行政院國家科學委員會專題研究計畫　成果報告

具抗發炎功效的青草之抗血管粥狀硬化功效評估及分子機轉探討

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中文摘要

發炎與免疫反應在引發血管粥狀硬化(atherogenesis)上扮演重要的角色。促發炎細胞激素及白血球的趨化作用可誘導血管粥狀硬化及粥瘤的形成。隨著分子病理的進步，最近有許多新的預防血管粥狀硬化藥劑的開發策略，包括:抗氧化劑及抗發炎劑等。

本報告主要探討本土青草中的香茹草及箭葉鳳尾蕨在試管中的自由基螯合能力及抗低密度脂蛋白的氧化能力，以及對白血球的氧化釋放的抑制等。另外，亦將使用巨噬細胞株探討箭葉鳳尾蕨的詳細抗發炎機轉。

香茹草及其主要成份 luteolin-7-glucoside 皆可顯著螯合 DPPH、超氧自由基、氫氧自由基並抑制銅離子誘導的低密度脂蛋白氧化及 *f*-MLP 誘發的白血球的氧化釋放(1)。

箭葉鳳尾蕨的水萃取物可抑制內毒素誘發的老鼠巨噬細胞株RAW264.7的促發炎細胞激素，NO 及 PGE_2 的釋放，並抑制 NF-κB 的活化(2)。箭葉鳳尾蕨亦具有強的抗氧化性，可螯合自由基並抑制銅離子誘導的 LDL 氧化，且其主要的抗氧化成份為 pterisoside 及 bockioside B (3, 4)。

本研究結果顯示香茹草及箭葉鳳尾蕨皆具抗氧化及抗發炎活性，且可藉由抑制低密度脂蛋白的氧化及白血球 ROS 的釋放，以抑制血管粥狀硬化的形成。
關鍵詞: 血管粥狀硬化、抗發炎、抗氧化、箭葉鳳尾蕨、香茹草、低密度脂蛋白

Abstract

Inflammation plays a fundamental role in the development and complications of atherosclerosis. Leukocyte recruitment and expression of pro-inflammatory cytokines characterize early atherogenesis and malfunction of inflammatory mediators mutes atheroma formation. Recently, several different pharmacological strategies for preventing atherosclerosis have been established, including antioxidants and anti-inflammatory agents.

Glossogyne tenuifolia is a native traditional anti-inflammatory herb in Taiwan. It has previously been shown that the ethanol extract of *G. tenuifolia* (GT) inhibited the LPS-induced inflammatory mediator release from murine macrophage cell line and human whole blood. In the present work, the ethanol extracts of *G. tenuifolia* and its major constituent, luteolin-7-glucoside, were shown to be scavengers of 1,1-diphenyl-2-picrylhydrazyl, superoxide, and hydroxyl radicals. Moreover, copper-induced low density lipoprotein oxidation was suppressed by GT and luteolin-7-glucoside as measured by decreased formation of malondialdehyde and conjugated diene as well as reduced electrophoretic mobility. GT and luteolin-7-glucoside were also against *f*-MLP induced reactive oxygen species (ROS) production in human polymorphonuclear neutrophils and peripheral blood mononuclear cells (1).

Sword brake fern (*Pteris ensiformis* Burm.) is an ingredient in most of the traditional herbal beverage formulas in Taiwan. The aim of this study is to elucidate the molecular pharmacological activity in the aqueous extract of sword brake fern (SBF). We found that SBF (0.05–0.25 mg/ml) dose dependently suppressed LPS-induced TNF-α, IL-1β, IL-6, NO and PGE_2 in activated RAW264.7 cells without exerting cytotoxicity. Further analysis of molecular mechanisms revealed that SBF prominently repressed LPS-induced iNOS (inducible nitric oxide synthase) and COX-2 (cyclooxygenase-2) promoter activities. Activation of the transcription factor NF-κB, which is one of the important pathways for transduction of LPS-stimulated inflammatory mediator producing signals, was suppressed by SBF in a dose-dependent manner, as demonstrated by both electrophoretic mobility shift assay (EMSA) and transfection with pNF-κB-Luc plasmid (2). DPPH scavenging activity-guided fractionation led to the isolation of two maltol glucosides and one pteriosin glucosides, pterisoside A (hyroxymaltol 7-*O*-caffeoyl-3-*O*-β-D-glucopyranoside), bockioside B (hyroxymaltol 7-*O*-coumaryl-3-*O*-β-D-glucopyranoside), and pterisoside B [2(*R*), 3(*R*)-pterosin L 3-*O*-β-$_D$-glucopyranoside], respectively from SBF. SBF (0.01~2 mg/ml) and these two maltol glucosides (1 ~ 10 μM) exhibited strong superoxide, hydroxyl radical, and hydrogen peroxide scavenging activities *in vitro*. Pterisoside A exhibits stronger antioxidant activity in every aspect than bockioside B (3- 4).

In summary, these data indicate that GT and SBF are potential ROS scavengers and may prevent atherosclerosis via inhibiting LDL oxidation or ROS production in human leukocytes. Moreover, luteolin-7-glucoside and pterisoside A may serve as the active principal for GT and SBF, respectively.
Keywords: atherosclerosis, antioxidant, anti-inflammatory, *Glossogyne tenuifolia*, *Pteris ensiformis*, LDL

Introduction

It has been well recognized that free radicals are formed in living organisms as a function of endogenous biochemical processes. Of particular relevance in aerobic systems are those reactive species derived from the metabolism of oxygen, which include hydrogen peroxide, singlet oxygen, superoxide radicals, and hydroxyl radicals, collectively known as the reactive oxygen species (ROS) (5). ROS are generated by several mechanisms, such as interaction of ionizing radiation with biological molecules, as well as produced by cellular respiration and phagocytic cells, namely, neutrophils and macrophages, which are crucial in the host innate immune system against invading microorganisms. Neutrophils and macrophages are readily mobilized to sites of infection and ingest microorganisms. Ingested bacteria are killed by ROS derived from superoxide produced by an activated, phagosome-bound NADPH-dependent oxidase (6, 7).

Superoxide anion is then quickly converted to hydrogen peroxide (H_2O_2) either spontaneously or via superoxide dismutase (SOD) (8). Although the production of ROS plays an important role in the killing of microorganism, excess production of these toxic metabolites damages the surrounding tissues during inflammation (9).

There is considerable evidence indicating that atherogenesis is initiated and promoted by lipid oxidation of low-density lipoprotein (LDL), ultimately leading to modification of apolipoprotein B such that the LDL particle becomes recognized by the macrophage scavenger receptor(s) and produces massive cholesterol loading and foam cell formation (10).

Glossogyne tenuifolia Cassini (Hsiang Ju grass) is a plant native to Penghu, Taiwan. It is a popular herb tea for preventing sunstroke and has a long history of being used as an antipyretic, hepatoprotective, and anti-inflammatory remedy in folk medicine among local residents. Our laboratory has previously demonstrated that the ethanol extract of *G. tenuifolia* and its active components, oleanolic acid and luteolin-7-glucoside, inhibited the LPS-induced NO, PGE2, IL-1β, IL-6, IL-12, and TNF-α synthesis in the murine macrophage cell line RAW 264.7 (11). The ethanol extract of *G. tenuifolia* also exhibited a strong inhibitory effect on the release of TNF-α, IL-6, and IFN-γ in activated human whole blood and on the secretion of hepatitis (12).

It is well-known that a variety of anti-inflammatory natural components also possess antioxidant activity (13-17). In view of these, the possibility that *G. tenuifolia* has free radical scavenging activity arose. To better understand whether dietary supplement of *G. tenuifolia* could prevent oxidative stress, the biochemical properties of the ethanol extract of *G. tenuifolia* and its major components toward free radical production, LDL oxidation, and ROS release in activated polymorphonuclear neutrophils (PMN) and peripheral blood mononuclear cells (PBMC) were investigated. Results of the present study facilitate our understanding of the antioxidant effects of *G. tenuifolia* and its bioactive constituents.

Sword brake fern (*Pteris ensiformis* Burm.) is one of the most popular components of herbal beverages in Taiwan. It has a long history of being used as a health improvement remedy; however, information regarding its biological action remains unclear. In this study, we focus on the immunomodulatory effect of the aqueous extract of sword brake fern by elucidating the detailed mechanisms in murine macrophage cell line, RAW264.7. To better understand whether SBF also possessed antioxidant activity as well, the bioactive constituents were isolated and identified using DPPH scavenging activity as an index. The scavenging activities of SBF and bioactive components against superoxide, H_2O_2 and hydroxyl radical, and the inhibitory effects on LDL oxidation were further studied *in vitro*.

Materials and Methods

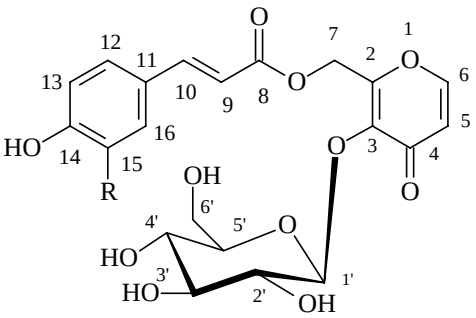
Materials. *G. tenuifolia* was obtained from the Kaohsiung DistrictAgricultural Improvement Station in Penghu, Taiwan, and the voucher specimens were deposited in the herbarium of Chia-Nan University of Pharmacy and Science.

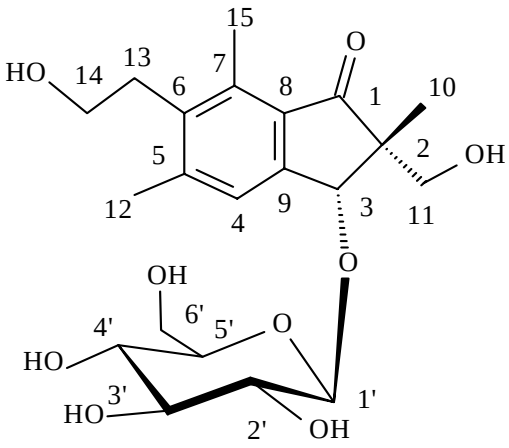
Sword brake fern (Pteris ensiformis Burm.) was obtained from the Taitung District Agricultural Research and Extension Station (Taitung, Taiwan) and the voucher specimens were deposited in the herbarium of Chia-Nan University of Pharmacy and Science.

Ethanol Extraction of G. tenuifolia. The dry above-ground part (5.46 kg) was extracted with ethanol (10 L) at room temperature four times followed by filtration. The flow-through was concentrated in vacuo to yield a dark-brown syrup (623.4 g), which was diluted to lower concentrations with ethanol.

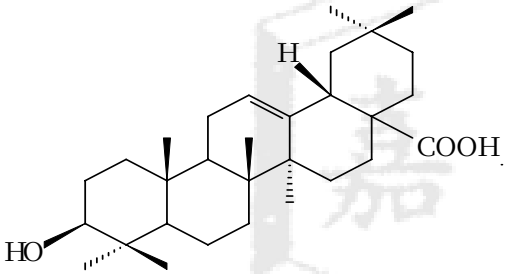
Extraction and Isolation of Active Compounds of Sword Brake Fern. The dry aboveground part (150g) was grinded in 1 L of deionized water by an engine room-driven blender for 5 min and then autoclaved for 20 min. After filtration twice, the flow through was then lyophilized to yield a dark-brown powder (5.74 g), which was redissolved in deionized water prior to use. To isolate the active compounds, dried whole plants (2.1 kg) of sword brake fern were cut into small pieces and extracted with MeOH (20 L×3) at room temperature. The combined MeOH extract was concentrated *in vacuo* to yield dark-brown syrup (208 g). The MeOH extract was then partitioned with ethyl acetate and water (1:1). The resulting aqueous fraction (98 g) was further partitioned with *n*-butanol and water, while the ethyl acetate fraction (102 g) was with methanol and hexane. The obtained *n*-butanol (50 g) and methanol fractions (46 g) showed significant scavenging activity toward 1,1-diphenyl-2-picrylhydrazyl (DPPH). The combined active fraction (96 g) was chromatographed over silica gel column (12ㄨ 110 cm, 2700 g) and eluted with $CHCl_3$-MeOH mixtures (20:1, 10:1, 8:1, 6:1, 4:1, each 1.5 L) to produce 9 fractions (P1-9). Among them, fractions P3-P5 showed significant activity in DPPH savenging. P5 (10.2 g) was further chromatographed over silica gel (1200 g) and eluted with $CHCl_3$-MeOH mixtures (10:1, 8:1, 6:1, 4:1, each 1.5 L) to produce compound **1** (ca. 0.5 g). Crystalline P3 (9.6 g) was filtered and washed with MeOH/water (1:2) to yield compound **2** (ca. 3.6 g). P-4 was further separated by preparative reverse-phase HPLC (Cosmosoil, 20×250 mm, MeOH: H2O = 1:1, flow rate 3 ml/min) to give compound 3 (ca. 10 mg).

Structures of Major Components of Sword Brake Fern and G. tenuifolia

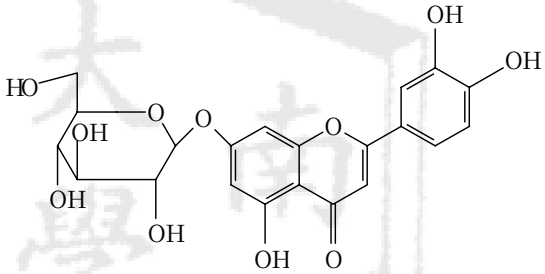




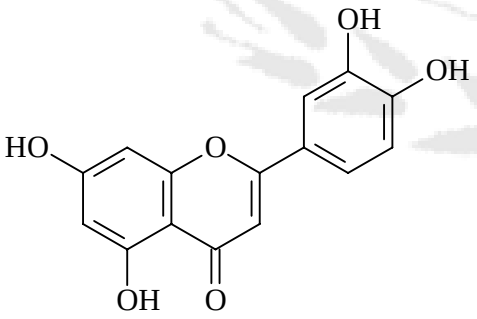
Pterisoside A (**1**) R=OH; Bockioside B (**2**) R=H Pterisoside B(**3**)



oleanolic acid



luteolin-7-glucoside



luteolin

Analysis of LDL Oxidation. Peroxidation of LDL was measured by the determination of thiobarbituric acid reactive substances (TBARS) and expressed as malondialdehyde (MDA) equivalents (20). The quantity of conjugated diene in LDL was assessed by monitoring the change at A234 (21). 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's instructions.

Anti-inflammatory Activity Analysis (2)

Free Radical Scavenging Activities Analysis (1)

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.

Results

1. Free Radical Scavenging Activities of GT and Major Consititutes (1)

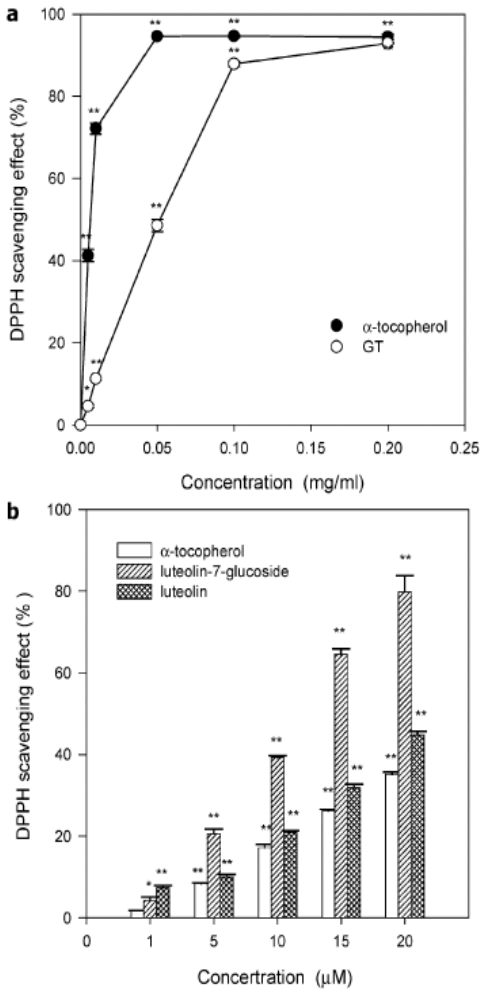


Figure 2. Ethanol extracts of *G. tenuifolia* (GT), luteolin, and luteolin-7-glucoside as stable free radical scavengers: (**a**) DPPH scavenging effects of GT and α-tocopherol; (**b**) DPPH scavenging effects of α-tocopherol, luteolin-7-glucoside, and its aglycon, luteolin. The reaction was performed in 1 mL of solution containing 0.1 mM of freshly prepared DPPH in methanol and various concentrations of GT, luteolin, or luteolin-7-glucoside. 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. Data represent the mean ± SEM ($n = 3$) of vehicle control. *, $p < 0.05$, and **, $p < 0.01$, represent significant differences compared with vehicle control.

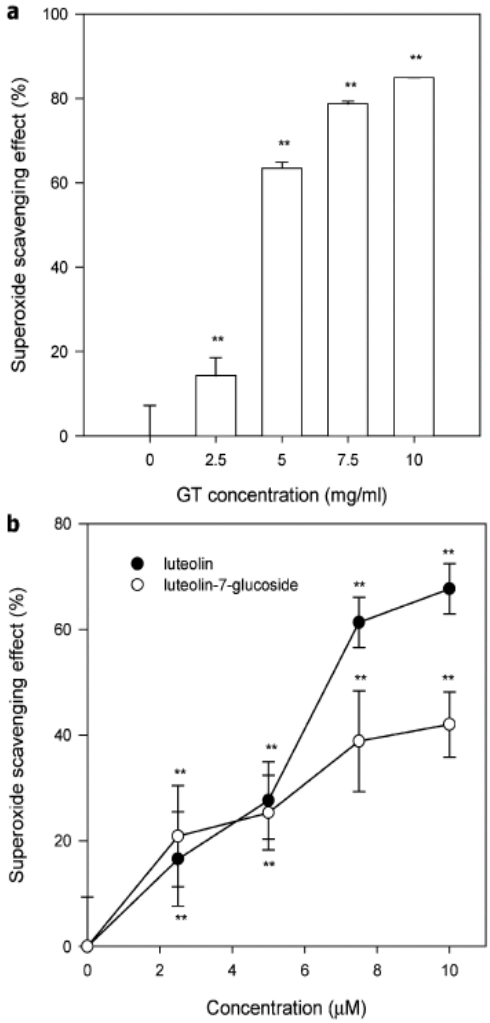


Figure 3. Ethanol extracts of *G. tenuifolia* (GT), luteolin, and luteolin-7-glucoside as superoxide scavengers: (**a**) superoxide scavenging effect of GT; (**b**) superoxide scavenging effects of luteolin and luteolin-7-glucoside. Xanthine and xanthine oxidase were used to generate superoxide radicals, and their inhibition by GT was recorded by a luminometer. The lucigenin-enhanced superoxide chemiluminescence during the first 15 s was averaged. The inhibitory efficiency in response to the CL of vehicle control was calculated. Data represent the mean ± SEM ($n = 3$) of vehicle control. *, $p < 0.05$, and **, $p < 0.01$, represent significant differences compared with vehicle control.

2. Inhibition of LDL Oxidation by GT and Major Constitutes (1)

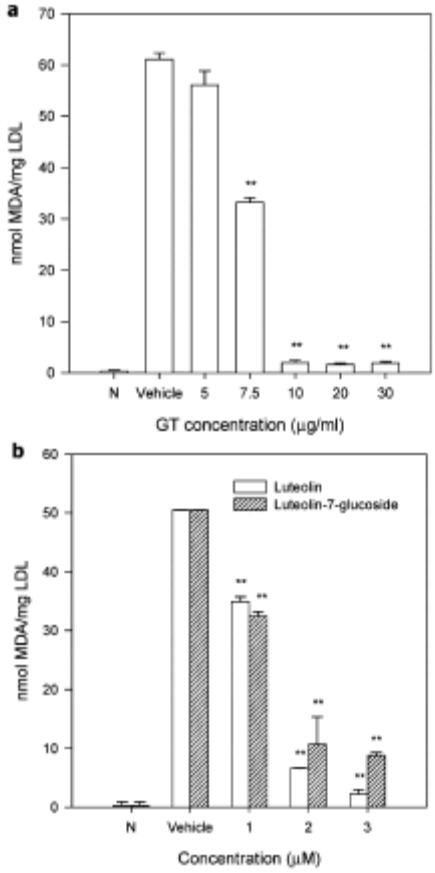


Figure 5. Ethanol extracts of *G. tenuifolia* (GT), luteolin, and luteolin-7-glucoside inhibit TBARS formation in copper-induced low-density lipoprotein (LDL): (**a**) GT inhibits TBARS formation in copper-induced LDL peroxidation; (**b**) luteolin and luteolin-7-glucoside inhibit TBARS formation in copper-induced LDL peroxidation. EDTA-free LDL (0.1 mg/mL) reacted with 10 μM Cu^{2+} in PBS in the presence of vehicle and different concentrations of GT, luteolin, or luteolin-7-glucoside at 37 °C for 3 h. Peroxidation of the LDL was expressed as nanomoles of malondialdehyde (MDA) equivalents per milligram of protein. Data represent the mean ± SEM (n = 3) of vehicle control. *, $p < 0.05$, and **, $p < 0.01$, represent significant differences compared with vehicle control.

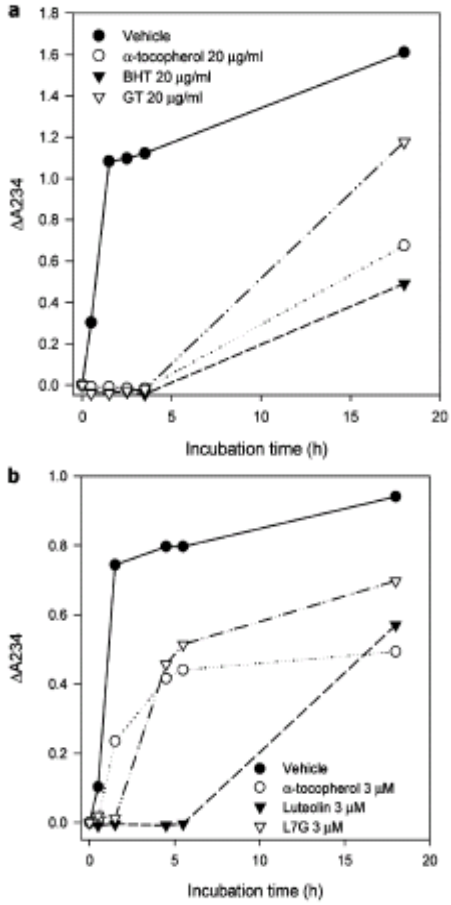


Figure 6. Ethanol extracts of *G. tenuifolia* (GT), luteolin, and luteolin-7-glucoside inhibit conjugated diene formation in copper-induced LDL: (**a**) EDTA-free LDL (0.1 mg/mL) reacted with 10 μM Cu^{2+} in PBS in the presence of vehicle, α-tocopherol, BHT, or GT (20 μg/mL) at 37 °C for the indicated period; (**b**) EDTA-free LDL (0.1 mg/mL) reacted with 10 μM Cu^{2+} in PBS in the presence of vehicle, α-tocopherol, luteolin, and luteolin-7-glucoside (L7G) (3 μM) at 37 °C for the indicated period. The formation of conjugated diene was measured by change in absorbance at 234 nm (ΔA_{234}). This experiment was repeated three times with similar results.

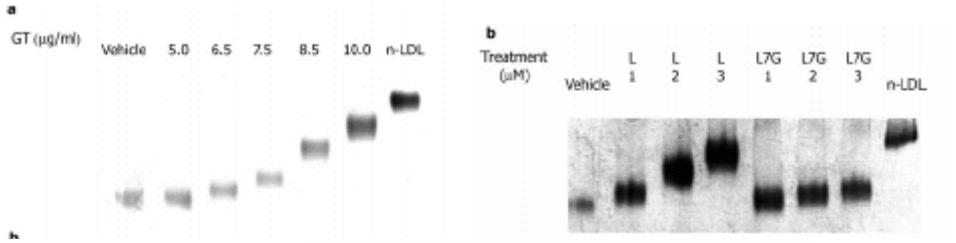


Figure 7. Ethanol extracts of *G. tenuifolia* (GT), luteolin, and luteolin-7-glucoside inhibit electrophoretic mobility in copper-induced LDL: (a) native LDL (*n*-LDL) (0.1 mg/mL) was oxidized with 10 μM Cu^{2+} in PBS in the presence of vehicle or indicated concentrations of GT for 24 h; (**b**) n-LDL (0.1 mg/mL) was oxidized with 10 μM Cu^{2+} in PBS in the presence of vehicle or indicated concentrations of luteolin (L) or luteolin-7-glucoside (L7G) for 24 h. Concentrated LDL (1–2 μL) was separated on Titan lipoprotein gel and stained with Fat Red 7B.

3. Inhibition of Oxidative Burst of PBMC and PMN by GT (1)

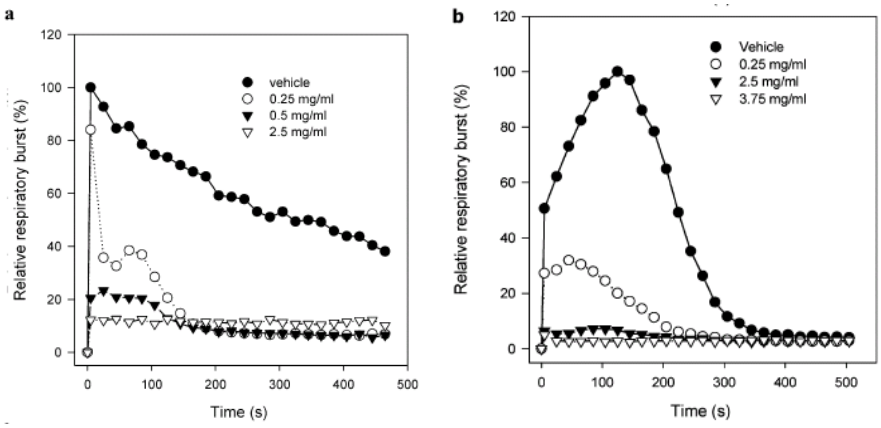


Figure 8. Ethanol extract of *G. tenuifolia* (GT) inhibits fMLP-induced ROS production in human leukocytes: (**a**) PMN; (**b**) PBMC. Each well containing 7×10^5 PMN or PBMC, 1 mM luminol, and vehicle or various concentrations of GT was incubated at room temperature for 15 min. Activator, 2 μM fMLP, was then added, and the light emission, expressed as relative light units (RLU), was monitored every 30 s for 5 s during a 10-min observation period.

4. *Effects of SBF on Proinflammatory Cytokine Synthesis in RAW264.7 Cells* (2)

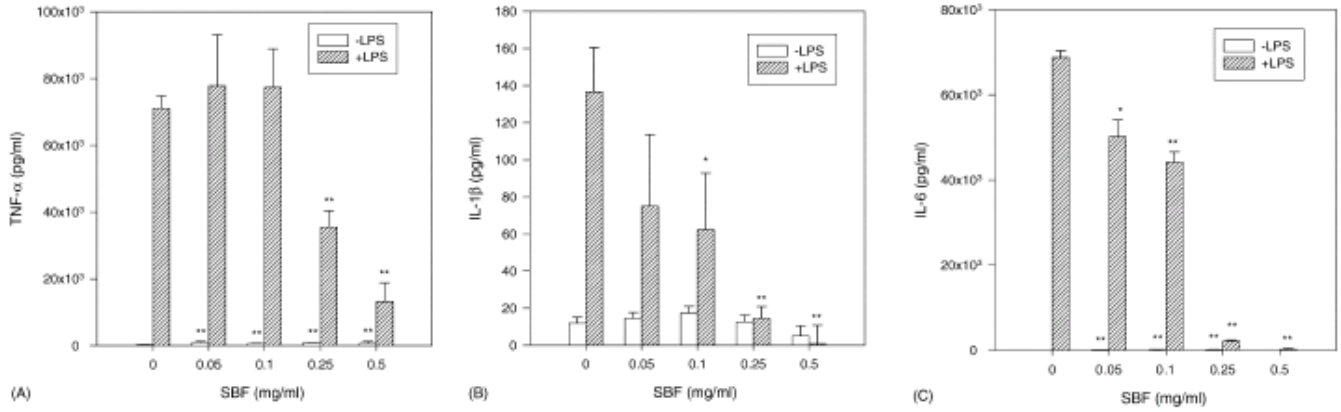


Fig. 1. Effects of the aqueous extract of sword brake fern (SBF) on the production of proinflammatory cytokines in RAW 264.7 macrophages. RAW264.7 cells were cultured with indicated concentrations of SBF alone or in combination with LPS (1 μg/ml) at 37 °C in a 96-well plate. Media were collected after 6 h of treatment for (A) TNF-α, (B) IL-1β and (C) IL-6 analysis. Data are expressed as the mean ± S.D. of three individual experiments. Statistically significant change of cytokine release ($^{*}p < 0.05$, $^{**}p < 0.01$), as compared with the group treated without SBF treatment.

5. *Effects of SBF on LPS-Induced NF-κB Activation in RAW264.7 Cells* (2)

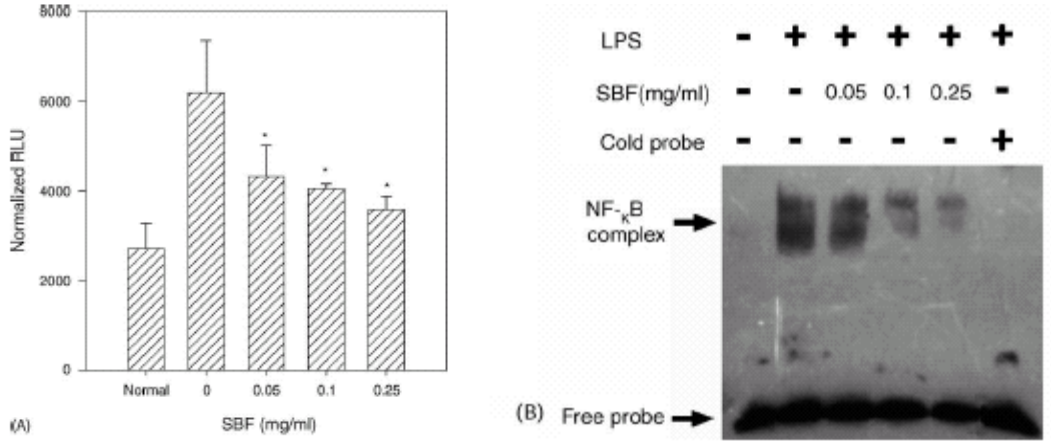


Fig. 5. Effect of the aqueous extract of sword brake fern (SBF) on LPS-induced NF-κB activation. (A) A reporter plasmid containing repetitive NF-κB sites in its enhancer element, pNF-κB-Luc, was transiently co-transfected into RAW264.7 cells with a pSV-β-galactosidase control plasmid for 15 h. Cells were treated with LPS (1 μg/ml) alone or in combination with indicated concentrations of SBF. Cells were then harvested after 4 h of incubation. The intensity of the luciferase activity measured in the lysates of the transient transfectants was normalized to their β-galactosidase activity, which was used as an internal control. Statistically significant inhibition of NF-κB activation ($^{*}p < 0.05$), as compared with group treated with LPS only. (B) RAW264.7 cells were treated with LPS (1 μg/ml) alone or in combination with indicated concentrations of SBF for 30 min. Nuclear extracts were prepared and analyzed for κB DNA binding using the electrophoretic mobility shift assay (EMSA). Competitive EMSA using an unlabeled NF-κB consensus sequence at 100-fold excess confirmed the specificity of NF-κB binding. Assays were repeated three times with a representative result shown.

6. *Free Radical Scavenging Activities of SBF* and Active Compounds(4)

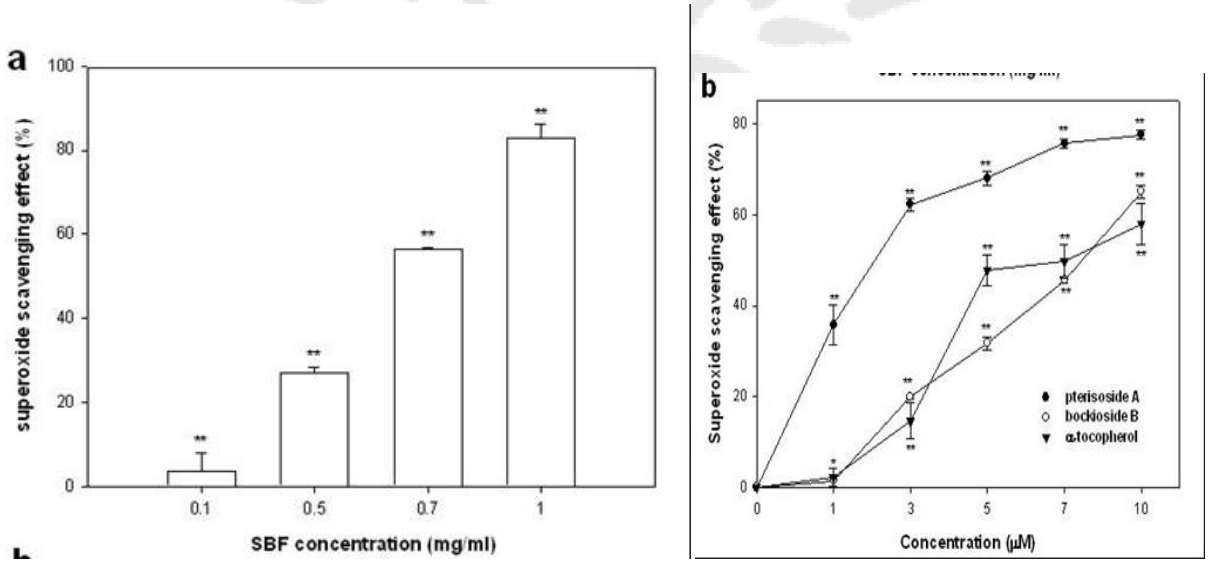


Fig. SBF, pterisoside A, and bockioside B superoxide scavengers: (a) superoxide scavenging effect of SBF; (b) superoxide scavenging effects of α-tocophenol, pterisoside A, and bockioside. Xanthine and xanthine oxidase were used to generate

superoxide radicals, and their inhibition by PE was recorded by a luminometer. The lucigenin-enhanced superoxide chemiluminescence during the first 15 s was averaged. The inhibitory efficiency in response to the CL of vehicle control was calculated. Data represent the mean ±SEM (n=3) of vehicle control. *, $p < 0.05$, and **, $p < 0.01$, represent significant differences compared with vehicle control.

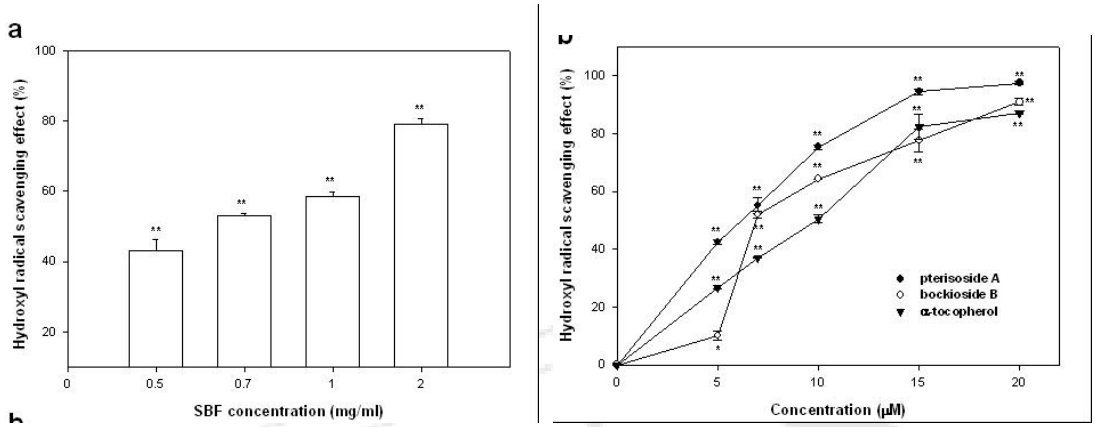


Fig. SBF, pterisoside A, and bockioside B as hydroxyl radical scavengers: (a) hydroxyl radical scavenging effect of SBF; (b) hydroxyl radical scavenging effects of -tocophenol, pterisoside A, and bockioside. Reaction by H2O2 and FeCl2 was used to generate hydroxyl radicals, and their inhibition by perisoside or bockioside was recorded by a luminometer. The hydroxyl-induced luminol chemiluminescence (CL) was monitored every 5 s during the first 1-min observation period. The inhibitory efficiency in response to the CL of vehicle control was calculated. Data represent the mean ± SEM (n=3) of vehicle control. *, p< 0.05, and **, p < 0.01, represent significant differences compared with vehicle control.

7. Inhibition of LDL Oxidation by SBF and Active Compounds (3)

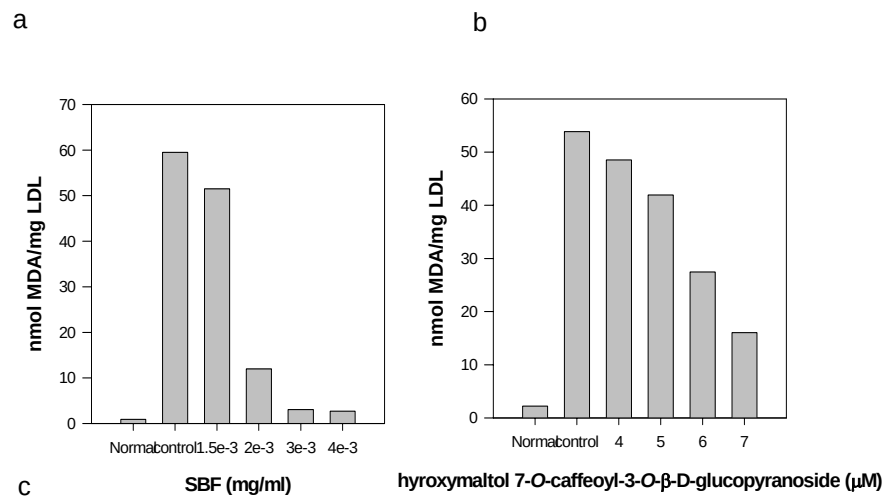


Fig. SBF and pterisoside inhibit TBARS formation in copper-induced low-density lipoprotein (LDL)

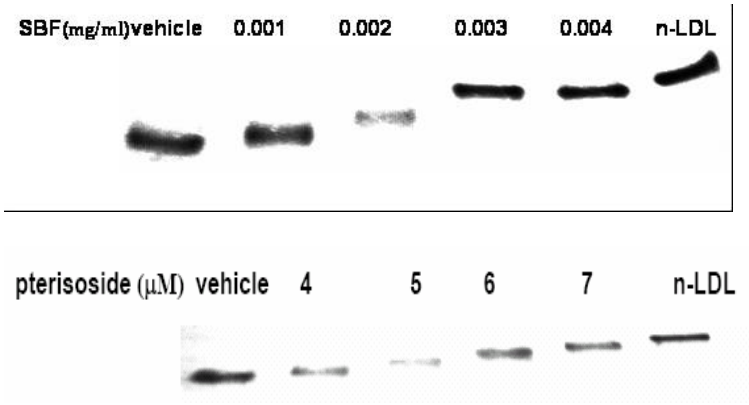


Fig. SBF and pterisoside inhibit electrophoretic mobility in copper-induced LDL

References

(1) Wu, M. J.;Huang, C.J.; Lian, T.; Kou, M. C.; Wang L. Antioxidant Activity of *Glossogyne tenuifolia*. *J. Agric. Food Chem.* **2005**, 53,6305-6312

(2) Wu, M. J.; Weng, C.Y.; Wang, L.; Lian, T. Immunomodulatory Mechanism of the Aqueous Extract of Sword Brake Fern (*Pteris ensiformis* Burm.). *J. Ethnopharmaco.* **2005**, 98, 73-81

(3)Wei, S.A.; Lian, T.W., Tu, Y.T., Wu, M. J. The antioxidant mechanisms against LDL oxidation by sword brake fern (Pteris ensifotmis Burm.) and its major components. 94 年度中國藥學年會, **2005**.

(4) Chen, Y.S.; Wu, M.J. Chang, F. R.; Wang, L.; Chen, J. L.; Wu, Y. C. Antioxidant Principles of Sword Brake Fern (Pteris ensiformis Burm.). Submitted to Phytochem., **2006**.

(5) Gutteridge, J. M. Biological origin of free radicals, and mechanisms of antioxidant protection. *Chem. Biol. Interact.* **1994**, 91, 133-140.

(6) Hurst, N. P. Molecular basis of activation and regulation of the phagocyte respiratory burst. *Ann. Rheum. Dis.* **1987**, 46, 265-72

(7) Rosen, H.; Klebanoff, S. Bactericidal activity of a superoxide anion-generating system. *J. Exp. Med.* **1979**, 149, 27-39

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

(9) Casimir, C. M.; Teahan, C. G. The respiratory burst of neutrtophils and its defciency. In: *Immunopharmacology of Neutrophils*; Hellewell, P.G.; Williams, T., Eds.; Academic Press: San Diego, CA., 1994, pp27-54.

(10) Meydani, M. Vitamin E and Atherosclerosis: Beyond Prevention of LDL Oxidation. *J. Nutr*. **2001**,131, 366S-368S.

(11) Wu, M. J.; Wang, L.; Ding, H. Y.; Weng, C. Y.; Yen, J. H. Glossogyne tenuifolia

Acts to Inhibit Inflammatory Mediator Production in a Macrophage Cell Line by Down-Regulating LPS-Induced NF-κB. *J. Biomed. Sci.*, **2004**, 11, 186-199.

(12) Wu, M. J.; Weng, C. Y.; Ding, H. Y.; Wu, P. J. Anti-Inflammatory and Antiviral Effects of Glossogyne tenuifolia. *Life Sci.*, **2005**, 76, 1135-1146

(13) Sandoval, M.,; Okuhama, N. N.; Zhang, X. J.; Condezo, L. A.; Lao, J.; Angeles', F. M.; Musah, R. A.; Bobrowski, P.; Miller, M. J. Anti-inflammatory and antioxidant activities of cat's claw (Uncaria tomentosa and Uncaria guianensis) are independent of their alkaloid content. *Phytomedicine.* **2002**, 9, 325-337

(14) Chamundeeswari, D.; Vasantha, J.; Gopalakrishnan, S.; Sukumar, E. Free radical scavenging activity of the alcoholic extract of Trewia polycarpa roots in arthritic rats. *J. Ethnopharmacol.* **2003**, 88, 51-6, 2003

(15) Lo, A. H.; Liang, Y. C.; Lin-Shiau, S. Y.; Ho, C. T.; Lin, J. K. Carnosol, an antioxidant in rosemary, suppresses inducible nitric oxide synthase through down-regulating nuclear factor-kappaB in mouse macrophages. *Carcinogenesis.* **2002**, 23, 983-991.

(16) Dharmasiri, M. G.; Jayakody, J. R.; Galhena, G.; Liyanage, S. S.; Ratnasooriya, W. D. Anti-inflammatory and analgesic activities of mature fresh leaves of Vitex negundo. *J. Ethnopharmacol.* **2003**, 87, 199-206

(17) Wu, M. J.; Yen, J. H.; Wang, L.; Weng, C. Y. Antioxidant Activity of Porcelainberry (Ampelopsis brevipedunculata (Maxim.) Trautv.). *Am. J. Chin. Med.* **2004**, 32, 681-693

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Antioxidant Activity of *Glossogyne tenuifolia*

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Glossogyne tenuifolia is a native traditional anti-inflammatory herb in Taiwan. It has previously been shown that the ethanol extract of *G. tenuifolia* (GT) inhibited the LPS-induced inflammatory mediator release from murine macrophage cell line and human whole blood. In the present work, the ethanol extracts of *G. tenuifolia* and its major constituent, luteolin-7-glucoside, were shown to be scavengers of 1,1-diphenyl-2-picrylhydrazyl, superoxide, and hydroxyl radicals. Moreover, copper-induced low-density lipoprotein oxidation was suppressed by GT and luteolin-7-glucoside as measured by decreased formation of malondialdehyde and conjugated diene as well as reduced electrophoretic mobility. GT and luteolin-7-glucoside were also against *N*-formyl-methionyl-leucyl-phenylalanine-induced reactive oxygen species (ROS) production in human polymorphonuclear neutrophils and peripheral blood mononuclear cells. In summary, these data indicated that GT is a potential ROS scavenger and may prevent atherosclerosis via inhibiting LDL oxidation or ROS production in human leukocytes. Moreover, luteolin-7-glucoside may serve as the active principal of GT.

KEYWORDS: ***Glossogyne tenuifolia*; reactive oxygen species; LDL; oxidation; luteolin; antioxidant**

INTRODUCTION

It has been well recognized that free radicals are formed in living organisms as a function of endogenous biochemical processes. Of particular relevance in aerobic systems are those reactive species derived from the metabolism of oxygen, which include hydrogen peroxide, singlet oxygen, superoxide radicals, and hydroxyl radicals, collectively known as the reactive oxygen species (ROS) (*1*). ROS are generated by several mechanisms, such as interaction of ionizing radiation with biological molecules, as well as produced by cellular respiration and phagocytic cells, namely, neutrophils and macrophages, which are crucial in the host innate immune system against invading microorganisms. Neutrophils and macrophages are readily mobilized to sites of infection and ingest microorganisms. Ingested bacteria are killed by ROS derived from superoxide produced by an activated, phagosome-bound NADPH-dependent oxidase (*2*, *3*). Superoxide anion is then quickly converted to hydrogen peroxide (H_2O_2) either spontaneously or via superoxide dismutase (SOD) (*4*). Although the production of ROS plays an important role in the killing of microorganism, excess production of these toxic metabolites damages the surrounding tissues during inflammation (*5*).

ROS have a huge potential for causing cellular components, such as DNA, protein, and lipid damages. It is widely acknowledged that the accumulation of oxidative damages in intracellular macromolecules is an essential element in the aging process and in certain degenerative diseases such as cancer, immune system decline, brain dysfunction, cataracts, and coronary heart disease (*6*). There is considerable evidence indicating that atherogenesis is initiated and promoted by lipid oxidation of low-density lipoprotein (LDL), ultimately leading to modification of apolipoprotein B such that the LDL particle becomes recognized by the macrophage scavenger receptor(s) and produces massive cholesterol loading and foam cell formation (*7*).

Lots of experimental and epidemiologic data have indicated that dietary antioxidants might reduce the risk of degenerative diseases by reduction of oxidative stress and inhibition of macromolecular oxidation. The antioxidants are not limited to well-known antioxidants such as ascorbic acid or α-tocopherol. Many natural components such as phenolic compounds are effective antioxidants (*8*).

Glossogyne tenuifolia Cassini (Hsiang Ju grass) is a plant native to Penghu, Taiwan. It is a popular herb tea for preventing sunstroke and has a long history of being used as an antipyretic, hepatoprotective, and anti-inflammatory remedy in folk medicine among local residents. Our laboratory has previously demonstrated that the ethanol extract of *G. tenuifolia* and its active components, oleanolic acid and luteolin-7-glucoside, inhibited the LPS-induced NO, PGE_2, IL-1β, IL-6, IL-12, and TNF-α synthesis in the murine macrophage cell line RAW 264.7 (*9*). The ethanol extract of *G. tenuifolia* also exhibited a strong inhibitory effect on the release of TNF-α, IL-6, and IFN-γ in activated human whole blood and on the secretion of hepatitis

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B virus surface antigen (HBsAg) in the hepatocellular carcinoma cell line PLC/PRF/5 (*10*).

It is well-known that a variety of anti-inflammatory natural components also possess antioxidant activity (*11*−*15*). In view of these, the possibility that *G. tenuifolia* has free radical scavenging activity arose. To better understand whether dietary supplement of *G. tenuifolia* could prevent oxidative stress, the biochemical properties of the ethanol extract of *G. tenuifolia* and its major components toward free radical production, LDL oxidation, and ROS release in activated polymorphonuclear neutrophils (PMN) and peripheral blood mononuclear cells (PBMC) were investigated. Results of the present study facilitate our understanding of the antioxidant effects of *G. tenuifolia* and its bioactive constituents.

MATERIALS AND METHODS

Materials. *G. tenuifolia* was obtained from the Kaohsiung District Agricultural Improvement Station in Penghu, Taiwan, and the voucher specimens were deposited in the herbarium of Chia-Nan University of Pharmacy and Science.

Ethanol Extraction and Fractionation of *G. tenuifolia*. The dry above-ground part (5.46 kg) was extracted with ethanol (10 L) at room temperature four times followed by filtration. The flow-through was concentrated in vacuo to yield a dark-brown syrup (623.4 g), which was diluted to lower concentrations with ethanol.

The crude ethanol extract of *G. tenuifolia* (GT) (623.4 g) was partitioned with hexane and 95% methanol. The 95% methanol layer was concentrated and then partitioned with ethyl acetate and water. The resulting aqueous solution was again partitioned with *n*-butanol and water. Oleanolic acid (17.1 g) was obtained by recrystallization of the insoluble particles existing in the hexane layer with acetone and methanol. Luteolin-7-glucoside (total, 4.92 g) existed widely in several partitions. It could be isolated from the ethyl acetate layer by passing through silica gel chromatography (70−230 mesh, Merck) followed by eluting with hexane/CH_2Cl_2 (1:1). It could also be obtained by recrystallization of the insoluble particles existing in ethyl acetate and water layers with acetone and methanol. These isolated compounds were identified by direct comparison (^{1}H, ^{13}C NMR[14]) with authentic samples (*9*). **Figure 1** shows the structures of oleanolic acid, luteolin-7-glucoside, and its aglycon, luteolin.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Scavenging Capacities of GT and Its Major Constituents. The ethanol extract of *G. tenuifolia* and its major constituents, namely, oleanolic acid and luteolin-7-glucoside, as well as its aglycon, luteolin, were evaluated for activities to scavenge the stable DPPH radical (0.1 mM) according to the method of Dinis et al. (*16*). The affinity of the test material to quench the DPPH free radical was evaluated according to the equation

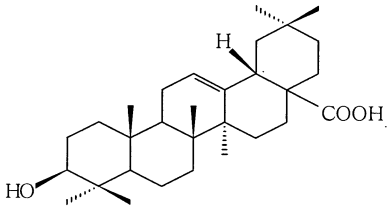
$$\text{scavenging \%} = \frac{A_c - A_s}{A_c} \times 100$$

where A_s and A_c are absorbance at 517 nm of the reaction mixture with samples and with vehicle control, respectively.

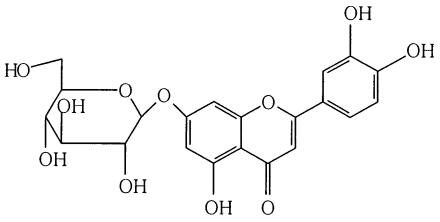
Superoxide Radical Scavenging Capacities of GT and Its Major Constituents. Superoxide scavenging effects of the ethanol extract of *G. tenuifolia* and its major constituents were measured using the modified lucigenin-enhanced chemiluminescence (CL) method (*17*). Briefly, the reaction was carried out in a mixture containing 80 μL of 1 mM lucigenin (in PBS) and 10 μL of vehicle, GT or its major constituents. 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). The superoxide-induced lucigenin CL during the first 15 s was averaged. The inhibitory efficiency in response to the CL of vehicle control was calculated.

Hydroxyl Radical Scavenging Capacities of GT and Its Major Constituents. The hydroxyl radical scavenging effects of the ethanol extract of *G. tenuifolia* and its major constituents were measured using a modified luminol-enhanced CL method (*17*). Briefly, the reaction was carried out in a 100 μL mixture containing 5 mM luminol (in PBS), ferrous chloride (40 μM), 1% H_2O_2, and vehicle, various concentrations of GT or its major constituents. The hydroxyl-induced luminol CL during the first minute was averaged. The inhibitory efficiency in response to the CL of vehicle control was calculated.

Oleanolic acid



luteolin-7-glucoside



luteolin

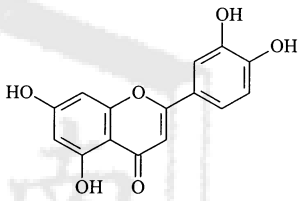


Figure 1. Structures of oleanolic acid, luteolin-7-glucoside, and luteolin.

Preparation and Oxidation of LDL. LDL (d = 1.019−1.063) was prepared from the plasma of healthy donors by sequential ultracentrifugation (*18*). Lipoprotein was desalted and concentrated by filtration (Centricon 4, Amicon, Beverly, MA) against PBS at 450g and 4 °C for 120 min. The protein concentration was measured according to the method of Bradford (*19*), using bovine serum albumin as a standard.

Oxidation of LDL was carried out by incubating EDTA-free LDL (0.1 mg/mL) with 10 μM Cu^{2+} in PBS in the presence of vehicle, different concentrations of the ethanol extract of *G. tenuifolia* or its major constituents, at 37 °C.

Analysis of LDL Oxidation. Peroxidation of LDL was measured by the determination of thiobarbituric acid reactive substances (TBARS) and expressed as malondialdehyde (MDA) equivalents (*20*). The quantity of conjugated diene in LDL was assessed by monitoring the change at A_{234} (*21*). To measure the electrophoretic mobility, the ox-LDL was concentrated by filtration (Centricon 4) at 450g and 4 °C for 120 min. About 1∼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's instructions.

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

Measurement of *N*-Formyl-methionyl-leucyl-phenylalanine (fMLP)-Induced ROS Production in Human PMN and PBMC. ROS production was measured using a modified luminol-enhanced CL method (*22*). The CL response of PMN and PBMC was measured using a microtiter plate luminometer within 5 h after blood collection. Each well, which contained 7 × 10^5 PMN or PBMC, 1 mM luminol, and vehicle, various concentrations of the ethanol extract of *G. tenufolia* or its major constituents, was incubated at room temperature for 15

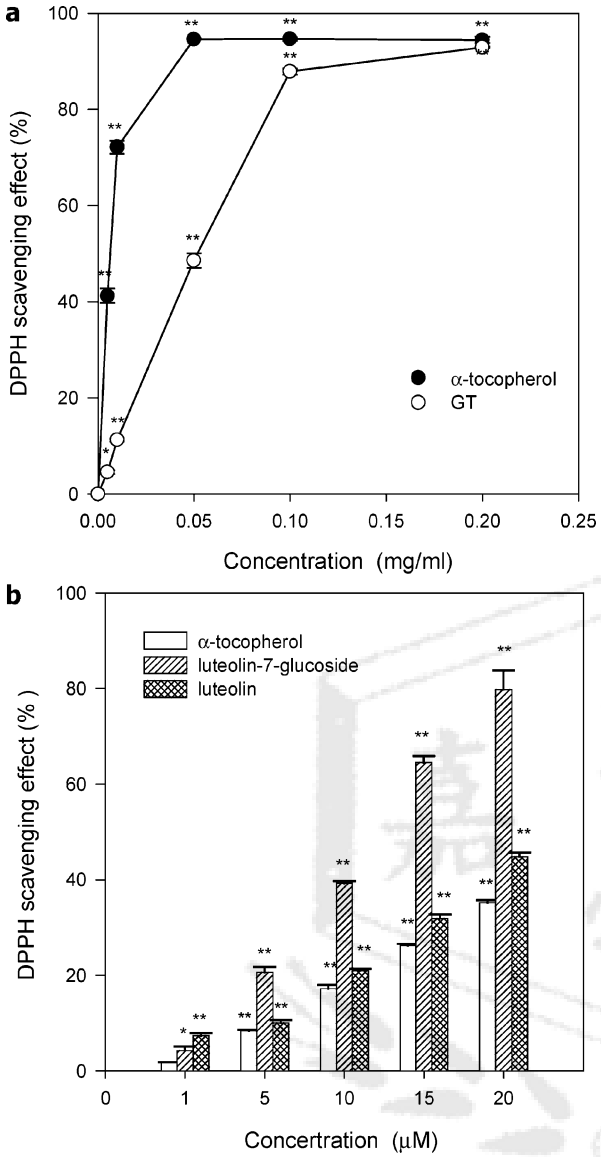


Figure 2. Ethanol extracts of *G. tenuifolia* (GT), luteolin, and luteolin-7-glucoside as stable free radical scavengers: (**a**) DPPH scavenging effects of GT and α-tocopherol; (**b**) DPPH scavenging effects of α-tocopherol, luteolin-7-glucoside, and its aglycon, luteolin. The reaction was performed in 1 mL of solution containing 0.1 mM of freshly prepared DPPH in methanol and various concentrations of GT, luteolin, or luteolin-7-glucoside. 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. Data represent the mean ± SEM ($n = 3$) of vehicle control. *, $p < 0.05$, and **, $p < 0.01$, represent significant differences compared with vehicle control.

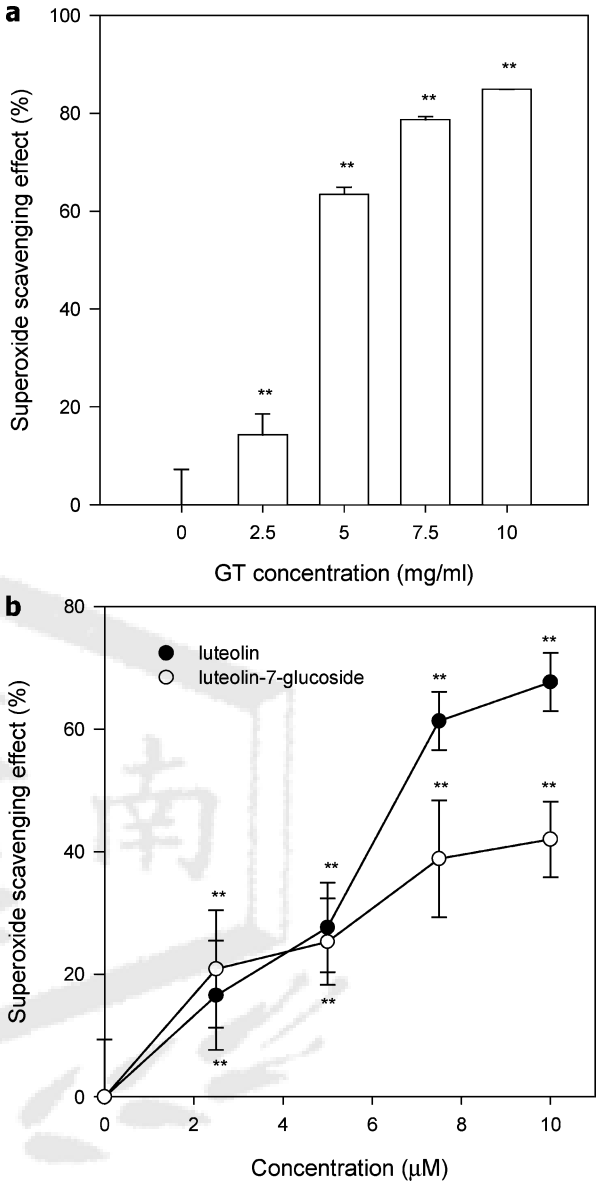


Figure 3. Ethanol extracts of *G. tenuifolia* (GT), luteolin, and luteolin-7-glucoside as superoxide scavengers: (**a**) superoxide scavenging effect of GT; (**b**) superoxide scavenging effects of luteolin and luteolin-7-glucoside. Xanthine and xanthine oxidase were used to generate superoxide radicals, and their inhibition by GT was recorded by a luminometer. The lucigenin-enhanced superoxide chemiluminescence during the first 15 s was averaged. The inhibitory efficiency in response to the CL of vehicle control was calculated. Data represent the mean ± SEM ($n = 3$) of vehicle control. *, $p < 0.05$, and **, $p < 0.01$, represent significant differences compared with vehicle control.

min. Activator, 2 μM fMLP, was then added, and the light emission, expressed as relative light units (RLU), was monitored every 30 s for 5 s during a 10-min observation period, and the kinetic curve was obtained.

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.

RESULTS

Free Radical Scavenging Activities of GT. To evaluate the possible antioxidant activity of GT, we started by investigating stable free radical (DPPH), superoxide ($O_2^{\bullet -}$), and hydroxyl radical ($^{\bullet}OH$) scavenging actions. **Figure 2a** demonstrates that the DPPH scavenging effect of GT was in a dose-dependent manner. The potency of GT was less than the positive control, α-tocopherol, at the same concentration. The estimated IC_{50} values for GT and α-tocopherol are approximately 55.2 and 6.7 μg/mL, respectively.

To further investigate the antioxidant activity of GT, lucigenin- and luminol-enhanced CL were employed to evaluate the superoxide and hydroxyl radical scavenging activities, respectively. Superoxide produced by xanthine/xanthine oxidase caused an increase in lucigenin-enhanced CL from a basal level of 100 to 3.61×10^5 RLU. The addition of GT (2.5−10 mg/mL) markedly inhibited superoxide radical induced lucigenin-enhanced CL in a dose-dependent manner with an IC_{50} of ∼5.18 mg/mL (**Figure 3a**).

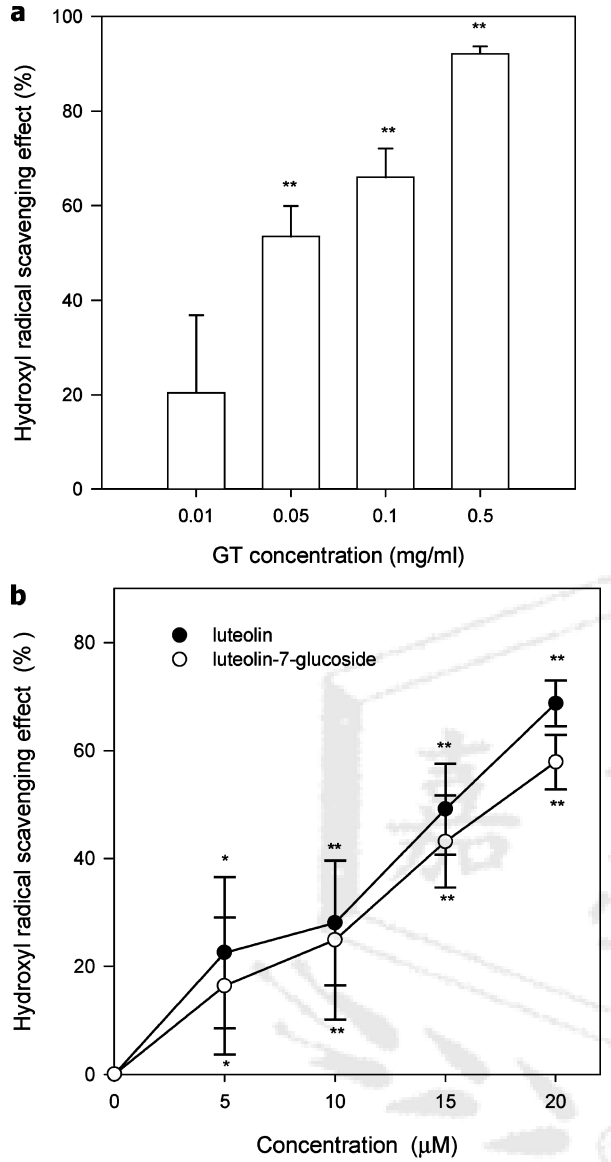


Figure 4. Ethanol extracts of *G. tenuifolia* (GT), luteolin, and luteolin-7-glucoside as hydroxyl radical scavengers: (**a**) hydroxyl radical scavenging effect of GT; (**b**) hydroxyl radical scavenging effects of luteolin and luteolin-7-glucoside. Reaction by H_2O_2 and $FeCl_2$ was used to generate hydroxyl radicals, and their inhibition by luteolin or luteolin-7-glucoside was recorded by a luminometer. The hydroxyl-induced luminol chemiluminescence (CL) was monitored every 5 s during the first 1-min observation period. The inhibitory efficiency in response to the CL of vehicle control was calculated. Data represent the mean ± SEM ($n = 12$) of vehicle control. *, $p < 0.05$, and **, $p < 0.01$, represent significant differences compared with vehicle control.

Hydroxyl radical (•OH), which is formed nonenzymatically from hydrogen peroxide (H_2O_2) in a metal-dependent reaction, is the most reactive and toxic oxygen radical known to date (*23*). CL, a highly sensitive method, has been widely used for monitoring •OH production (*24*). In our experiment, hydroxyl radicals generated by H_2O_2- and $FeCl_2$-mediated Fenton reaction caused an increase of luminol CL from 87 to 6.1×10^5 RLU. **Figure 4a** shows that the addition of GT (0.01−0.5 mg/mL) exerted a dose-dependent scavenging activity on Fenton-mediated hydroxyl radicals with an IC_{50} of ∼60 μg/mL.

Free Radical Scavenging Activities of the Major Constituents of GT. We have previously identified oleanolic acid and luteolin-7-glucoside as the major constituents of GT (*9*). To test whether they are the active antioxidant principals, we performed the aforementioned tests. **Figure 2b** demonstrates that luteolin-7-glucoside possessed higher DPPH scavenging potency than its aglycon, luteolin, and a positive control, α-tocopherol. The estimated IC_{50} values for luteolin-7-glucoside and luteolin are 12.2 and 22.8 μM, respectively.

Lucigenin-enhanced CL showed that xanthine/xanthine oxidase-generated superoxide could be inhibited by luteolin and luteolin-7-glucoside (up to 10 μM) dose-dependently. Luteolin (>8 μM) showed a higher inhibitory potency than luteolin-7-glucoside did (**Figure 3b**). The estimated IC_{50} values are 11.0 and 7.1 μM for luteolin-7-glucoside and luteolin, respectively.

Figure 4b shows that luteolin inhibited Fenton-mediated hydroxyl radical production slightly but insignificantly stronger than luteolin-7-glucoside did. The estimated IC_{50} values are 17.6 and 15.0 μM for luteolin-7-glucoside and luteolin, respectively. On the other hand, oleanolic acid did not have any detectable DPPH, superoxide, or hydroxyl radical scavenging action (data not shown). This result indicated that the flavonoid instead of triterpenoid is the component responsible for the ROS scavenging activity of GT.

Inhibition of LDL Oxidation by GT. It is recognized that oxidatively modified low-density lipoproteins (ox-LDL) play an important role in the generation and progression of the atherosclerotic plaque (*25*). In vitro inhibition of the copper-catalyzed oxidation of LDL is a widely used strategy for the evaluation of antioxidant activity of natural components (*26*). To study the effect of GT as an antioxidant in preventing copper-induced oxidation of LDL, three different approaches were employed to measure changes in several parameters known to be associated with LDL oxidation: formation of TBARS and conjugated dienes during lipid peroxidation and increase in the electrophoretic mobility of LDL due to apolipoprotein B100 modification (*27*). As shown in **Figure 5a**, incubation of LDL (0.1 mg/mL) with Cu^{2+} (10 μM) at 37 °C for 3 h was responsible for the MDA formation increase from 0.35 ± 0.17 to 61.06 ± 1.28 nmol/mg of LDL. GT treatment produced dose-dependent reduction in MDA formation. GT (10 μg/mL) inhibited the MDA formation by ∼97.3% as compared with those of the vehicle group with an estimated IC_{50} of 7.5 μg/mL.

Oxidation of LDL is accompanied by an increase in absorbance at 234 nm, due to the formation of conjugated dienes in constituent polyenoic fatty acids (*28*). As shown in **Figure 6a**, LDL treated with GT, BHT, and α-tocopherol (20 μg/mL) showed a longer resistance to copper-initiated LDL oxidation, and this was indicated by a prolonged lag phase of lipid oxidation.

The ability of GT to inhibit the alteration in the surface charge of the apolipoprotein B100 when LDL was incubated with copper ions was monitored by observing the effects on electrophoretic mobility by agaorse gel electrophoresis. **Figure 7a** shows that treatment of native LDL (0.1 mg/mL) with copper (10 μM) at 37 °C overnight increased the electrophoretic mobility. LDL treated with GT (6.5−10 μg/mL) significantly reduced copper-induced LDL oxidation dose-dependently as indicated by decreased mobility shift. GT (10 μg/mL) reduced the electrophoretic mobility by ∼70%, as compared with the vehicle group.

Inhibition of LDL Oxidation by Major Constituents in GT. To study the possible phytochemicals present in GT responsible for inhibiting LDL oxidation, we analyzed copper-induced (10 μM) LDL oxidation in the presence of luteolin-7-

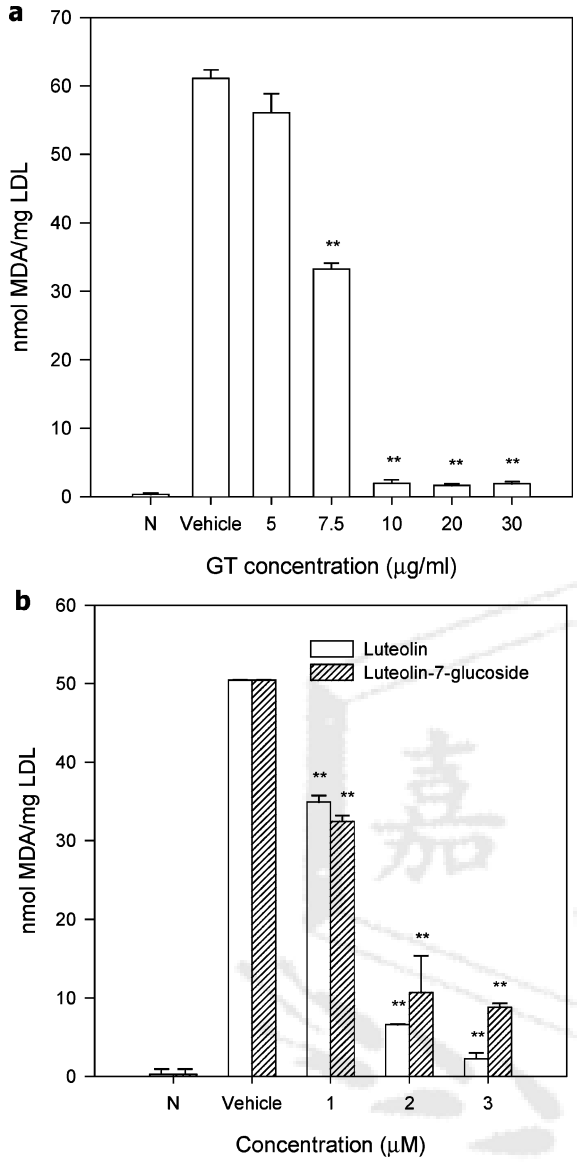


Figure 5. Ethanol extracts of *G. tenuifolia* (GT), luteolin, and luteolin-7-glucoside inhibit TBARS formation in copper-induced low-density lipoprotein (LDL): (**a**) GT inhibits TBARS formation in copper-induced LDL peroxidation; (**b**) luteolin and luteolin-7-glucoside inhibit TBARS formation in copper-induced LDL peroxidation. EDTA-free LDL (0.1 mg/mL) reacted with 10 μM Cu^{2+} in PBS in the presence of vehicle and different concentrations of GT, luteolin, or luteolin-7-glucoside at 37 °C for 3 h. Peroxidation of the LDL was expressed as nanomoles of malondialdehyde (MDA) equivalents per milligram of protein. Data represent the mean ± SEM ($n = 3$) of vehicle control. *, $p < 0.05$, and **, $p < 0.01$, represent significant differences compared with vehicle control.

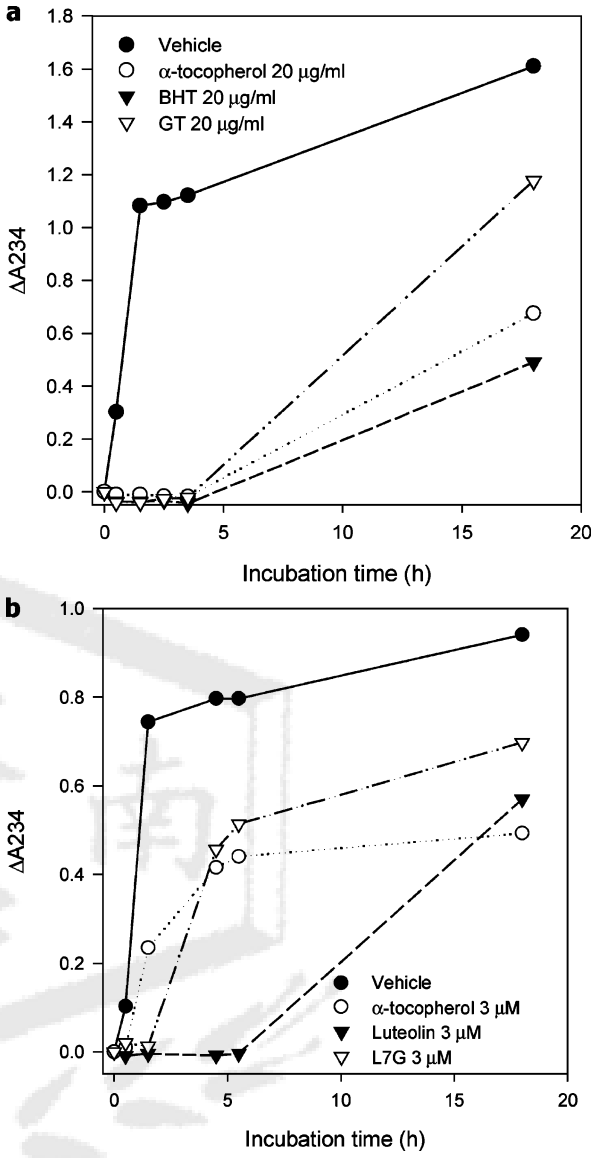


Figure 6. Ethanol extracts of *G. tenuifolia* (GT), luteolin, and luteolin-7-glucoside inhibit conjugated diene formation in copper-induced LDL: (**a**) EDTA-free LDL (0.1 mg/mL) reacted with 10 μM Cu^{2+} in PBS in the presence of vehicle, α-tocopherol, BHT, or GT (20 μg/mL) at 37 °C for the indicated period; (**b**) EDTA-free LDL (0.1 mg/mL) reacted with 10 μM Cu^{2+} in PBS in the presence of vehicle, α-tocopherol, luteolin, and luteolin-7-glucoside (L7G) (3 μM) at 37 °C for the indicated period. The formation of conjugated diene was measured by change in absorbance at 234 nm (ΔA_{234}). This experiment was repeated three times with similar results.

glucoside, luteolin, and oleanolic acid. As shown in **Figure 5b**, the MDA formation is inhibited by luteolin-7-glucoside and luteolin with estimated IC_{50} values of 1.52 and 1.34 μM, respectively. **Figure 6b** also demonstrates that the lag stage of diene formation were prolonged when luteolin-7-glucoside, luteolin, and α-tocopherol (3 μM) was added during LDL oxidation. The inhibitory effect was in the order of luteolin being greater than luteolin-7-glucoside followed by α-tocopherol. **Figure 7b** shows that luteolin-7-glucoside and luteolin reduced copper-induced LDL net charge change as compared with the vehicle group. Luteolin-7-glucoside and luteolin (3 μM) significantly reduced the electrophoretic mobility by about 41 and 63%, respectively, as compared with the vehicle group. The pattern of activities for luteolin and luteolin-7-glucoside was generally consistent with those seen for the lag-phase measurements. However, no detectable protection of LDL from copper-mediated oxidation could be found for the other major constituent, oleanolic acid (data not shown).

Inhibition of Oxidative Burst in PMN or PBMC by GT. 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 whether GT could prevent ROS production by activated human leukocytes, we set up an in vitro method with luminol-enhanced CL to measure the ROS production induced by fMLP in PMN or PBMC. The chemoattractive peptide fMLP activates an oxidative burst by its binding to a membrane receptor and activating a signal transduction pathway that leads to an

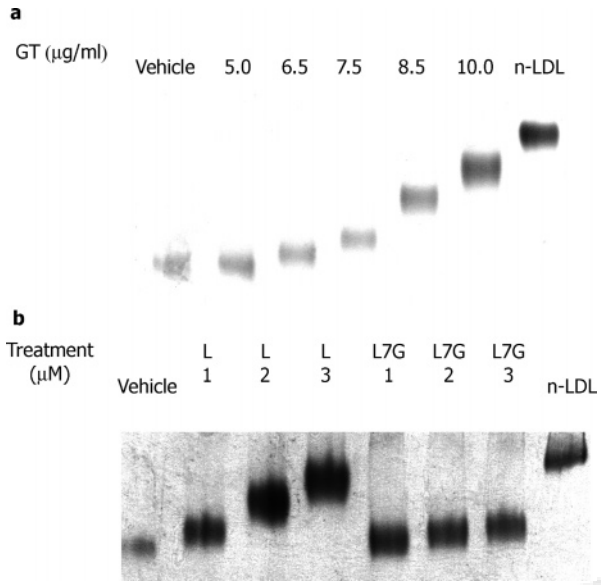


Figure 7. Ethanol extracts of *G. tenuifolia* (GT), luteolin, and luteolin-7-glucoside inhibit electrophoretic mobility in copper-induced LDL: (**a**) native LDL (n-LDL) (0.1 mg/mL) was oxidized with 10 μM Cu^{2+} in PBS in the presence of vehicle or indicated concentrations of GT for 24 h; (**b**) n-LDL (0.1 mg/mL) was oxidized with 10 μM Cu^{2+} in PBS in the presence of vehicle or indicated concentrations of luteolin (L) or luteolin-7-glucoside (L7G) for 24 h. Concentrated LDL (1–2 μL) was separated on Titan lipoprotein gel and stained with Fat Red 7B.

oxidative burst being induced (29). **Figure 8a** shows the kinetic profile of fMLP-stimulated ROS production in PMN. GT (0.25−2.5 mg/mL) significantly inhibited ROS generation in a dose-dependent manner. The reaction curve of fMLP-induced ROS production in PBMC (**Figure 8b**) showed a slower and longer response than fMLP-stimulated PMN. GT (0.25−3.75 mg/mL) treatment dose-dependently inhibited ROS production in PBMC.

Inhibition of Oxidative Burst in PMN or PBMC by Major Constituents in GT. To elucidate whether luteolin-7-glucoside can inhibit ROS production in phagocytes, the aforementioned procedure was performed, and the peak CL values were used for calculating IC_{50}. **Table 1** demonstrates that both luteolin-7-glucoside and luteolin exhibited slightly stronger inhibitory effects against fMLP-triggered ROS production in PBMC than in PMN.

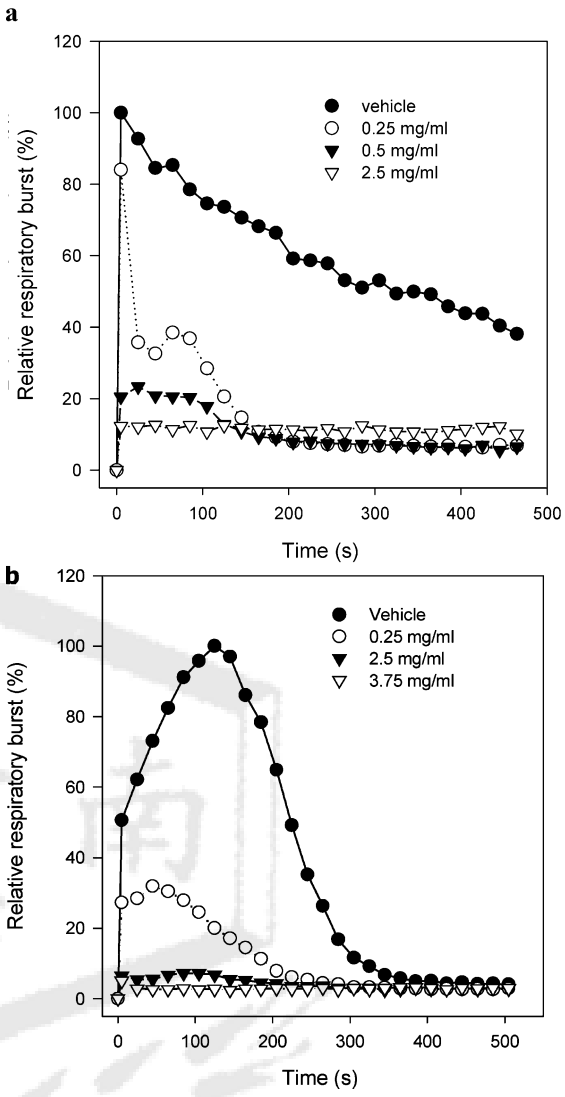


Figure 8. Ethanol extract of *G. tenuifolia* (GT) inhibits fMLP-induced ROS production in human leukocytes: (**a**) PMN; (**b**) PBMC. Each well containing 7×10^5 PMN or PBMC, 1 mM luminol, and vehicle or various concentrations of GT was incubated at room temperature for 15 min. Activator, 2 μM fMLP, was then added, and the light emission, expressed as relative light units (RLU), was monitored every 30 s for 5 s during a 10-min observation period.

Table 1. Effects of Luteolin-7-glucoside and Luteolin on the Inhibition of FMLP-Induced ROS Production in Human Leukocytes

IC_{50} (μM) for fMLP-induced ROS[a]	PMN	PBMC
luteolin-7-glucoside	14.8 ± 0.4	6.4 ± 0.2
luteolin	11.7 ± 0.2	5.2 ± 0.2

[a] Each well containing 7×10^5 PMN or PBMC, 1 mM luminol, and vehicle or various concentrations of luteolin or luteolin-7-glucoside was incubated at room temperature for 15 min. Activator, 2 μM fMLP, was then added, and the light emission, expressed as relative light units (RLU), was monitored every 30 s for 5 s during a 10-min observation period and the peak CL values were used to calculate IC_{50}.

DISCUSSION

Herbs have played a significant role in maintaining human health and improving the quality of life for thousands of years. Many active phytochemicals, including flavonoids, terpenoids, lignans, sulfides, polyphenolics, carotenoids, coumarins, saponins, plant sterols, curcumins, and phthalides, have been identified (*30*). Numerous in vitro studies have shown that some of the phytochemicals are potent antioxidants, metal chelators, or free radical scavengers, which may account for their health-promoting properties (*31*). To evaluate the antioxidant potency of *G. tenuifolia*, scavenging effects against free radicals including DPPH, superoxide, and hydroxyl radical were analyzed in this study. Our current results demonstrate that the ethanol extracts of *G. tenuifolia* and its major flavonoid, luteolin-7-glucoside, as well as its aglycon, luteolin, are strong DPPH scavengers with IC_{50} values of 55.2 μg/mL,12.2 μM, and 22.8 μM, respectively.

It is generally accepted that luminol-enhanced CL can be used to measure the ROS production in vivo (*32*) and in neutrophils (*29*) or of hydroxyl radicals in a cell-free system (*33*). On the other hand, lucigenin reacts only with superoxide, hydrogen peroxide, or singlet oxygen (*34*, *35*). Using lucigenin- and luminol-enhanced CL, we discovered herein that GT is a superoxide and hydroxyl radical scavenger in vitro with IC_{50} values of 5.18 and 0.06 mg/mL, respectively. There is consider-

able difference between superoxide and hydroxyl radical scavenging potencies of GT in vitro. On the other hand, luteolin-7-glucoside, one of the major constituents of GT, exhibited compatible superoxide and hydroxyl radical scavenging effects with IC_{50} values of 11.0 and 17.6 μM, respectively. It is possible that the free radical scavenging effects of GT are attributable to synergistic interactions among all natural phytochemicals. The discrepancy between superoxide and DPPH/hydroxyl radical scavenging potencies of GT may be caused by adverse effects of other unidentified phytochemicals, which could disrupt the superoxide scavenging effect of luteolin-7-glucoside.

We also found that luteolin-7-glucoside exerted a stronger DPPH quenching activity than its aglycon, luteolin. On the other hand, luteolin-7-glucoside had weaker or compatible superoxide and hydroxyl radical scavenging potencies than luteolin. The scavenging potencies of flavonoids against free radicals are thought to be closely linked to their structures and to depend on the nature of the radicals. The reaction mechanism between free radicals and flavonoids is influenced by the presence of glycosidic moieties, the position of glycosylation, and the number and positions of hydroxy groups in flavonoids. Thus, the antioxidant effectiveness will be determined by a combination of all structural elements.

A substantial body of evidence has indicated oxidation of LDL to be one of the major mechanisms for the pathogenesis of atherogenesis. Some flavonoids possess antioxidant properties and have been shown to be potent inhibitors of LDL oxidation in vitro (*26*). From our current data, GT demonstrated abilities to inhibit copper-induced peroxidation of LDL in a dose-dependent manner (7.5−30 μg/mL) during the propagation phase (**Figure 5a**) and to extend the lag phase to oxidation at 20 μg/mL (**Figure 6a**). Moreover, GT (6.5−10 μg/mL) inhibited an increase in the net negative surface charge of apolipoprotein B 100 as measured by decreased electrophoretic mobility (**Figure 7a**). The effective concentration of GT for inhibiting copper-induced LDL oxidation was slightly lower than that for scavenging hydroxyl radical (IC_{50} of 60 μg/mL) and differed by 2 orders of magnitude as compared with that for scavenging superoxide (IC_{50} of 5.18 mg/mL). It has been demonstrated that in vitro copper-mediated oxidation of LDL was not involved in the generation of detectable superoxide or H_2O_2 (*36*). These results suggest the contribution of metal-sequestering rather than superoxide scavenging functions to the antioxidant activity of GT against LDL oxidation. We also found that luteolin-7-glucoside demonstrated a dose-dependent reduction of LDL oxidation. It has been reported that luteolin-7-glucoside had copper-chelating properties, which suggest a potential role of metal chelating in the antioxidant effects of GT (*37*).

We also found that luteolin-7-glucoside was significantly less effective in inhibiting copper-mediated LDL oxidation than its aglycon, luteolin. It has been reported that the reactivities of flavonoids in protecting LDL against copper-induced oxidation are dependent on their structural properties in terms of the response to copper ions, whether chelation or oxidation, their partitioning abilities between the aqueous compartment, and the lipophilic environment within the LDL particle, as well as their hydrogen-donating antioxidant properties (*38*). It is possible that the higher effectiveness of luteolin in inhibiting LDL oxidation may be attributed to its higher hydrophobicity compared with luteolin-7-glucoside.

Due to its chemical structure, luminol can cross biological membranes and therefore allow for the detection of both extra- and intracellular production of ROS (*39*). In this paper, fMLP stimulated PMN to generate ROS, which exhibited biphasic shapes as was observed by other authors in experiments with human blood neutrophils. It has been speculated that the first and second peaks of the CL reaction would reflect extra- and intracellular ROS production, respectively (*40*). GT and luteolin-7-glucoside showed concentration-dependent inhibition of fMLP-induced ROS production in PMN and PBMC, indicating that they could serve as potent antioxidants against oxidative stress in human primary cells.

Previously, we have demonstrated that GT inhibited pro-inflammatory cytokine release from human whole blood and PBMC and HBsAg expression in PLC/PRF/5 cells (*10*), which suggests that the health promotion effect of GT may be the result of combinatory activities, including antioxidant, anti-inflammatory, and antiviral. It has been proposed that the additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their antioxidant and anticancer activities (*41*). In conclusion, we demonstrated for the first time that the ethanol extract of *G. tenuifolia* exhibited strong ROS scavenging activity in both cell-free and cell-based systems. In addition, GT also exhibited antioxidant activity against copper-induced LDL oxidation. Overall, the results demonstrate that the antioxidant activity of GT relates in part to its constituent, luteolin-7-glucoside, which acts as free radical and copper scavenger.

ABBREVIATIONS USED

GT, ethanol extract of *Glossogyne tenuifolia*; CL, chemiluminescence; LDL, low-density lipoprotein; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde.

LITERATURE CITED

(1) Gutteridge, J. M. Biological origin of free radicals, and mechanisms of antioxidant protection. *Chem.−Biol. Interact.* **1994**, *91*, 133−140.

(2) Hurst, N. P. Molecular basis of activation and regulation of the phagocyte respiratory burst. *Ann. Rheum. Dis.* **1987**, *46*, 265−272.

(3) Rosen, H.; Klebanoff, S. Bactericidal activity of a superoxide anion-generating system. *J. Exp. Med.* **1979**, *149*, 27−39.

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

(5) Casimir, C. M.; Teahan, C. G. The respiratory burst of neutrophils and its deficiency. In *Immunopharmacology of Neutrophils*; Hellewell, P. G., Williams, T., Eds.; Academic Press: San Diego, CA., 1994; pp 27−54.

(6) Halliwell, B.; Gutteridge, J. M.; Cross, C. E. Free radicals, antioxidants, and human disease: where are we now? *J. Lab. Clin. Med.* **1992**, *119*, 598−620.

(7) Meydani, M. Vitamin E and atherosclerosis: beyond prevention of LDL oxidation. *J. Nutr.* **2001**, *131*, 366S−368S.

(8) Frankel, E. N. Natural phenolic antioxidants and their impact on health. In *Antioxidant Food Supplements in Human Health*; Lester, P., Hiramastu, M., Yoshikawa, T., Eds.; Academic Press: San Diego, CA, 1999; pp 385−392.

(9) Wu, M. J.; Wang, L.; Ding, H. Y.; Weng, C. Y.; Yen, J. H. *Glossogyne tenuifolia* acts to inhibit inflammatory mediator production in a macrophage cell line by down-regulating LPS-induced NF-κB. *J. Biomed. Sci.* **2004**, *11*, 186−199.

(10) Wu, M. J.; Weng, C. Y.; Ding, H. Y.; Wu, P. J. Anti-inflammatory and antiviral effects of *Glossogyne tenuifolia*. *Life Sci.* **2005**, *76*, 1135−1146.

(11) Sandoval, M.; Okuhama, N. N.; Zhang, X. J.; Condezo, L. A.; Lao, J.; Angeles, F. M.; Musah, R. A.; Bobrowski, P.; Miller, M. J. Anti-inflammatory and antioxidant activities of cat's claw (*Uncaria tomentosa* and *Uncaria guianensis*) are independent of their alkaloid content. *Phytomedicine* **2002**, *9*, 325−337.

(12) Chamundeeswari, D.; Vasantha, J.; Gopalakrishnan, S.; Sukumar, E. Free radical scavenging activity of the alcoholic extract of *Trewia polycarpa* roots in arthritic rats. *J. Ethnopharmacol.* **2003**, *88*, 51−56.

(13) Lo, A. H.; Liang, Y. C.; Lin-Shiau, S. Y.; Ho, C. T.; Lin, J. K. Carnosol, an antioxidant in rosemary, suppresses inducible nitric oxide synthase through down-regulating nuclear factor-kappaB in mouse macrophages. *Carcinogenesis.* **2002**, *23*, 983−991.

(14) Dharmasiri, M. G.; Jayakody, J. R.; Galhena, G.; Liyanage, S. S.; Ratnasooriya, W. D. Anti-inflammatory and analgesic activities of mature fresh leaves of *Vitex negundo*. *J. Ethnopharmacol.* **2003**, *87*, 199−206.

(15) Wu, M. J.; Yen, J. H.; Wang, L.; Weng, C. Y. Antioxidant activity of porcelainberry (*Ampelopsis brevipedunculata* (Maxim.) Trautv.). *Am. J. Chin. Med.* **2004**, *32*, 681−693.

(16) 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*, 161−169.

(17) Lei, D.; Chan, C. P.; Wang, Y. J.; Wang, T. M.; Lin, B. R.; Huang, C. H.; Lee, J. J.; Chen, H. M.; Jeng, J. H.; Chang, M. C. Antioxidative and antiplatelet effects of aqueous inflorescence *Piper betle* extract. *J. Agric. Food Chem.* **2003**, *51*, 2083−2088.

(18) Havel, R. J.; Eder, H. A.; Bragdon, J. H. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **1955**, *34*, 1345−1353.

(19) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein−dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(20) Hermann, M.; Gmeiner, B. Altered susceptibility to in vitro oxidation of LDL in LDL complexes and LDL aggregates. *Ann. N. Y. Acad. Sci.* **1993**, *683*, 363−364.

(21) Esterbauer, H.; Striegl, G.; Puhl, H.; Rotheneder, M. Continuous monitoring of in vitro oxidation of human low-density lipoprotein. *Free Radical Res. Commun.* **1989**, *6*, 67−75.

(22) Pavelkova, M.; Kubala, L. Luminol-, isoluminol- and lucigenin-enhanced chemiluminescence of rat blood phagocytes stimulated with different activators. *Luminescence* **2004**, *19*, 37−42.

(23) Halliwell, B. Reactive oxygen species and the central nervous system. *J. Neutrochem.* **1992**, *59*, 1609−1623.

(24) 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*, 376−380.

(25) Steinbrecher, U. P.; Zhang, H.; Lougheed, M. Role of oxidatively modified LDL in atherosclerosis. *Free Radical Biol. Med.* **1990**, *9*, 155−168.

(26) Frankel, E. N.; Kanner, J.; German, J. B.; Parks, E.; Kinsella, J. E. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* **1993**, *341*, 454−457.

(27) Steinbrecher, U. P.; Witztum, J. L.; Parthasarathy, S.; Steinberg, D. Decrease in reactive amino groups during oxidation or endothelial cell modification of LDL: correlation with changes in receptor-mediated catabolism. *Arteriosclerosis* **1987**, *7*, 135−143.

(28) Pinchuk, I.; Lichtenberg, D. Continuous monitoring of intermediates and final products of oxidation of low-density lipoprotein by means of UV-spectroscopy. *Free Radical Res.* **1996**, *24*, 351−360.

(29) 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**, *3*, 277−285.

(30) Craig, W. J. Health-promoting properties of common herbs. *Am. J. Clin. Nutr.* **1999**, *70*, 491S−499S.

(31) Cotell, N.; Bernier, J. L.; Catteau, J. P.; Pommery, J.; Wallet, J. C.; Gaydou, E. M. Antioxidant properties of hydroxy-flavones. *Free Radical Biol. Med.* **1996**, *20*, 35−43.

(32) Oldenburg, B.; van Kats-Renaud, H.; Koningsberger, J. C.; van Berge Henegouwen, G. P.; van Asbeck, B. S. Chemiluminescence in inflammatory bowel disease patients: a parameter of inflammatory activity. *Clin. Chim. Acta.* **2001**, *310*, 151−156.

(33) Yildiz, G.; Demiryurek, A. T. Ferrous iron-induced luminol chemiluminescence: a method for hydroxyl radical study. *J. Pharmacol. Toxicol. Methods* **1998**, *39*, 179−184.

(34) Edwards, S. W. Luminol- and lucigenin-dependent chemiluminescence of neutrophils: role of degranulation. *J. Clin. Lab. Immunol.* **1987**, *22*, 35−39.

(35) Weber, G. F. The measurement of oxygen-derived free radicals and related substances in medicine. *J. Clin. Chem. Clin. Biochem.* **1990**, *28*, 569−603.

(36) Lynch, S. M.; Frei, B. Mechanisms of copper- and iron-dependent oxidative modification of human low-density lipoprotein. *J. Lipid Res.* **1993**, *34*, 1745−1753.

(37) Brown, J. E.; Rice-Evans, C. A. Luteolin-rich artichoke extract protects low-density lipoprotein from oxidation in vitro. *Free Radical Res.* **1998**, *29*, 247−255.

(38) Brown, J. E.; Khodr, H.; Hider, R. C.; Rice-Evans, C. A. Structural dependence of flavonoid interactions with Cu^{2+} ions: implications for their antioxidant properties. *Biochem. J.* **1998**, *330*, 1173−1178.

(39) Ushijima, Y.; Totsune, H.; Nishida, A.; Nakano, M. Chemiluminescence from human polymorphonuclear leukocytes activated with opsonized zymosan. *Free Radical Biol. Med.* **1997**, *22*, 401−409.

(40) Kopprasch, S.; Kohl, M.; Graessler, J. Different effects of exogenous horseradish peroxidase on luminol-amplified chemiluminescence induced by soluble and particulate stimuli in human neutrophils. *J. Biolumin. Chemilumin.* **1998**, *13*, 267−271.

(41) Liu, R. H. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *Am. J. Clin. Nutr.* **2003**, *78*, 517S−20S.

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Immunomodulatory mechanism of the aqueous extract of sword brake fern (*Pteris ensiformis* Burm.)

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Abstract

Sword brake fern (*Pteris ensiformis* Burm.) is an ingredient in most of the traditional herbal beverage formulas in Taiwan; however, no information is available to explain its bioactivity. The aim of this study is to elucidate the molecular pharmacological activity in the aqueous extract of sword brake fern (SBF). We found that SBF (0.05–0.25 mg/ml) slightly induced TNF-α, IL-6, NO (nitric oxide) and PGE_2 (prostaglandin E_2) production in unstimualted murine macrophages, RAW264.7 cells. Furthermore, SBF (0.05–0.25 mg/ml) dose-dependently suppressed LPS-induced TNF-α, IL-1β, IL-6, NO and PGE_2 in activated RAW264.7 cells without exerting cytotoxicity. Further analysis of molecular mechanisms revealed that SBF prominently repressed LPS-induced iNOS (inducible nitric oxide synthase) and COX-2 (cyclooxygenase-2) promoter activities. Activation of the transcription factor NF-κB, which is one of the important pathways for transduction of LPS-stimulated inflammatory mediator producing signals, was suppressed by SBF in a dose-dependent manner, as demonstrated by both electrophoretic mobility shift assay (EMSA) and transfection with pNF-κB-Luc plasmid. These results suggest that SBF attenuates inflammatory mediator synthesis of activated macrophages partially through a NF-κB-dependent pathway. The immunomodulatory activity of SBF supports its traditional health promotion effect.

Keywords: TNF-α; Inducible nitric oxide synthase; Prostaglandin E2; Cyclooxygenase-2; Nuclear factor-κB

1. Introduction

Activation of macrophages is a key event in innate immunity for the initiation and propagation of defensive reactions against pathogens. When stimulated by pathologic stimuli or injury, macrophages release TNF-α, interleukin (IL)-1, IL-6 and other inflammatory mediators. TNF-α is secreted in response to various stimuli, including lipopolysaccharide (LPS) and exerts a wide variety of effects. The role of TNF-α includes activation and chemotaxis of leukocytes; induction of the expression of adhesion molecules, such as intercellular adhesion, or ICAM, on neutrophils and endothelial cells; and regulation of the secretion of other proinflammatory cytokines (Beutler and Cerami, 1988). Nearly each biological property of TNF-α has also been observed with IL-1. These include fever (Dinarello et al., 1986), sleep, hemodynamic shock (Okusawa et al., 1988), the induction of prostaglandin and collagenase synthesis (Dayer et al., 1985). Activated monocytes also produce IL-6 in response to IL-1 and TNF-α stimulation. IL-6 acts as a B-cell growth factor, thereby promoting antibody formation and release (Barton, 1996).

The release of proinflammatory cytokines is essential for host survival from infection, and is also required for the repair of tissue injury (Tracey et al., 1988; Tracey, 1991; Glauser, 1996; Dinarello, 1998). These beneficial effects, however, are critically dependent on the magnitude of the immune response, because large amounts of macrophage-derived proinflammatory cytokines can also cause collateral damage to normal cells and are potentially lethal when the release is sufficient to cause systemic exposure (Tracey et al., 1988; Tracey, 1991; Glauser, 1996; Dinarello, 1998). Thus, inhibit-

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ing the overproduction of proinflammatory cytokines is an important therapeutic goal for drug development.

Nitric oxide (NO) is synthesized from L-arginine by constitutive and inducible nitric oxide synthase (cNOS and iNOS) in numerous mammalian cells and tissues (Nathan and Xie, 1994). 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 bacterial lipopolysaccharide (LPS) or immunological stimuli (IFN-γ). The sustained NO production by iNOS has been implicated as a mediator of inflammation. It has been reported that excess production of NO by macrophages and other cells exposed to endotoxin may contribute to septic shock (Kilbourn and Griffith, 1992), cerebral injury (Beckman, 1994), myocardical ischemia (Matheis et al., 1992), local or systemic inflammatory disorders, diabetes and other diseases (Rees et al., 1990; Stefanovic-Racic et al., 1993; McDaniel et al., 1996). Although iNOS induction may not be a primary event in all of these diseases, the cytotoxic and proinflammatory effects of NO generated by iNOS contribute to their pathophysiology. Therefore, inhibition of iNOS is potentially beneficial.

Prostanoids are produced when arachidonic acid is released from plasma membranes by phospholipases and metabolized by cyclooxygenases (COXs). It is now known that two isoforms of COX mediate prostanoid production (Xie et al., 1999). COX-1 produces physiological levels of prostanoids and is constitutively expressed under normal conditions in most tissues (Vane, 1994). COX-2, the inducible isoform of the enzyme, is induced in many cells under the stimulation of inflammatory mediators such as lipopolysaccharides and cytokines (Pang and Hoult, 1996; Pang and Knox, 1997). Accumulating evidence suggests that the induction and regulation of COX-2 may be key elements in the pathophysiological process of a number of inflammatory disorders.

Macrophage-derived inflammatory mediators are involved at multiple steps of both immune and inflammatory responses, and the transcriptional factor, NF-κB, appears to play a pivotal role in their coordinated upregulation (Chen et al., 1999). 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 ubiquitination 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 (Chen et al., 1995). The identification of NF-κB as a key factor in the pathogenesis of inflammation suggests that NF-κB-targeted therapeutics might be effective in treating human inflammatory diseases. A variety of pharmacologic agents have been described to inhibit NF-κB at one or more activation steps of the signaling pathway (Zingarelli et al., 2003).

Sword brake fern (*Pteris ensiformis* Burm.) is one of the most popular components of herbal beverages in Taiwan. It has a long history of being used as a health improvement remedy; however, information regarding its biological action remains unclear. In this study, we focus on the immunomodulatory effect of the aqueous extract of sword brake fern by elucidating the detailed mechanisms in murine macrophage cell line, RAW264.7. The present study shows that the aqueous extract of sword brake fern slightly induces inflammatory mediator release in unstimulated RAW264.7 cells. Furthermore, it inhibits the release of proinflammatory cytokine, TNF-α, IL-1β and IL-6, as well as secondary mediators, NO and PGE_2 in LPS-activated RAW264.7 cells. It also suppressed iNOS and COX-2 promoter activities as well as NF-κB activation in activated macrophages.

2. Materials and methods

2.1. Preparation of the aqueous extract of sword brake fern (SBF)

Sword brake fern (*Pteris ensiformis* Burm.) was obtained from the Taitung District Agricultural Research and Extension Station (Taitung, Taiwan) and the voucher specimens were deposited in the herbarium of Chia-Nan University of Pharmacy and Science. The dry aboveground part (150 g) was grinded in 1 L of deionized water by an engine room-driven blender for 5 min and then autoclaved for 20 min. After filtration twice, the flow through was then lyophilized to yield a dark-brown powder (5.74 g), which was redissolved in deionized water prior to use.

2.2. Cell culture

RAW264.7 cells were purchased from Bioresources Collection and Research Center (Hsinchu, Taiwan) and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (HyClone, Logan, UT, USA), 2 mM glutamine, 1% non-essential amino acid, 1 mM pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA). Cells were maintained in a humidified incubator at 37 °C in 5% CO_2. Cells were treated with various concentrations of BSF followed by 1 μg/ml LPS (*Escherichia coli* O55:B5, Fluka Chemie AG, Buchs, Switzerland) 30 min later. Supernatants were collected after LPS treatment for 6 and 24 h for cytokine and NO/PGE_2 determination, respectively.

2.3. Cytokine measurements

Cytokine concentrations in the supernatants were determined by ELISA kits that were specific against murine cytokines. Levels of TNF-α, IL-1β and IL-6 were measured using ELISA kits from Pierce Endogen (Rockford, IL, USA). Assays were performed according to the manufacturer's instructions.

2.4. Cell viability

Cell viability was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) to purple formazan (Carmichael et al., 1987). Cells were incubated with MTT (0.5%) for 4 h at 37 °C. The medium was removed by aspiration, and formazan crystals were dissolved in DMSO. The extent of the reduction of MTT was quantitated by measurement of the absorbance at 550 nm.

2.5. Determination of nitrite and prostaglandin E_2 (PGE_2)

Nitrite production, an indicator of nitric oxide (NO) synthesis, was determined by the Griess reaction (Nims et al., 1996). The supernatant of cell culture was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid). The absorbance at 550 nm was measured and nitrite concentration was determined using sodium nitrite as a standard. Culture medium was used as diluent and as blank to avoid medium phenol red interference. The level of PGE_2 in the supernatant of the culture medium was measured using an ELISA kit (Cayman Chemical, Ann Arbor, MI, USA). Assays were performed according to the manufacturer's instruction.

2.6. Plasmids

To generate the mouse iNOS promoter reporter plasmid, piNOS/GL3, the promoter fragment from −1035 to +84 relative to the transcription start, was produced by PCR using genomic DNA from BABL/cByJ mice (National Laboratory Animal Center, Taipei, Taiwan) as a template, with two primers: 5′-GCGCCTCGAGCACGAGGCTGAGCTGACTTT-3′and 5′-CCGGAAGCTTACCAAGGTGGCTGAGAAGTT-3′. The resulting iNOS promoter fragment was digested with *Hind*III and *Xho*I, gel-purified, and ligated into the *Hind*III–*Xho*I sites of the polyclonal site of pGL3 (Promega, Madison, WI, USA) to construct piNOS/GL3. The promoter sequence was confirmed by DNA sequencing.

The murine COX-2 promoter plasmid (pCOX2/GL3) containing a 731-bp promoter fragment, −724 to +7 relative to the transcription start, was constructed as described previously (Wu et al., 2004). A luciferase reporter plasmid, pNF-κB-Luc, which contains six κB *cis*-acting elements was purchased from Stratagene (La Jolla, CA). A transfection control plasmid, pSV-β-galactosidase, was purchased from Promega.

2.7. Transfection and transient expression of luciferase reporter gene constructs

RAW264.7 cells were plated in six-well plates at a density of 1×10^5 per well and allowed to reach 40–60% confluence. Constructs were co-introduced into RAW264.7 cells with control plasmid, pSV-β-galactosidase, by liposomal-mediated transfection (Lipofectin, Invitrogen Life Technologies) at a DNA/lipid ratio of 2 μg of each plasmid DNA/6 μl of lipid. Transfection efficiency was typically 50% or greater.

2.8. Luciferase and β-galactosidase assays

Fifteen hours after initiation of transfection, cells were treated with LPS (1 μg/ml) alone or in combination with the indicated reagents. To determine the promoter activities of iNOS and COX-2, cells were harvested after 12 and 18 h of treatment, respectively. To determine the NF-κB-mediated promoter activity, cells were harvested after 4 h of treatment. Luciferase activities were measured by the Luciferase Assay System (Promega). β-Galactosidase activities were measured from different aliquots of the same lysates by the Mammalian β-Gal Assay Reagent Kit (Pierce Endogen). The intensity of the luciferase reactions measured in the lysates of the transient transfectants was normalized to their β-galactosidase activity, which was used as an internal control.

2.9. Electrophoretic mobility shift assay (EMSA)

RAW264.7 cells were grown in 6-well plates and stimulated with LPS (1 μg/ml) in the presence or absence of SBF (0.05–0.25 mg/ml) for 30 min. Nuclear extracts were prepared by NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce Endogen). 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′-AGTTGAGGGGACTTTCCCAGGC-3′) for 20 min at room temperature and resolved in an 8% non-denaturing 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. Specificity of the DNA–protein complex was confirmed by competition with 100-fold excess of an unlabeled NF-κB probe.

2.10. Statistical analysis

The results were analyzed by Student's unpaired *t*-test, and a *p*-value of <0.05 was taken to be significant.

3. Results

3.1. Effects of the aqueous extract of sword brake fern (SBF) on TNF-α, IL-1β and IL-6 release

Murine macrophage-like cell line, RAW264.7, has been used to characterize the action of various immunomodulatory components at the molecular level previously (Cho et al.,

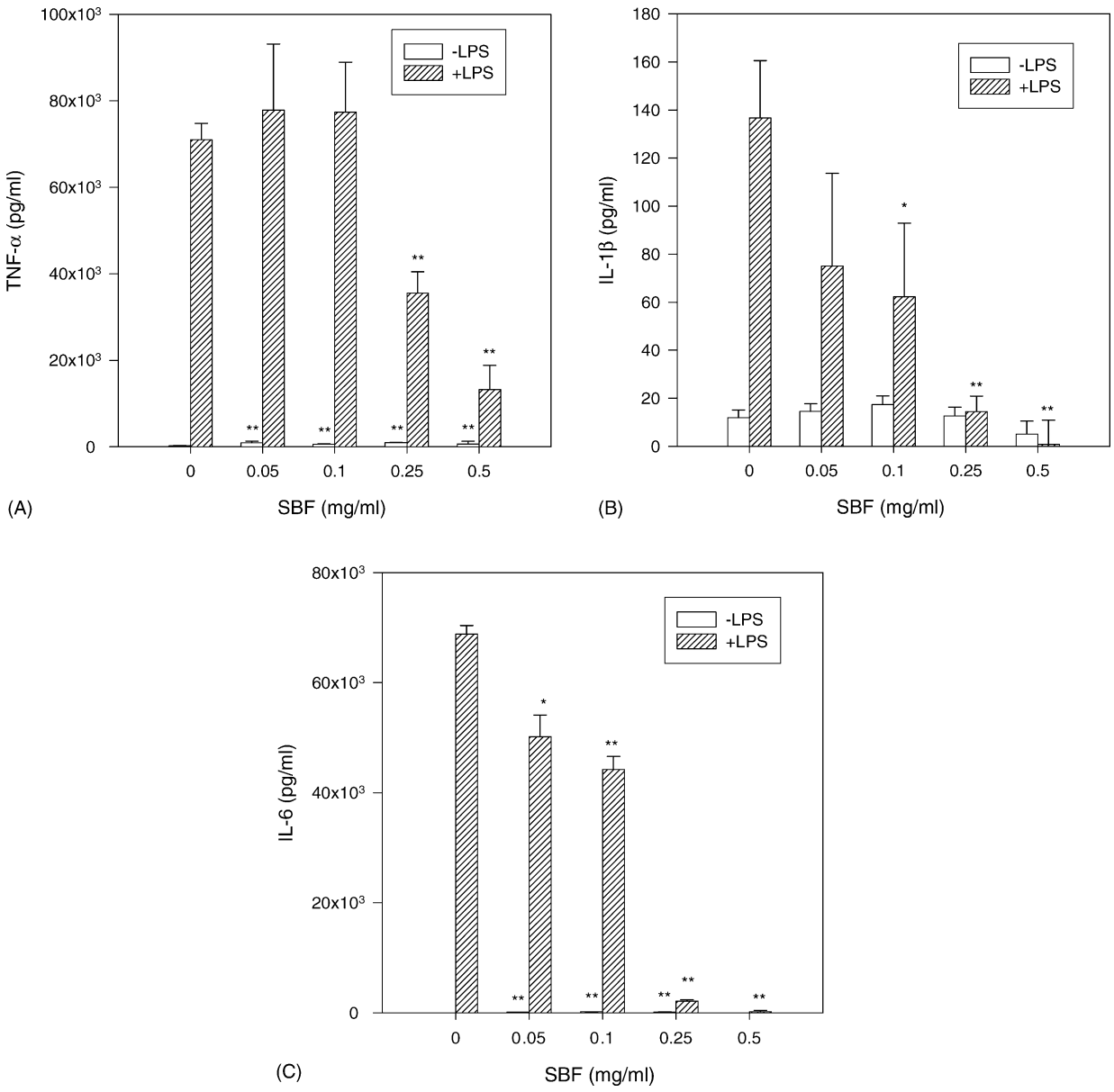

Fig. 1. Effects of the aqueous extract of sword brake fern (SBF) on the production of proinflammatory cytokines in RAW 264.7 macrophages. RAW264.7 cells were cultured with indicated concentrations of SBF alone or in combination with LPS (1 μg/ml) at 37 °C in a 96-well plate. Media were collected after 6 h of treatment for (A) TNF-α, (B) IL-1β and (C) IL-6 analysis. Data are expressed as the mean ± S.D. of three individual experiments. Statistically significant change of cytokine release ($^{*}p < 0.05$, $^{**}p < 0.01$), as compared with the group treated without SBF treatment.

1999; Choi et al., 2001; Wu et al., 2004). To study the immunomodulatory effect of the aqueous extract of sword brake fern (SBF), proinflammatory cytokine release, TNF-α, IL-1β and IL-6, was first measured in unstimulated and LPS-stimulated RAW264.7 cells. Fig. 1 demonstrated that unstimulated RAW264.7 cells cultured for 6 h produced 245 ± 27 and 12 ± 3 pg/ml of TNF-α and IL-1β, respectively, as well as negligible quantity of IL-6. Treatment of cells with SBF (0.05–0.5 mg/ml) for 6 h significantly induced TNF-α and IL-6 release up to 975 ± 55 and 168 ± 29 pg/ml, respectively ($p < 0.01$) (Fig. 1(A) and (C)). However, no significant alteration in IL-1β production could be found (Fig. 1(B)).

Stimulation of RAW264.7 cells with LPS (1 μg/ml) for 6 h caused a substantial increase in the release of proinflammatory cytokines (70987 ± 3855, 137 ± 24 and 68834 ± 1545 pg/ml for TNF-α, IL-1β and IL-6, respectively). When RAW264.7 cells were stimulated with LPS (1 μg/ml) in the presence of SBF (0.05–0.5 mg/ml), dose-dependent inhibitions of cytokine release were observed (Fig. 1(A)–(C)). When SBF concentration was 0.25 mg/ml, the inhibitory effects of cytokine release reached 50, 89 and 97% ($p < 0.05$); and the IC_{50}s of approximately 0.25, 0.05 and 0.16 mg/ml were observed for TNF-α, IL-1β and IL-6, respectively.

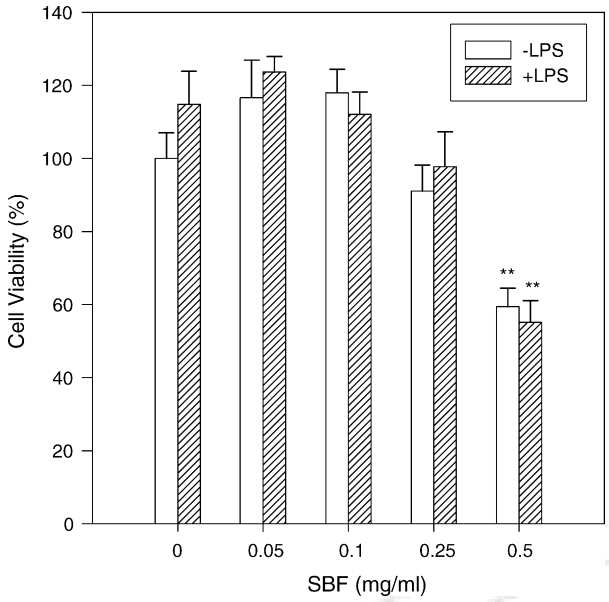

Fig. 2. Effect of the aqueous extract of sword brake fern (SBF) on cell viability i ± n RAW264.7 macrophages. RAW264.7 cells were cultured with indicated concentrations of SBF alone or in combination with LPS (1 μg/ml) at 37 °C in a 96-well plate for 24 h. Cell viability was determined by MTT assay. Data are expressed as the mean ± S.D. of three individual experiments. Statistically significant inhibition of cell viability ($^{**}p < 0.01$), as compared with normal group.

To evaluate whether the inhibitory effects of cytokine release were due to cytotoxicity, cell viabilities of macrophages were evaluated by MTT test. Fig. 2 showed that SBF (0.05–0.25 mg/ml) did not cause significant cytotoxicity either in the presence or absence of LPS (1 μg/ml). This result implies that SBF (<0.25 mg/ml) inhibited LPS-stimulated proinflammatory cytokine release without causing cell death. However, 0.5 mg/ml of SBF exerted significant cytotoxicity ($p < 0.01$). Therefore, the abolishment of proinflammatory cytokine release by 0.5 mg/ml of SBF in LPS-treated cells might be caused by both anti-inflammatory activity and cytotoxicity.

3.2. Effect of SBF on NO production

Nitric oxide (NO) synthesized by activated inflammatory cells regulates the functions of other cells involved in the inflammatory process and appears to act as a secondary mediator of some actions of proinflammatory cytokines. The effects of SBF on NO production in unstimulated macrophages and LPS-stimulated macrophages are shown in Fig. 3(A). Unstimulated macrophages produced undetectable level of nitrite. Treatment of unstimulated macrophages with SBF (0.05 and 0.1 mg/ml) for 24 h slightly induced nitrite production to 2.0 ± 0.7 and 2.5 ± 1.0 μM, respectively ($p < 0.01$). However, 0.25 mg/ml of SBF did not exhibit significant increase of nitrite release. When cells were activated with LPS (1 μg/ml) for 24 h, substantial increase in nitrite production was observed (21.34 ± 1.40 μM). Co-treatment of cells

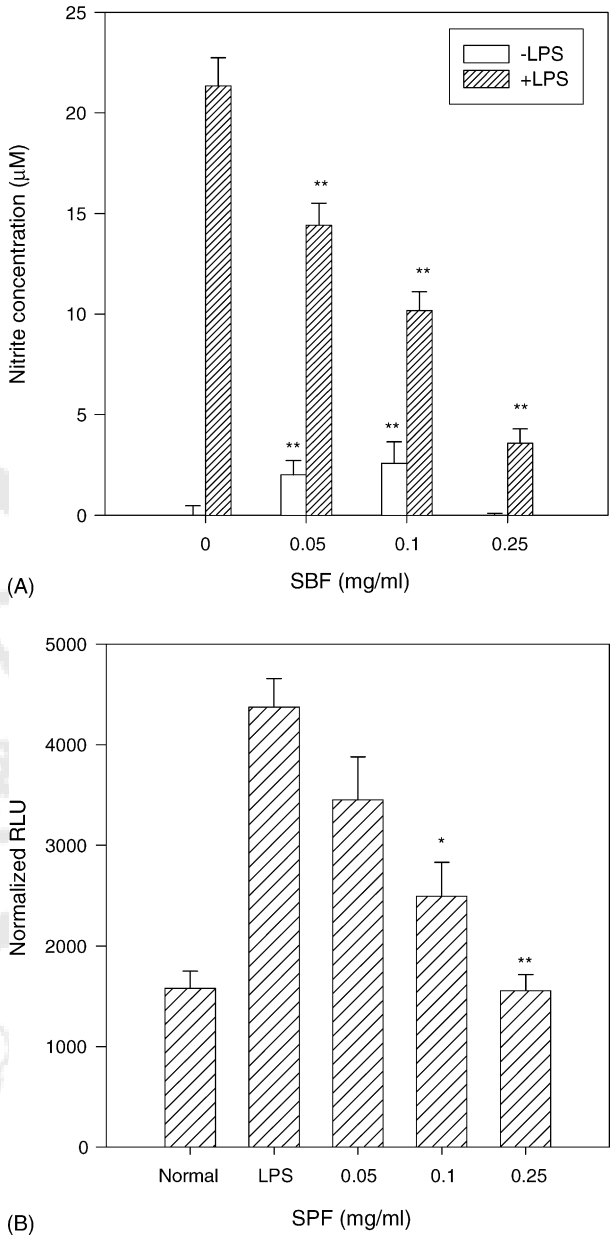

Fig. 3. Effects of the aqueous extract of sword brake fern (SBF) on nitrite formation and iNOS promoter activity in RAW 264.7 macrophages. (A) RAW 264.7 macrophages were cultured with indicated concentrations of SBF alone or in combination with LPS (1 μg/ml) at 37 °C for 24 h in a 96-well plate. Nitrite release in the medium, an indicator of nitric oxide (NO) synthesis, was determined by the Griess reaction as described in Section 2. Data are expressed as the mean ± S.D. of three individual experiments. Statistically significant change of nitrite formation ($^{**}p < 0.01$), as compared with the groups without SBF treatment. (B) An iNOS promoter-containing reporter plasmid, piNOS/GL3, was transiently co-transfected into RAW264.7 cells with the pSV-β-galactosidase control plasmid for 15 h. Cells were then treated with LPS (1 μg/ml) alone or in combination with indicated concentrations of SBF. Cells were then harvested after 12 h of incubation. Luciferase activity measured in the lysates of the transient transfectants was normalized to their β-galactosidase activities, used as an internal control. Statistically significant inhibition ($^{*}p < 0.05$, $^{**}p < 0.01$), as compared with the group treated with LPS only. Assays were repeated three times with a representative result shown.

with LPS and 0.05, 0.1 and 0.25 mg/ml of SBF evoked a concentration-dependent inhibition of nitrite release by 32.5, 52.3 and 83.2%, respectively ($p < 0.01$).

3.3. Effect of SBF on iNOS promoter activity

To determine whether the NO-inhibitory effect of SBF was through directly repressing the iNOS promoter activity, we generated a murine iNOS promoter fragment spanning nucleotide −1035 to +84 by PCR. The fragment was then cloned into the polycloning site of the luciferase reporter plasmid, pGL3, to construct piNOS/GL3. The activity of the iNOS promoter was measured as a function of luciferase activity, normalized to β-galactosidase levels which reflect equal transfection, following co-transfections of piNOS/GL3 and pSV-β-gal control plasmids into RAW264.7 cells as described in Section 2. It was found that incubation of transfected cells with LPS (1 μg/ml) for 12 h markedly increased the iNOS promoter activity by 2.76-fold compared with that of control group. The iNOS promoter activity was significantly inhibited by concurrent treatment of the cells with SBF (0.1 and 0.25 mg/ml) in a dose-dependent manner (Fig. 3(B)). This result suggests that SBF inhibits NO release partially through downregulating iNOS promoter activity in activated macrophages.

3.4. Effect of SBF on PGE_2 release and COX-2 promoter activity

Prostanoids are produced when arachidonic acid is released from plasma membranes by phospholipases and metabolized by cyclooxygenases (COXs). During an inflammatory response, inducible cyclooxygenase (COX-2) expression increases dramatically, resulting in high level of prostaglandin E_2 (PGE_2). To examine whether SBF inhibits PGE_2 release in activated macrophages, culture supernatants were analyzed using commercial ELISA kits. Unstimulated RAW264.7 cells maintained in culture medium for 24 h produced a basal amount of PGE_2 (69 ± 6 pg/ml). Treatment of unstimulated macrophages with SBF (0.05, 0.1 and 0.25 mg/ml) for 24 h slightly activated PGE_2 synthesis to 351 ± 139, 580 ± 69 and 691 ± 120 pg/ml, respectively ($p < 0.01$).

Treatment of RAW264.7 cells with LPS (1 μg/ml) for 24 h, the medium concentration of PGE_2 increased substantially (26065 ± 268 pg/ml) (Fig. 4(A)). Co-treatment of RAW24.7 cells with LPS (1 μg/ml) and 0.05, 0.1 and 0.25 mg/ml of SBF dose-dependently reduced PGE2 production by 12.1, 18.3 and 26.3%, respectively ($p < 0.05$, 0.05 and 0.01, respectively).

We next sought to determine whether SBF decreased PGE_2 production through repressing the promoter activity of the COX-2 gene. RAW264.7 cells were transfected with pCOX2/GL3, a reporter vector containing murine COX-2 pormoter (Wu et al., 2004). It was found that incubation of transfected cells with LPS (1 μg/ml) for 18 h increased the luciferase activity by 7.35-fold compared with that of control group. Concurrent treatment of the cells with 0.05, 0.1 and 0.25 mg/ml of SBF inhibited LPS-stimulated COX-2 promoter activity by 48.7, 61.3 and 79.7%, respec-

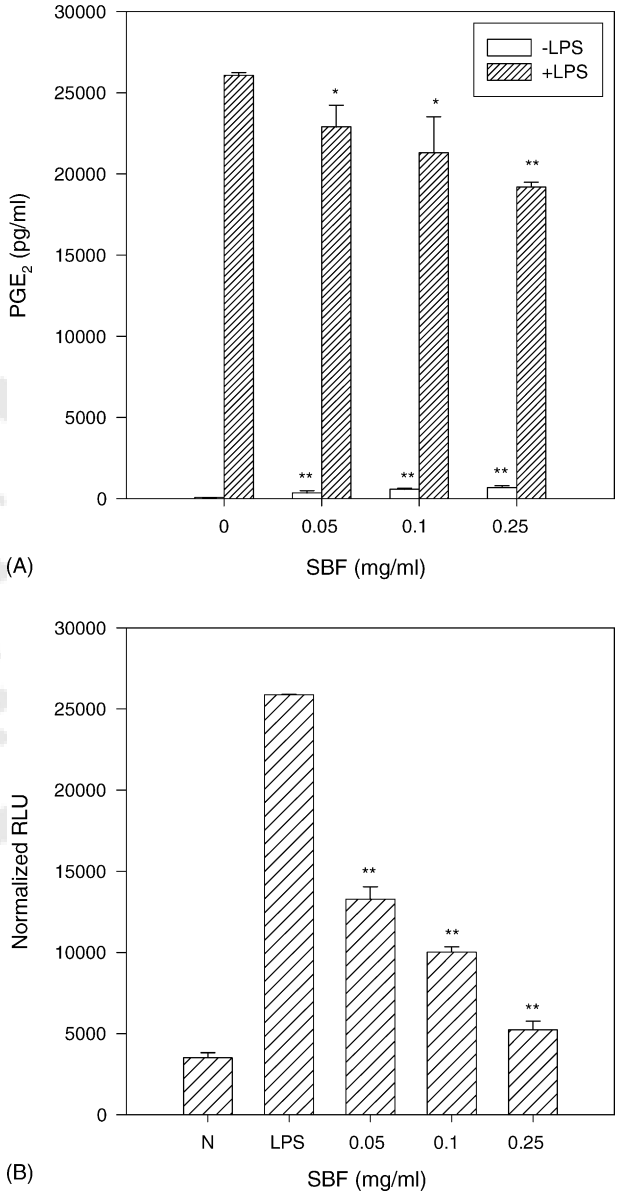

Fig. 4. Effects of the aqueous extract of sword brake fern (SBF) on PGE_2 production and COX-2 promoter activity in RAW264.7 macrophages. (A) RAW264.7 cells were treated with LPS (1 μg/ml) alone or in combination with indicated concentrations of SBF for 24 h. The culture media were then collected for PGE_2 assay. Data are expressed as the mean ± S.D. of three individual experiments. Statistically significant change of PGE_2 ($^{**}p < 0.01$), as compared with the groups without SBF treatment. (B) A COX-2 promoter-containing reporter plasmid, pCOX2/GL3, was transiently co-transfected into RAW264.7 cells with the pSV-β-galactosidase control plasmid for 15 h. Cells were then treated with LPS (1 μg/ml) alone or in combination with indicated concentrations of SBF. Cells were harvested after 18 h of incubation. Luciferase activity measured in the lysates of the transient transfectants was normalized to their β-galactosidase activities, used as an internal control. Statistically significant inhibition ($^{**}p < 0.01$), as compared with the group treated with LPS alone. Assays were repeated three times with a representative result shown.

tively ($p < 0.01$) (Fig. 4(B)). This result indicates that SBF suppressed COX-2 promoter activity thus inhibiting PGE_2 release in activated macrophages.

3.5. Effect of SBF on NF-κB activation

A search for common pathways involved in the regulated induction of diverse inflammatory gene production has focused on transcriptional control mechanisms, and has identified NF-κB as a likely converging point of various immune and inflammatory responses (Sun and Andersson, 2002). To investigate whether SBF is able to attenuate LPS-induced NF-κB-mediated promoter activity, RAW264.7 cells were transfected with pNF-κB-Luc, a reporter plasmid containing six κB *cis*-acting elements. It was found that incubation of transfected RAW264.7 cells with LPS for 4 h increased luciferase activity by 2.27-fold compared with that of control group. Concurrent treatment of the cells with 0.05, 0.1 and 0.25 mg/ml of SBF significantly suppressed promoter activity by 30.1, 34.6 and 42.0%, respectively ($p < 0.05$) (Fig. 5(A)).

To gain more insight into the mechanisms of SBF-mediated inhibition of NF-κB-mediated promoter activity, we analyzed the κB DNA binding activity present in nuclear extracts of unstimulated or LPS-stimulated macrophages, either in the absence or presence of SBF. As expected, 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 (Fig. 5(B)). 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. Nuclear extract from macrophages stimulated by LPS in the presence of SBF (0.05–0.25 mg/ml) showed decreased κB-binding activity (Fig. 5(B)).

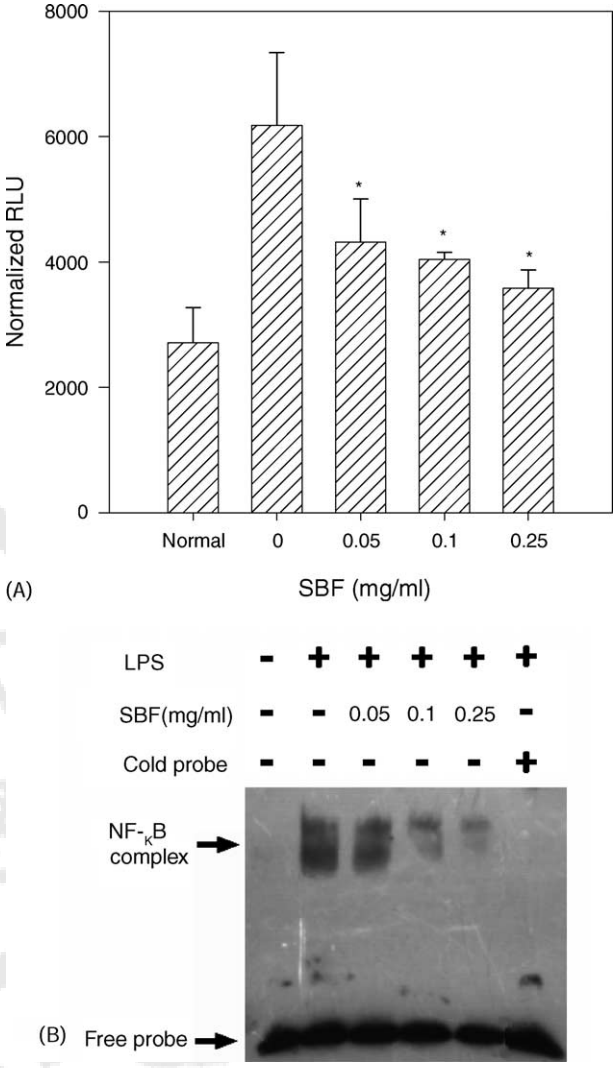

Fig. 5. Effect of the aqueous extract of sword brake fern (SBF) on LPS-induced NF-κB activation. (A) A reporter plasmid containing repetitive NF-κB sites in its enhancer element, pNF-κB-Luc, was transiently co-transfected into RAW264.7 cells with a pSV-β-galactosidase control plasmid for 15 h. Cells were treated with LPS (1 μg/ml) alone or in combination with indicated concentrations of SBF. Cells were then harvested after 4 h of incubation. The intensity of the luciferase activity measured in the lysates of the transient transfectants was normalized to their β-galactosidase activity, which was used as an internal control. Statistically significant inhibition of NF-κB activation ($^*p < 0.05$), as compared with group treated with LPS only. (B) RAW264.7 cells were treated with LPS (1 μg/ml) alone or in combination with indicated concentrations of SBF for 30 min. Nuclear extracts were prepared and analyzed for κB DNA binding using the electrophoretic mobility shift assay (EMSA). Competitive EMSA using an unlabeled NF-κB consensus sequence at 100-fold excess confirmed the specificity of NF-κB binding. Assays were repeated three times with a representative result shown.

4. Discussion

In this report, we have shown for the first time the novel bioactivity and mechanism of the immunomodulatory effect of the aqueous extract of sword brake fern (SBF; *P. ensiformis* Burm.), one of the most common ingredients of herbal beverages in Taiwan. It has been demonstrated that macrophages may play a role in antitumor activity in part due to the production of effector molecules such as NO and TNF-α (Hirazumi and Furusawa, 1999). These macrophage-derived mediators have been recognized for their cytostasis and/or cytotoxic properties against tumor cells (Stuehr and Nathan, 1989; Keller et al., 1990). In addition, IL-6 plays an essential role in the final differentiation of B-cells into plasma cells (Saggio et al., 1997) and functions as a late-acting killer helper factor in the differentiation of cytotoxic T-lymphocytes (CTL) (Okada et al., 1988). In this study, we demonstrated that SBF (0.05–0.25 mg/ml) is able to activate murine macrophages, RAW264.7 cells, resulting in increased release of TNF-α, IL-6, nitric oxide and PGE_2 production. The induction magnitudes of inflammatory mediator are consistently less than LPS, suggesting the possible beneficial effect of SBF on innate immunity.

Moderate level of inflammatory mediators is essential for host survival from infection, while overproduction has

deleterious effects. As a result, syntheses of inflammatory mediators must be tightly governed (Luheshi, 1988; Glauser, 1996). In the present study, we demonstrated that SBF (0.05–0.25 mg/ml) significantly decreased the production of TNF-α, IL-1β, IL-6, NO and PGE_2 in LPS-activated RAW264.7 cells in concentration-dependent manners. These activities were not attributable to cytotoxicity as assessed by the MTT assay. Reactive metabolites of NO, especially $ONOO^-$ induce oxidative stress and inactivate proteins through oxidation and nitrosylation (Fredstrom, 2002). It is conceivable that the observed activities of SBF can also reduce the oxidative stress during anti-inflammatory process.

Expression of the iNOS gene can be regulated at different levels (Nathan and Xie, 1994; Rao, 2000). A murine iNOS-promoter-mediated luciferase vector, which includes TNF-responsive elements, κB binding sites and nuclear factor-IL-6 binding sites, was constructed and transiently transfected into RAW264.7 cells (Lowenstein et al., 1993). We found that LPS-stimulated iNOS promoter-mediated luciferase activity was associated with the high-level production of NO. SBF addition at the same time as activation attenuated iNOS promoter activity and resulted in the repression of NO release in a concentration-dependent manner. This result suggests that iNOS gene expression is downregulated transcriptionally by SBF in LPS-activated macrophages.

Prostaglandin E_2 (PGE_2) is generated by the sequential metabolism of arachidonic acid by cyclooxygenase and prostaglandin E synthase (Needleman et al., 1986; Smith, 1992). We found that the COX-2 promoter was inducible by LPS in RAW264.7 cells, as a consequence of which PGE_2 production was enhanced. In contrast to what observed for the parallel inhibition on iNOS promoter activity and on NO release, SBF attenuated LPS-induced COX-2 promoter activity more effectively than PGE_2 production. This result indicates that SBF might exert adverse effects on COX-2 enzyme activity or other levels of COX-2 expression, such as post-transcriptional, translational or post-translational steps.

It is well known that the expressions of several genes involved in immune and inflammatory responses are regulated at the transcriptional level by the nuclear factor-κB (NF-κB) (Yamamoto and Gaynor, 2001). In transient transfection experiments, LPS-stimulated luciferase expression through κB-response elements was correlated with high-level activities of iNOS and COX-2 promoters in LPS-treated macrophages. Activation of luciferase expression was abolished by treating LPS-stimulated cells with SBF in a dose-dependent manner. Electrophoretic mobility shift assay (EMSA) also revealed that SBF concentration-dependently decreased κB DNA binding activity in LPS-activated RAW264.7 cells. This result provides evidence that SBF inhibits NF-κB activation, thereby inhibiting the induction of iNOS and COX-2 gene expression and proinflammatory cytokine synthesis.

In conclusion, this research provides the first evidence that SBF induces TNF-α, IL-6, NO and PGE_2 slightly in unstimulated macrophages and inhibits their production in a dose-dependent manner in LPS-activated macrophages. These effects may account for the immunomodulatory activities of SBF. It is also plausible that consumption of SBF may be able to enhance innate immunity, to reduce the oxidative stress from NO and to protect against cardiovascular and chronic inflammatory diseases.

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References

Barton, B.E., 1996. The biological effects of interleukin 6. Medicinal Research Review 6, 87–109.

Beckman, J.S., 1994. Peroxynitrite versus hydroxyl radical: the role of nitric oxide in superoxide-dependent cerebral injury. Annals of the New York Academy of Sciences 738, 69–75.

Beutler, B., Cerami, A., 1988. Tumor necrosis, cachexia, shock, and inflammation: a common mediator. Annual Review of Biochemistry 57, 505–518.

Carmichael, J., DeGraff, W.G., Gazdar, A.F., Minna, J.D., Mitchell, J.B., 1987. Evaluation of a tetrazolium-based colorimetric assay: assessment of chemosensitivity testing. Cancer Research 47, 936–945.

Chen, F., Castrnova, V., Shi, X., Dermers, L.M., 1999. New insights into the role of nuclear factor-kappaB, a ubiquitus transcription factor in the initiation of diseases. Clinical Chemistry 45, 7–17.

Chen, Z., Hagler, J., Palombella, V.J., Melandri, F., Scherer, D., Ballard, D., Maniatis, T., 1995. Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. Genes & Development 9, 1586–1597.

Cho, J.Y., Kim, A.R., Yoo, E.S., Baik, K.U., Park, M.H., 1999. Immunomodulatory effect of arctigenin, a lignan compound, on tumour necrosis factor-alpha and nitric oxide production, and lymphocyte proliferation. The Journal of Pharmacy and Pharmacology 51, 1267–1273.

Choi, C.Y., You, H.J., Jeong, H.G., 2001. Nitric oxide and tumor necrosis factor-alpha production by oleanolic acid via nuclear factor-kappaB activation in macrophages. Biochemical and Biophysical Research Communications 288, 49–55.

Dayer, J.M., Beutler, B., Cerami, A., 1985. Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E2 production by human synovial cells and dermal fibroblasts. The Journal of Experimental Medicine 162, 2163–2168.

Dinarello, C.A., 1998. Interleukin-1, interleukin-1 receptors and interleukin-1 receptor antagonist. International Reviews of Immunology 16, 457–499.

Dinarello, C.A., Cannon, J.G., Wolff, S.M., Bernheim, H.A., Beutler, B., Cerami, A., Figari, I.S., Palladino Jr., M.A., O'Connor, J.V., 1986. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. The Journal of Experimental Medicine 163, 1433–1450.

Fredstrom, S., 2002. Nitric oxide, oxidative stress, and dietary antioxidants. Nutrition 18, 537–539.

Glauser, M.P., 1996. The inflammatory cytokines: new developments in the pathophysiology and treatment of septic shock. Drugs 52 Supplement 2, 9–17.

Hirazumi, A., Furusawa, E., 1999. An immunomodulatory polysaccharide-rich substance from the fruit juice of *Morinda citrifolia* (noni) with antitumour activity. Phytotherapy Research 13, 380–387.

Keller, R., Keist, R., Wechsler, A., Leist, T.P., Van der Meide, P.H., 1990. Mechanisms of macrophage-mediated tumor cell killing: a comparative analysis of the roles of reactive nitrogen intermediates and tumor necrosis factor. International Journal of Cancer 46, 682–686.

Kilbourn, R.G., Griffith, O.W., 1992. Overproduction of nitric oxide in cytokine-mediated and septic shock. Journal of the National Cancer Institute 84, 827–831.

Lowenstein, C.J., Alley, E.W., Raval, P., Snowman, A.M., Snyder, S.H., Russell, S.W., Murphy, W.J., 1993. Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide. In: Proceedings of the National Academy of Sciences of the United States of America, vol. 90, pp. 9730–9734.

Luheshi, G.N., 1988. Cytokines and fever: mechanisms and sites of action. Annals of the New York Academy of Sciences 856, 83–89.

Matheis, G., Sheman, M.P., Buckberg, G.D., Haybron, D.M., Young, H.J., Ignarro, L.J., 1992. Role of L-arginine-nitric oxide pathway in myocardial reoxygenation injury. The American Journal of Physiology 262, H616–H620.

McDaniel, M.L., Kwon, G., Hill, J.R., Marshall, C.A., Corbett, J.A., 1996. Cytokines and nitric oxide in islet inflammation and diabetes. In: Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, NY) vol. 211, pp. 24–32.

Nathan, C., Xie, Q.W., 1994. Nitric oxide synthase: roles, tolls, and controls. Cell 78, 915–918.

Needleman, P., Turk, J., Jakschik, B., Morrison, A., Lefkowith, J., 1986. Arachidonic acid metabolism. Annual Review of Biochemistry 55, 69–102.

Nims, R.W., Cook, J.C., Krishna, M.C., Christodoulou, D., Poore, C.M., Miles, A.M., Grisham, M.B., Wink, D.A., 1996. Colorimetric assays for nitric oxide and nitrogen oxide species formed from nitric oxide stock solutions and donor compounds. Methods in Enzymology 268, 93–105.

Okada, M.M., Kitahara, S., Kishimoto, T., Matsuda, T., Hirano, T., Kishimoto, T., 1988. IL-6/BSF-2 functions as a killer helper factor in the in vitro induction of cytotoxic T cells. Journal of Immunology 141, 1543–1549.

Okusawa, S., Gelfand, J.A., Ikejima, T., Connolly, R.J., Dinarello, C.A., 1988. Interleukin 1 induces a shock-like state in rabbits: synergism with tumor necrosis factor and the effect of cyclooxygenase inhibition. The Journal of Clinical Investigation 81, 1162–1172.

Pang, L.H., Hoult, J.R.S., 1996. Induction of cyclooxygenase and nitric oxide synthase in endotoxin-activated J774 macrophages is differentially regulated by indomethacin: enhanced cyclooxygenase-2 protein expression but reduction of inducible nitric oxide synthase. European Journal of Pharmacology 317, 151–155.

Pang, L.H., Knox, A.J., 1997. Effect of interleukin-1β, tumour necrosis factor-α and interferon-γ on the induction of cyclo-oxygenase-2 in cultured human airway smooth muscle cells. British Journal of Pharmacology 121, 579–587.

Rao, K.M., 2000. Molecular mechanisms regulating iNOS expression in various cell types. Journal of Toxicology and Environmental Health Part B: Critical Reviews 3, 27–58.

Rees, D.D., Cellek, S., Palmer, R.M., Moncada, S., 1990. Dexamethasone prevents the induction by endotoxin of a nitric oxide synthase and the associated effects on vascular tone: an insight into endotoxin shock. Biochemical and Biophysical Research Communications 173, 541–547.

Saggio, I., Ciapponi, L., Savino, R., Ciliberto, G., Perricaudet, M., 1997. Adenovirus-mediated gene transfer of a human IL-6 antagonist. Gene Therapy 4, 839–845.

Smith, W., 1992. Prostanoid biosynthesis and mechanisms of action. American Journal of Physiology 263, F181–F191.

Stefanovic-Racic, S., Stadler, J., Evan, C.H., 1993. Nitric oxide and arthritis. Arthritis and Rheumatism 36, 1036–1044.

Stuehr, D.J., Nathan, C.F., 1989. Nitric oxide: a macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. The Journal of Experimental Medicine 169, 1543–1555.

Sun, Z., Andersson, R., 2002. NF-kappaB activation and inhibition: a review. Shock 18, 99–106.

Tracey, K.J., 1991. Tumor necrosis factor (cachectin) in the biology of septic shock syndrome. Circulatory Shock 35, 123–128.

Tracey, K.J., Wei, H., Manogue, K.R., Fong, Y., Hesse, D.G., Nguyen, H.T., Kuo, G.C., Beutler, B., Cotran, R.S., Cerami, A., Lowry, S.F., 1988. Cachectin/tumor necrosis factor induces cachexia, anemia, and inflammation. The Journal of Experimental Medicine 167, 1211–1227.

Vane, J.R., 1994. Pharmacology: towards a better aspirin. Nature 367, 215–216.

Wu, M.-J., Wang, L., Ding, H.Y., Weng, C.-Y., Yen, J.H., 2004. *Glossogyne tenuifolia* acts to inhibit inflammatory mediator production in a macrophage cell line by downregulating LPS-induced NF-kB. Journal of Biomedical Science 11, 186–199.

Xie, W.L., Chipman, J.G., Robertson, D.L., Erikson, R.L., Simmons, D.L., 1999. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. In: Proceedings of the National Academy of Sciences of the United States of America, vol. 88, pp. 2692–2696.

Yamamoto, Y., Gaynor, R.B., 2001. Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. The Journal of Clinical Investigation 107, 135–142.

Zingarelli, B., Sheehan, M., Wong, H., 2003. Nuclear factor-[kappa]B as a therapeutic target in critical care medicine. Critical Care Medicine 31, S105–S111.