expression. This result also indicates that although closely related in structure, the antioxidant and antiinflammatory efficiencies of the selected flavonol compounds differ significantly. It has been proposed that the additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their biological functions (*37*). Therefore, consumption of various sources of fruits and vegetables is highly recommended for health maintenance.

ABBREVIATIONS USED

CL, chemiluminescence; COX-2, cyclooxygenase-2; EMSA, electrophoreic mobility shift assay; *f*MLP, *N*-formyl-methionyl-leucyl-phenylalanine; LPS, lipopolysaccharide; NO, nitric oxide; PGE_2, prostaglandin E_2; PMN, polymorphonuclear neutrophils; RNS, reactive nitrogen species; ROS, reactive oxygen species.

LITERATURE CITED

(1) Gutteridge, J. M. Biological origin of free radicals and mechanisms of antioxidant protection. *Chem.-Biol. Interact.* **1994**, *91* (2−3), 133−40.

(2) Babior, B. M. The respiratory burst of phagocytes. *J. Clin. Invest.* **1984**, *73* (3), 599−601.

(3) Nathan, C.; Xie, Q. W. Nitric oxide synthases: roles, tolls, and controls. *Cell* **1994**, *78* (6), 915−8.

(4) Chen, F.; Castranova, V.; Shi, X.; Demers, L. M. New insights into the role of nuclear factor-κB, a ubiquitous transcription factor in the initiation of diseases. *Clin. Chem.* **1999**, *45* (1), 7−17.

(5) Aruoma, O. I.; Kaur, H.; Halliwell, B. Oxygen free radicals and human diseases. *J. R. Soc. Health* **1991**, *111* (5), 172−7.

(6) Emerit, J.; Edeas, M.; Bricaire, F. Neurodegenerative diseases and oxidative stress. *Biomed. Pharmacother.* **2004**, *58* (1), 39−46.

(7) Nijveldt, R. J.; van Nood, E.; van Hoorn, D. E.; Boelens, P. G.; van Norren, K.; van Leeuwen, P. A. Flavonoids: a review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.* **2001**, *74* (4), 418−25.

(8) Kandaswami, C.; Middleton, E. Free radical scavenging and antioxidant activity of plant flavonoids. In *Free Radicals in Diagnostic Medicine*; Amstron, D., Ed.; Plenum Press: New York, 1994.

(9) Furusawa, M.; Tanaka, T.; Ito, T. Antioxidant activities of hydroxyl flavonoids. *J. Health Sci.* **2005**, *51* (3), 376−8.

(10) Hu, J. P.; Calomme, M.; Lasure, A.; De Bruyne, T.; Pieters, L.; Vlietinck, A.; Vanden Berghe, D. A. Structure−activity relationship of flavonoids with superoxide scavenging activity. *Biol. Trace Elem. Res.* **1995**, *47* (1−3), 327−31.

(11) Mikamo, E.; Okada, Y.; Semma, M.; Ito, Y.; Morimoto, T.; Nakamura, M. Studies on structural correlation with antioxidant activity of flavonoids. *Nippon Shokuhin Kagaku Gakkaishi* **2000**, *7*, 97−101.

(12) Burda, S.; Oleszek, W. Antioxidant and antiradical activities of flavonoids. *J. Agric. Food Chem.* **2001**, *49* (6), 2774−9.

(13) Hollman, P. C.; Katan, M. B. Absorption, metabolism, and health effects of dietary flavonoids in man. *Biomed. Pharmacother.* **1997**, *51* (8), 305−10.

(14) Sekher Pannala, A.; Chan, T. S.; O'Brien, P. J.; Rice-Evans, C. A. Flavonoid B-ring chemistry and antioxidant activity: fast reaction kinetics. *Biochem. Biophys. Res. Commun.* **2001**, *282* (5), 1161−8.

(15) Dinis, T. C.; Maderia, V. M.; Almeida, L. M. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch. Biochem. Biophys.* **1994**, *315* (1), 161−9.

(16) Marquele, F. D.; Di Mambro, V. M.; Georgetti, S. R.; Casagrande, R.; Valim, Y. M.; Fonseca, M. J. Assessment of the antioxidant activities of Brazilian extracts of propolis alone and in topical pharmaceutical formulations. *J. Pharm. Biomed. Anal.* **2005**, *39* (3−4), 455−62.

(17) Cheng, Z.; Yan, G.; Li, Y.; Chang, W. Determination of antioxidant activity of phenolic antioxidants in a Fenton-type reaction system by chemiluminescence assay. *Anal. Bioanal. Chem.* **2003**, *375* (3), 376−80.

(18) Wu, M. J.; Huang, C. L.; Lian, T. W.; Kou, M. C.; Wang, L.

Antioxidant activity of *Glossogyne tenuifolia*. *J. Agric. Food Chem.* **2005**, *53* (16), 6305−12.
(19) Kladna, A.; Aboul-Enein, H. Y.; Kruk, I. Enhancing effect of melatonin on chemiluminescence accompanying decomposition of hydrogen peroxide in the presence of copper. *Free Radical Biol. Med.* **2003**, *34* (12), 1544−54.
(20) Nemeth, K.; Furesz, J.; Csikor, K.; Schweitzer, K.; Lakatos, S. Luminol-dependent chemiluminescence is related to the extracellularly released reactive oxygen intermediates in the case of rat neutrophils activated by formyl-methionyl-leucyl-phenylalanine. *Haematologia* **2002**, *31* (4), 277−85.
(21) Chen, Y. C.; Shen, S. C.; Lee, W. R.; Hou, W. C.; Yang, L. L.; Lee, T. J. Inhibition of nitric oxide synthase inhibitors and lipopolysaccharide induced inducible NOS and cyclooxygenase-2 gene expressions by rutin, quercetin, and quercetin pentaacetate in RAW 264.7 macrophages. *J. Cell. Biochem.* **2001**, *82* (4), 537−48.
(22) O'Leary, K. A.; de Pascual-Tereasa, S.; Needs, P. W.; Bao, Y. P.; O'Brien, N. M.; Williamson, G. Effect of flavonoids and vitamin E on cyclooxygenase-2 (COX-2) transcription. *Mutat. Res.* **2004**, *551* (1−2), 245−54.
(23) Sun, Z.; Andersson, R. NF-κB activation and inhibition: a review. *Shock* **2002**, *18* (2), 99−106.
(24) Cho, S. Y.; Park, S. J.; Kwon, M. J.; Jeong, T. S.; Bok, S. H.; Choi, W. Y.; Jeong, W. I.; Ryu, S. Y.; Do, S. H.; Lee, C. S.; Song, J. C.; Jeong, K. S. Quercetin suppresses proinflammatory cytokine production through MAP kinases and NF-κB pathway in lipopolysaccharide-stimulated macrophage. *Mol. Cell. Biochem.* **2003**, *243* (1−2), 153−60.
(25) Comalada, M.; Ballester, I.; Bailon, E.; Sierra, S.; Xaus, J.; Galvez, J.; Medina, F. S.; Zarzuelo, A. Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: Analysis of the structure−activity relationship. *Biochem. Pharmacol.* **2006**, *72* (8), 1010−21.
(26) Kandaswami, C.; Middleton, E., Jr. Free radical scavenging and antioxidant activity of plant flavonoids. *Adv. Exp. Med. Biol.* **1994**, *366*, 351−76.
(27) Cao, G.; Sofic, E.; Prior, R. L. Antioxidant and prooxidant behavior of flavonoids: structure−activity relationships. *Free Radical Biol. Med.* **1997**, *22* (5), 749−60.
(28) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure−antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20* (7), 933−56.
(29) Pannala, A. S.; Rice-Evans, C. A.; Halliwell, B.; Singh, S. Inhibition of peroxynitrite-mediated tyrosine nitration by catechin polyphenols. *Biochem. Biophys. Res. Commun.* **1997**, *232* (1), 164−8.
(30) Cos, P.; Ying, L.; Calomme, M.; Hu, J. P.; Cimanga, K.; Van Poel, B.; Pieters, L.; Vlietinck, A. J.; Vanden Berghe, D. Structure−activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. *J. Nat. Prod.* **1998**, *61* (1), 71−6.
(31) Middleton, E., Jr.; Kandaswami, C.; Theoharides, T. C. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* **2000**, *52* (4), 673−751.
(32) Ferrali, M.; Signorini, C.; Caciotti, B.; Sugherini, L.; Ciccoli, L.; Giachetti, D.; Comporti, M. Protection against oxidative damage of erythrocyte membrane by the flavonoid quercetin and its relation to iron chelating activity. *FEBS Lett.* **1997**, *416* (2), 123−9.
(33) Cheng, I. F.; Breen, K. On the ability of four flavonoids, baicilein, luteolin, naringenin, and quercetin, to suppress the Fenton reaction of the iron−ATP complex. *Biometals* **2000**, *13* (1), 77−83.
(34) Furuno, K.; Akasako, T.; Sugihara, N. The contribution of the pyrogallol moiety to the superoxide radical scavenging activity of flavonoids. *Biol. Pharm. Bull.* **2002**, *25* (1), 19−23.
(35) Hodnick, W. F.; Duval, D. L.; Pardini, R. S. Inhibition of mitochondrial respiration and cyanide-stimulated generation of reactive oxygen species by selected flavonoids. *Biochem. Pharmacol.* **1994**, *47* (3), 573−80.
(36) Hong, J.; Sang, S.; Park, H. J.; Kwon, S. J.; Suh, N.; Huang, M. T.; Ho, C. T.; Yang, C. S. Modulation of arachidonic acid metabolism and nitric oxide synthesis by garcinol and its derivatives. *Carcinogenesis* **2006**, *27* (2), 278−86.
(37) Liu, R. H. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *Am. J. Clin. Nutr.* **2003**, *78* (Suppl. 3), 517−20.

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