Anti-Inflammatory Activity of Sugiol, A Diterpene Isolated from *Calocedrus formosana* Bark

Kuo-Ping Chao[1]
Kuo-Feng Hua[2]
Hsien-Yeh Hsu[2]
Yu-Chang Su[3]
Shang-Tzen Chang[4]

Abstract

Sugiol is a diterpene which was isolated and purified from alcohol extracts of the bark of *Calocedrus formosana* Florin (Cupressaceae). Although sugiol has low inhibitory activity against the DPPH radical, it could effectively reduce intracellular reactive oxygen species (ROS) production in lipopolysaccharide (LPS)-stimulated macrophages. The present study investigated the potential anti-inflammatory activity of sugiol, and the relationship between signal transduction and inflammatory cytokines *in vitro*. A dose of 30 μM of sugiol was effectively inhibitory for proIL-1β, IL-1β and TNF-α production, suggesting that sugiol is bioactive against inflammation. Moreover, sugiol reveals a capacity for suppressing the activation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38) activated by LPS-stimulation in J774A.1 murine macrophages. A low dosage of 10 μM of sugiol completely inhibited ERK1/2 phosphorylation, while 30 μM effectively inhibited JNK1/2 and p38 phosphorylation in LPS-stimulated macrophages. In addition, sugiol significantly inhibited LPS-induced ROS production. Our studies suggest that sugiol's efficacy in inhibiting the inflammatory cytokines of IL-1β and TNF-α could be attributed to a reduction of the ROS that leads to a decrease in the phosphorylation of MAPKs.

Key words
Calocedrus formosana · Cupressaceae · sugiol · anti-inflammatory activity · TNF-α · IL-1β · MAPKs

Abbreviations

LPS:	lipopolysaccharide
ROS:	reactive oxygen species
MAPK:	mitogen-activated protein kinase
ERK:	extracellular signal-regulated kinase
JNK:	C-Jun NH_2-terminal protein kinase
p38:	p38 mitogen-activated protein kinase

Introduction

Calocedrus formosana Florin (Cupressaceae) is an endemic tree that grows at elevations of 800 – 1500 meters in Taiwan's central mountains. Sugiol is abundant in the bark of *C. formosana*, but in the forestry industry the bark has always been discarded. Many studies have investigated the bioactivity of sugiol, including a strong antitumor activity on EBV-EA induction [1], and weak antimicrobial and aldose reductase (AR) inhibitory activities [2], [3]. Many natural products exhibiting a good anti-inflammation

Affiliation
[1] Department of Biological Science and Technology, Chung Hwa College of Medical Technology, Tainan, Taiwan
[2] Faculty of Medical Technology, Institute of Biotechnology in Medicine, National Yang-Ming University, Taipei, Taiwan
[3] Division of Wood Cellulose, Taiwan Forestry Research Institute, Taipei, Taiwan
[4] School of Forestry and Resource Conservation, National Taiwan University, Taipei, Taiwan

Correspondence
Dr. Shang-Tzen Chang · School of Forestry and Resource Conservation · National Taiwan University · No. 1, Section 4 Roosevelt Road · Taipei 106 · Taiwan · Republic of China · Phone: +886-2-236-30231-3196 · Fax: +886-2-236-54520 · E-mail: peter@ntu.edu.tw

Received June 22, 2004 · **Accepted** November 15, 2004

Bibliography
Planta Med 2005; 71: 300–305 · © Georg Thieme Verlag KG Stuttgart · New York
DOI 10.1055/s-2005-864094
ISSN 0032-0943

capacity have been isolated from plants, but there have been few discussions about their molecular mechanism. Results from our preliminary study demonstrated that the *n*-hexane fractions of alcoholic extracts from *C. formosana*, from which large amounts of sugiol were isolated, exhibited a significant anti-inflammatory capacity (unpublished results). In this study, we have isolated sugiol (Fig. **1**) and investigated its biological functions, including antioxidant and anti-inflammatory activities in macrophages.

Innate immunity is triggered by pathogen-associated molecular patterns shared by groups of microbial pathogens, which can be recognized by pattern recognition receptors in host cells [4]. The lipopolysaccharide (LPS), or endotoxin, represents a well-known pathogen-associated molecular pattern, localized on Gram-negative bacteria cell walls [5]. LPS activates macrophages by binding to toll-like receptor 4 and stimulates the production of inflammatory cytokines, including TNF-α and IL-1β proteins. While mediation of inflammation against pathogen infection by TNF-α and IL-1β proteins could be beneficial to the host, overexpression of these cytokines may cause serious disease, including septic shock. Hence, suppression of TNF-α and IL-1β protein production could aid in the treatment of septic shock.

It is well known that the mitogen-activated protein kinases (MAPKs) play an important role in regulating cell growth and survival, including mitogenic and stress responses. Our recent study revealed that LPS stimulated reactive oxygen species (ROS) production, MAPKs activation, and proIL-1β/IL-1β protein expression in murine macrophages [6]. This prompted us to investigate whether sugiol affects LPS-induced signal transduction in the regulation of cytokine production. Herein we present evidence that sugiol inhibited LPS-induced TNF-α and IL-1β expression as well as ROS and MAPKs activation in macrophages. This is the first report to demonstrate that sugiol has an anti-inflammatory activity in macrophages.

Materials and Methods

Materials

The samples of *C. formosana* were collected in September 2003 from the Lien Hua-Chin Research Center located in Nantou County in central Taiwan. The species was identified by Mr. Yen-Ray Hsui of the Taiwan Forestry Research Institute, and voucher specimens (reg. #WCCL-04-B006) were deposited at the laboratory of wood chemistry (School of Forestry and Resource Conservation, National Taiwan University). Murine macrophage J774A.1 cells were obtained from ATCC (Rockville, MD); LPS (from *Escherichia coli* 0111:B4), SB203580, curcumin (> 95%), monoclonal anti-MAP kinase, activated (diphosphorylated ERK1/2) antibody, monoclonal anti-JNK kinase, activated (diphosphorylated JNK) antibody, and monoclonal anti-p38 MAP kinase, activated (diphosphorylated p38) antibody were obtained from Sigma Co. Mouse TNF-α and IL-1β enzyme-linked immunosorbent assay (ELISA) kits were purchased from R & D Systems, Inc. (Minneapolis, MN). Anti-IL-1β polyclonal antibody, anti-ERK1 polyclonal antibody, anti-JNK1 polyclonal antibody, anti-p38 polyclonal antibody, anti-rabbit IgG-HRP, and anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The other chemicals and solvents used in this study were of the highest quality available.

Extraction and purification

The bark particles of *C. formosana* (10 kg d. w.) were treated with ethanol (95% v/v, 10 days repeated 3 times) at room temperature, and then the extracts were concentrated to give the alcoholic extracts (AE; ca. 410 g). 205 g of AE were successively extracted with *n*-C_6H_{14}, CH_2Cl_2, EtOAc, BuOH and H_2O, which then yielded about 64.8 g of *n*-C_6H_{14}-soluble fraction. The *n*-hexane fraction was applied on the top of a 160 g silica gel column, then eluted with *n*-hexane/EtOAc (95/5, 90/10, 85/15, 80/20, 75/25, 70/30, 60/40, 40/60, 20/80, 0/100) followed by elution with EtOAc/ethanol (100/0, 90/10, 80/20, 70/30, 60/40, 50/50, 40/60, 20/80, 0/100) to give 22 subfractions. Each eluted volume of subfraction was 1000 mL, except for the H1 subfraction, which was 4000 mL. Each collected subfraction was rotary evaporated at 50 °C to dryness, resulting in a white precipitate (678 mg) from subfraction H5. Recrystallization of this precipitate with ethanol gave sugiol which was then purified by semi-preparative HPLC (Hitachi L-7100 pump, a UV detector model Jasco UV 975, l = 254 nm) on a Zorbax SIL column (25 cm in length, 4.6 mm i.d., 5.0 μm). The pure sugiol (> 98%) was obtained at t_R = 5.14 min. The separation conditions were as follows: flow rate, 2 mL/min; mobile phase, EtOAc/EtOH = 1/1. The structure of sugiol was confirmed by comparison of its physical and spectral data (optical rotation, EI-MS, ^{1}H-NMR) with previously reported values [7]. The optical rotation was determined using a Horiva STA-300 polarimeter. $[\alpha]_D^{25}$: + 28.3° (*c* 1.0, $CHCl_3$). The NMR spectra were recorded on a Bruker Avance-300 MHz FT-NMR. ^{1}H-NMR ($CDCl_3$): δ = 0.99 (3H, s, H-18), 1.00 (3H, s, H-19), 1.19 (3H, s, H-20), 1.20 (3H, d, *J* = 7.0 Hz, H-16), 1.24 (3H, d, *J* = 7.0 Hz, H-17), 1.83 (dd, *J* = 4.5, 13.0 Hz, H-5), 2.18 (dd, *J* = 13.0, 18.0 Hz, H-6β), 2.62 (1H, dd, *J* = 4.5, 1.8 Hz, H-6α), 3.13 (1H, sept, *J* = 7.0 Hz, H-15), 6.68 (1H, s, H-11), 7.89 (1H, s, H-14). The mass spectra (MS) were obtained on a Finnigan MAT-95S mass spectrometer. EI-MS: *m/z* calcd. for $C_{20}H_{28}O_2$: 300; found: 300.

Cell culture

The J774A.1 cells were propagated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine (Life Technologies, Inc., MD) and cultured in a 37 °C, 5% CO_2 incubator. Sugiol was dissolved in dimethyl sulfoxide (DMSO) and the final DMSO concentration was 0.1% in all cultures containing this agent; the same amount of vehicle was added to the control cultures.

MTT assay for cell viability

The cytotoxicity of sugiol was assessed using the microculture tetrazolium (MTT) assay [8]. After culturing on 96 well plates for 24 h, the cells were washed twice and incubated with 100 μL of 1 mg/mL of MTT for 2 h at 37 °C. The medium was discarded and 100 μL lysis buffer were then added. After 30 min incubation, the absorbance was measured at 570 nm using a microplate reader.

Measurement of cytokine production

To investigate the inhibitory effect of sugiol on TNF-α and proIL-1β/IL-1β protein production from LPS-stimulated J774A.1 cells, the cells were pretreated with sugiol (5 to 30 μM) for 30 min at

37 °C, followed by LPS (1 μg/mL) treatment for the indicated times. The levels of TNF-α and IL-1β protein in supernatants and proIL-1β protein in cell lysates were quantified by ELISA and by Western blot, respectively [6]. Histograms represent quantification by PhosphorImager® of proIL-1β in J774A.1 cell samples with ImageQuaNT® software from Molecular Dynamics.

Measurement of MAPK activation

To investigate the inhibitory effect of sugiol on MAPK activation in LPS-stimulated J774A.1, the cells were pretreated with sugiol (5 to 30 μM) for 30 min at 37 °C, followed by LPS (1 μg/mL) treatment for 15 min. The phosphorylation levels of ERK1/2, JNK1/2, and p38 were quantified by Western blot with antibody against diphosphorylated ERK1/2, antibody against diphosphorylated JNK, and antibody against diphosphorylated p38, respectively [6]. Histograms represent quantification by PhosphorImager® of phospho-ERK1/2, phospho-JNK1/2, and phospho-p38 in J774A.1 cell samples with ImageQuaNT® software from Molecular Dynamics.

Measurement of intracellular ROS production

Intracellular ROS stimulated by LPS was measured by detecting the fluorescent intensity of either 2′,7′-dichlorofluorescein diacetate (DCFH) or the improved analogue carboxyl-DCFH (CM-DCFH) (Molecular Probes, Inc., Eugene, OR) oxidized product, DCF (or CM-DCF), as described [9]. Briefly, 0.5 – 1.0 × 10^6 J774A.1 cells/mL were grown in serum- and phenol red-free RPMI medium (starvation medium) for 24 h, and then were preincubated with 2 μM CM-DCFH, and sugiol or 10 mM NAC, at 37 °C for 30 min in the dark. To these were added fresh starvation medium containing LPS for additional incubation for the indicated times. The relative fluorescent intensity of the fluorophore CM-DCF, which was formed by peroxide oxidation of the non-fluorescent precursor, was detected at an excitation wavelength of 485 nm and an emission wavelength of 530 nm with a fluorometer, Cytofluor 2300 (Millipore Inc., Bedford, MA). CM-DCFH with starvation medium was used as a blank control [6].

DPPH (1,1-diphenyl-2-picrylhydrazyl) assay

Sugiol's reductive activities on the DPPH assay were measured as follows. The reaction mixture contained 1000 μL of 0.1 mM DPPH-ethanol solution, 450 μL of 0.05 M Tris-HCl buffer (pH 7.4), and 50 μL of test samples or control (ethanol). After incubation for 30 min, the reduction in DPPH was measured by reading the optical absorption at 517 nm [10]. Curcumin was used as a positive reference in this experiment [11]. The inhibition ratio (%) was calculated using the following equation: % Inhibition = [(absorbance of control – absorbance of test sample)/absorbance of control] × 100.

Statistical analysis

Statistical differences between the experimental groups were examined by analysis of variance, and statistical significance was determined at $p < 0.05$. The experiments were conducted three times, or as indicated, and all data are expressed as mean ± S.D.

Results

Sugiol (> 98 % pure), colorless crystals, was purified from the precipitates of subfraction H5 and the yield was about 50 mg. Fig. **1** shows the structure of sugiol, and it was characterized as described previously [7].

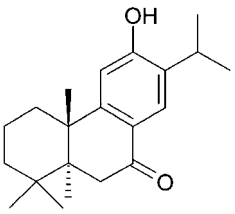

Fig. **1** Chemical structure of sugiol from *C. formosana*.

To investigate whether sugiol exhibits immunomodulation activity in macrophages, J774A.1 cells were pretreated with the indicated concentrations of sugiol for 30 min and challenged with LPS for 6 h. As shown in Fig. **2A**, bioactive TNF-α protein was secreted from J774A.1 cells in response to LPS challenge as measured by ELISA. Interestingly, LPS-induced TNF-α secretion was dramatically reduced in sugiol pretreated macrophages. Specifically, 60 ng/mL TNF-α was secreted from LPS-stimulated cells, and LPS-induced TNF-α secretion was reduced to 30 and 20 ng/mL by 20 and 30 μM sugiol, respectively. SB203580 (positive control), a pharmacological antagonist that inhibits the activation of p38, had a significant effect on TNF-α secretion [12]. Furthermore, no cytotoxic effect was observed after cells were treated with sugiol plus LPS for 24 h as measured by MTT assay (data not shown). These results indicate that the inhibition of TNF-α secretion by sugiol is not due to cell death. In addition, cells were pretreated with sugiol for 30 min, followed by LPS challenge for 24 h. As shown in Fig. **2B**, sugiol showed a dose-dependent inhibitory effect on LPS-induced IL-1β secretion. We found that 135 pg/mL IL-1β protein was secreted by LPS-stimulated cells, and by using 5, 10, 20, and 30 μM sugiol, this was reduced to 90, 85, 60, and 45 pg/mL respectively. 1 μM SB20380 reduced LPS-induced IL-1β protein secretion to 40 pg/mL.

The molecular mechanism of IL-1β protein secretion was then investigated by detection of proIL-1β protein (IL-1β precursor, 34 kD) expression via Western blotting analysis. As shown in Fig. **3**, incubation of J774A.1 with LPS for 6 h significantly increased the expression of proIL-1β protein 5-fold compared with untreated control cells. Sugiol, at concentrations of 20 and 30 μM, reduced LPS-induced proIL-1β protein expression 4- and 2.5-fold, respectively. In addition, SB203580 reduced LPS-induced proIL-1β expression 1.8-fold at 1 μM.

Our previous study demonstrated that MAPKs play important roles in the regulation of proIL-1β/IL-1β expression in LPS-stimulated J774A.1 cells [6]. Therefore, we investigated whether LPS-mediated activation of one or more of these MAPKs is altered in the J774A.1 macrophages pre-exposed to sugiol. As demonstrated in Fig. **4**, LPS strongly induced activation of ERK1/2, JNK1/2, and p38 in J774A.1 cells. Interestingly, LPS-mediated activation of ERK1/2, JNK1/2, and p38 was greatly inhibited in the J774A.1 cells previously exposed to sugiol. Specifically, LPS-induced ERK1/2 phosphorylation, and sugiol showed a significant inhibitory effect on ERK1/2 phosphorylation at concentrations above 10 μM, yet SB203580 had no observed effect. In addition, the LPS-induced phosphorylated JNK1/2 was significantly inhibited by sugiol concentrations ranging from 10 to 30 μM, and comple-

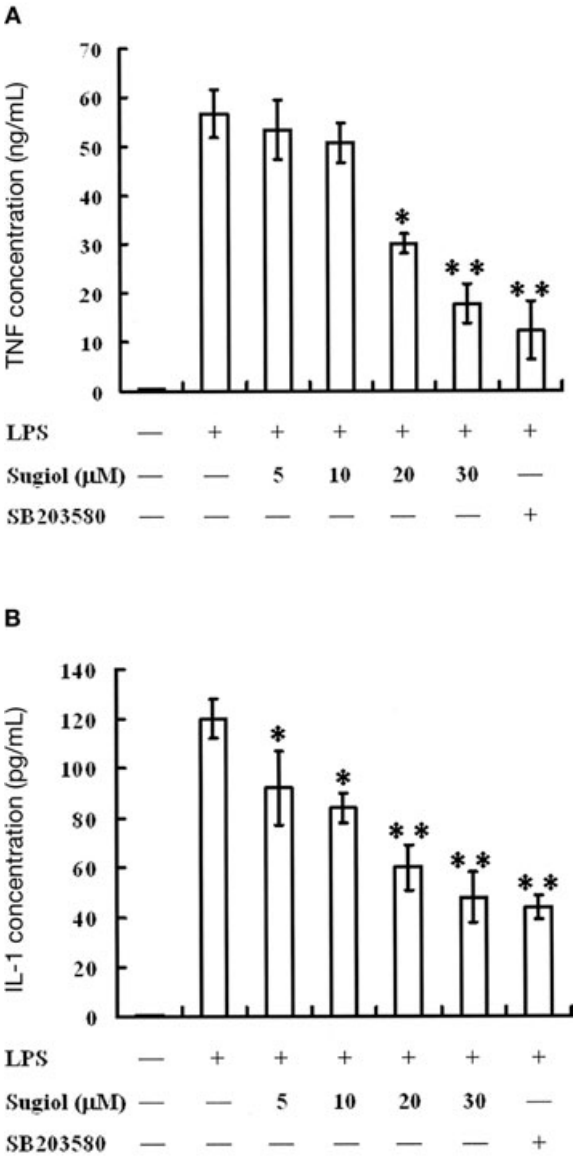

Fig. **2** Sugiol inhibits TNF-α and IL-1β protein expression in LPS-stimulated macrophages. J774A.1 cells were pretreated with the indicated concentration of sugiol for 30 min prior to incubation with 1 μg/mL LPS for indicated time. Culture media were assayed by using a specific ELISA. **A** Dose-dependent incubation of sugiol on LPS-induced TNF-α. **B** Dose-dependent incubation of sugiol on LPS-induced IL-1β. SB203580 (1 μM) was used as a positive control. The error bar for all experiments was mean ± SD (n = 3).

tely inhibited at 30 μM. Finally, sugiol, at concentrations above 20 μM, reduced LPS-induced p38 phosphorylation to the basal level, and SB203580 also inhibited p38 phosphorylation. These results indicate that sugiol inhibits LPS-induced MAPKs activation in a concentration-dependent manner.

To investigate the antioxidant activity of sugiol, DPPH and intracellular ROS production assays were conducted. We found that at dosages over 100 μg/mL sugiol had the lowest inhibitory activity against the DPPH radical, where inhibition was always below 10%, (Fig. **5A**). The inhibitory activity of curcumin was better than that of sugiol. Furthermore, LPS stimulation of cells rapidly induced significant ROS when compared with that of LPS-untreated control samples. In contrast, pretreatment with NAC (10 mM), a potent antioxidant, quickly reduced the release of LPS-induced ROS. Interestingly, we found that cells pretreated with sugiol significantly reduced LPS-stimulated ROS production in the initial 30 min, falling at almost constant values (Fig. **5B**).

Discussion

Pathogen infection causes significant mortality mediated by the up-regulation of inflammatory cytokines (i.e., TNF-α and IL-1β) or by other factors. The inflammatory cytokine TNF-α, in particular, plays a complex and central role in responses to infection [13]. Sugiol is a diterpene, which abounds in woody plants [7], [14]. Our results demonstrate that sugiol inhibited the production of TNF-α and IL-1β protein in LPS-stimulated macrophages. A number of studies show that natural products in herbal medicines can have a remarkable effect on anti-inflammation. For instance, the methanolic extracts of *Cordyceps pruinosa* have been proven to have a strong inhibitory capacity on LPS-induced TNF and IL-1β expression in murine macrophage cell line RAW 264.7 [15]. A similar inhibitory capacity has been observed in the diterpene tanshinone IIA that was isolated from *Salvia miltiorrhiza* [16]. In this study, we found that sugiol could effectively inhibit IL-1β and TNF-α proteins expression in LPS-stimulated J774A.1 cells in a dose-dependent manner (Figs. **2A** and **B**). Our results suggest that the inhibitory capacity of sugiol in LPS-induced TNF-α and IL-1β expression may offer an advantage in protecting the host from endotoxic shock. However, the effect of sugiol on LPS-induced cytokines production *in vivo* needs further investigation.

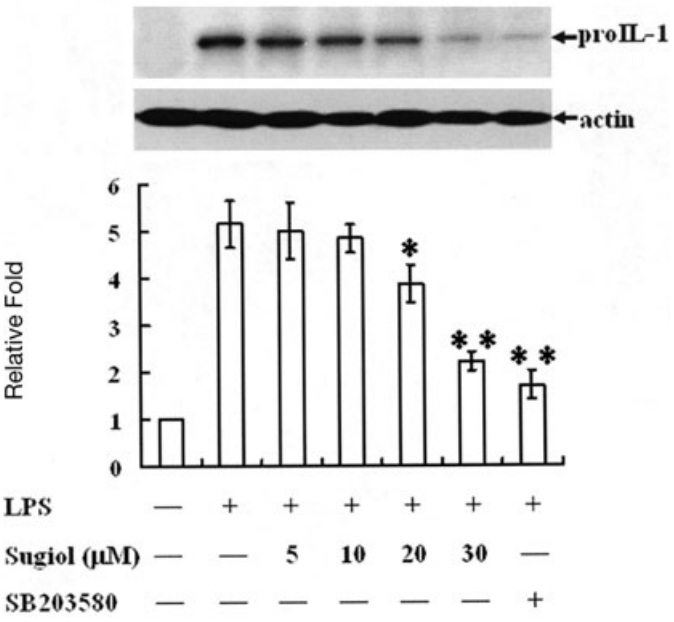

Fig. **3** Sugiol inhibits proIL-1β protein expression in LPS-stimulated macrophages. J774A.1 cells were pretreated with the indicated concentration of sugiol for 30 min prior to incubation with 1 μg/mL LPS for 6 h. ProIL-1β protein expression level was analyzed by Western blot. The histogram represents quantification by PhosphorImager® of LPS-stimulated proIL-1β in J774A.1 cell sample using ImageQuaNT® software from Molecular Dynamics. SB203580 (1 μM) was used as a positive control. One of four experiments is presented. * $p < 0.05$; ** $p < 0.01$ versus LPS alone.

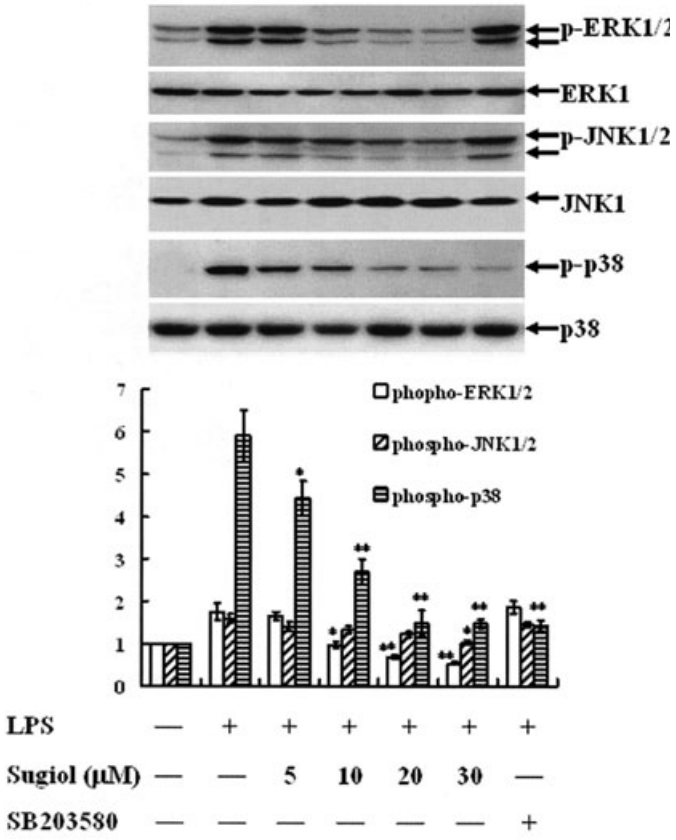

Fig. **4** Sugiol inhibits MAPKs activation in LPS-stimulated macrophages. J774A.1 cells were pretreated with the indicated concentration of sugiol for 30 min prior to incubation with 1 μg/mL LPS for 15 min. The phosphorylation levels of ERK1/2, JNK1/2, and p38 were analyzed by Western blot with anti-diphosphorylated ERK1/2, anti-diphosphorylated JNK1/2 or anti-diphosphorylated p38 monoclonal antibody, respectively. The histogram represents quantification by PhosphorImager® of LPS-stimulated ERK1/2, JNK1/2 and p38 phosphorylation in J774A.1 cell sample using ImageQuaNT® software from Molecular Dynamics. SB203580 (1 μM) was used as a positive control. One of three experiments is presented. * $p < 0.05$; ** $p < 0.01$ versus LPS alone.

Next, to assess whether the inhibitory effect on IL-1β protein production was regulated by transcriptional regulation, we used Western blotting to detect proIL-1β (IL-1β precursor) protein expression. The inhibitory effect of sugiol on LPS-induced proIL-1β protein expression was dose-dependent and required more than 20 μM for a significant inhibitory effect (Fig. **3**). However, sugiol at a concentration of 5 μM showed a significant inhibitory effect on IL-1β protein secretion (Fig. **2B**). These results suggest that sugiol inhibits LPS-induced IL-1β secretion at the transcriptional level, and perhaps at the post-transcriptional, interleukin-1 converting enzyme-catalyzed level [6].

Nevertheless, our results show that sugiol has a low antioxidant ability that is not consistent in different bioassays (Fig. **5A** and Fig. **5B**). In a tissue culture system, sugiol displayed a strong anti-oxidation activity as analyzed by CM-DCF. However, in a cell-free system we observed no significant anti-oxidation activity of sugiol as analyzed by DPPH. This result indicates that if a DPPH assay system is used, we may overlook some biologically significant anti-oxidation components. Sugiol's bioactivity is comparable with that of NAC at 10 mM, which is an inhibitor of ROS [6]. This illustrates that the bioactivity of sugiol antioxidant analysis *ex vivo* is more distinct than in the cell-free model.

To gain further insights into mechanisms of sugiol-mediated inhibition of LPS-induced TNF-α and proIL-1β/IL-1β protein expression, we then examined the intracellular signaling molecules involved in the LPS signaling pathways in sugiol-treated macrophages. By utilizing certain specific pharmacological antagonists such as PD98059, SP600125, and SB203580 that inhibit the activation of MEK1, JNK, and p38, respectively, we have demonstrated that MAPKs, including ERK1/2, JNK1/2, and p38 play important roles in LPS-induced proIL-1β/IL-1β protein expression [6]. This prompted us to investigate whether sugiol affects LPS-induced MAPKs activation. The results showed that sugiol inhibited ERK1/2, JNK1/2, and p38 phosphorylation in LPS-stimulated macrophages. Hence, we suggest that the inhibitory effect

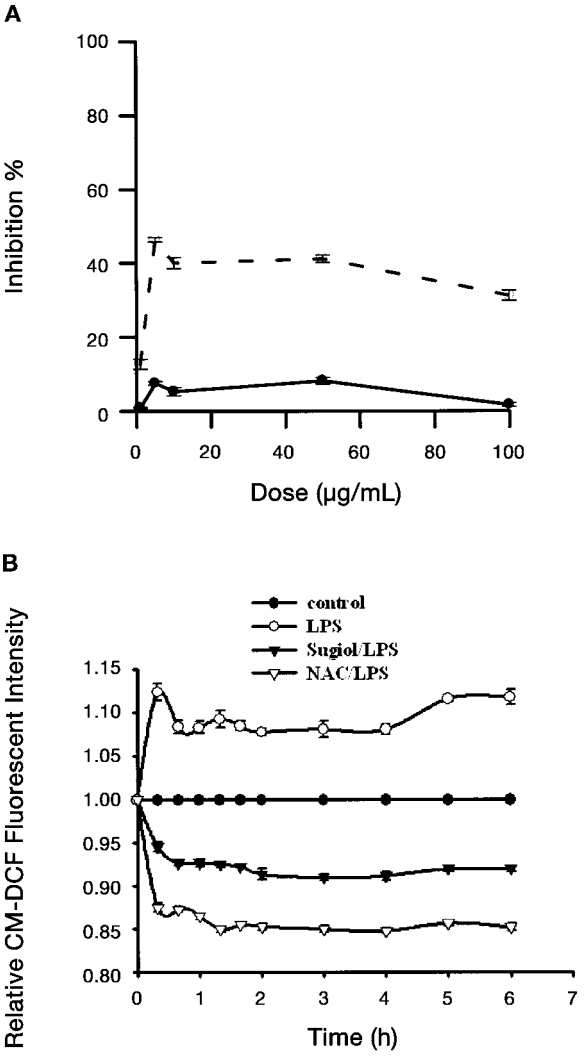

Fig. **5** Antioxidant activity of sugiol. **A** Free-radical scavenging activity of sugiol measured using the DPPH assay: (○) curcumin; (●) sugiol. Results are mean ± SD (n = 3). **B** Reduced intracellular ROS capacity of sugiol in LPS-stimulated macrophages. J774A.1 cells were pre-incubated with CM-DCFH (2 μM) and sugiol or 10 mM NAC for 30 min, followed by substitution with medium. Cells were treated with 1 μg/mL LPS for the indicated time points. The relative fluorescence intensity of fluorophore CM-DCF was detected as described in Materials and Methods. The data are representative experiments (n = 6).

of sugiol on LPS-induced cytokines production may result from the inhibition of MAPKs activation.

According to our previous studies, LPS activated all three MAPKs including ERK1/2, JNK1/2 and p38 in J774A.1 cells within 15 – 30 min [6]. Sugiol, unlike other specific MAPK inhibitors, has a broad inhibitory activity on ERK1/2, JNK1/2, and p38. This led us to investigate whether sugiol affected the signal molecules upstream of MAPKs. In a previous study [6], NAC, an ROS scavenger, inhibited LPS-induced MAPKs activation as well as proIL-1β/IL-1β expression, which indicated that ROS was upstream of MAPKs and regulated proIL-1β/IL-1β expression in LPS-stimulated macrophages [6]. In the present study, we found that sugiol significantly inhibited by ROS production in LPS-stimulated macrophages. Our experiments showed that the pretreatment of macrophages with sugiol effectively decreased ROS production and prevented LPS-induced production of the three MAPKs as well as proIL-1/IL-1 expression. Our results indicate that sugiol-mediated inhibition of TNF-α and proIL-1β/IL-1β protein expression and MAPKs phosphorylation may be, at least in part, due to the scavenger activity of ROS [17].

Acknowledgements

This study was supported by a grant (NSC-92-2313-B-002-042) from the National Science Council of Taiwan.

References

[1] Iwamoto M, Minami T, Tokuda H, Ohtsu H, Tanaka R. Potential antitumor promoting diterpenoids from the stem bark of *Thuja standishii*. Planta Medica 2003; 69: 69 – 72

[2] Tezuka Y, Kasimu R, Basnet P, Namba T, Kadota S. Aldose reductase inhibitory constituents of the root of *Salvia miltiorrhiza* Bunge. Chem Pharm Bull 1997; 45: 1306 – 11

[3] Politi M, Braca A, Tommasi ND, Morelli I, Manunta A, Battinelli L, Maxxanti G. Antimicrobial diterpenes from the seeds of *Cephalotaxus harringtonia* var. *drupacea*. Planta Medica 2003; 69: 468 – 70

[4] Medzhitov R, Janeway CA. Innate immunity: the virtues of a nonclonal system of recognition. Cell 1997; 91: 295 – 8

[5] Christian RH, Chris Whitfield. Lipolysaccharide endotoxins. Annu Rev Biochem 2002; 71: 635 – 700

[6] Hsu HY, Wen MH. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. J Biol Chem 2002; 277: 22131 – 9

[7] Wang SY, Wu JH, Shyur LF, Kuo YH, Chang ST. Antioxidant activity of abietane-type diterpenes from *Taiwania crytomerioides* Hayata. Holzforschung 2002; 56: 487 – 92

[8] Wang YY, Khoo KH, Chen ST, Lin CC, Wong CH, Lin CH. Studies on the immuno-modulating and antitumor activities of *Ganoderma lucidum* (Reishi) polysaccharides: functional and proteomic analyses of a fucose-containing glycoprotein fraction responsible for the activities. Bioorg Med Chem 2002; 10: 1057 – 62

[9] Wan CP, Myung E, Lau BH. An automated micro-fluorometric assay for monitoring oxidative burst activity of phagocytes. J Immunol Methods 1993; 159: 131 – 8

[10] Wang SY, Wu JH, Cheng SS, Lo CP, Chang HN, Shyur , Chang ST. Antioxidant activity of extracts from *Calocedrus formosana* leaf, bark and heartwood. J. Wood Sci 2004; 50: 422 – 6

[11] Priyadarsini KI, Maity DK, Naik GH, Kumar MS, Unnikrishnan MK, Satav JG, Mohan H. Role of phenolic O-H and methylene hydrogen on the free radical reactions and antioxidant activity of curcumin. Free Radic Biol Med. 2003; 35: 475 – 84

[12] Ropert C, Almeida IC, Closel M, Travassos LR, Ferguson MA, Cohen P, Gazzinelli RT. Requirement of mitogen-activated protein kinases and I kappa B phosphorylation for induction of proinflammatory cytokines synthesis by macrophages indicates functional similarity of receptors triggered by glycosylphosphatidylinositol anchors from parasitic protozoa and bacterial lipopolysaccharide. J Immunol 2001; 166: 3423 – 31

[13] Ware CF. The TNF superfamily. Cytokine Growth Factor Rev 2003; 14: 81 – 4

[14] Ulubelen A, Topçu G, Sönmez U, Choudhary MI, Atta-Ur-Rahman . Abietane diterpenes from *Salvia napifolia*. Phytochemistry 1995; 40: 861 – 4

[15] Kim KM, Kwon YG, Chung HT, Yun YG, Pae HO, Han JA, Ha KS, Kim TW, Kim YM. Methanol extracts of *Cordyceps pruinosa* inhibits *in vitro* and *in vivo* inflammatory mediators by suppressing NF-κB activation. Toxicology and Applied Pharmacology 2003; 190: 1 – 8

[16] Jang SI, Jeong SI, Kim KJ, Kim HJ, Yu HH, Park R, Kim HM, You YO. Tanshinone IIA from *Salvia miltiorrhiza* inhibits inducible nitric oxide synthase expression and production of TNF-α, IL-1β and IL-6 protein in activated raw 264.7 cells. Planta Medica 2003; 69: 1057 – 9

[17] Haddad JJ, Land SC. Redox/ROS regulation of lipopolysaccharide-induced mitogen-activated protein kinase (MAPK) activation and MAPK-mediated TNF-alpha biosynthesis. Br J Pharmacol 2002; 135: 520 – 36