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Hepatoprotective phytocompounds from *Cryptomeria japonica* are potent modulators of inflammatory mediators

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Abstract

Cryptomeria japonica is an important plantation conifer tree in Asia. This study aimed to characterize the anti-inflammatory and hepatoprotective activities of the phytocompounds from *C. japonica* wood on LPS- or TPA-induced activation of proinflammatory mediators and CCl_4-induced acute liver injury in mice. A CJH7-2 fraction was purified from *C. japonica* extracts following bioactivity-guided fractionation, and it exhibited significant activities on inhibition of NO production and iNOS expression as well as up-regulating HO-1 expression in LPS-stimulated macrophages. CJH7-2 also potently inhibits COX-2 enzymatic activity ($IC_{50} = 5$ μg/mL) and TPA-induced COX-2 protein expression in mouse skin (1 mg/200 μL/site). CJH7-2 (10 mg/kg BW) can prevent CCl_4-induced liver injury and aminotransferases activities in mice. Chemical fingerprinting analysis showed that terpenes are the major bioactive compounds in the CJH7-2 fraction. This is the first study to demonstrate that chemical constituents from the wood extract of *C. japonica* possess anti-inflammatory activities *in vitro* and *in vivo* that may play a role in hepatoprotection.

Keywords: *Cryptomeria japonica*; Taxodiaceae; Hepatoprotection; Cycloxygenase-2; Nitric oxide; Inducible nitric oxide synthase; Heme oxygenase-1; Terpenoids

1. Introduction

Recently, there has been much interest in the role of improper activation or up-regulation of iNOS or COX-2 in the pathogenesis of inflammatory disorders, including toxin-induced liver damage (Chen et al., 2004; Tipoe et al., 2006). The iNOS-catalyzed oxidative deamination of L-arginine to produce NO following exposure to pro-inflammatory cytokines (e.g., TNF) or endotoxins (e.g., lipopolysaccharide, LPS), could trigger disadvantageous cellular responses and may result in inflammation and sepsis (Bultinck et al., 2006). COX-2 is another important inflammatory mediator through its rate-limiting synthesis of the precursors of prostaglandins and thromboxanes (Serhan and Oliw, 2001). Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin and celecoxib, which inhibit COX-2 activity, are associated with reduced incidence of various cancers (Davies et al., 2002; Marnett and DuBois, 2002). Moreover, homozygous deletion of the COX-2 gene in mice reduces hepatocellular toxicity caused by LPS administration (Dinchuk et al., 1995). Heme oxygenase (HO) is the rate-limiting enzyme in the catabolism of heme into biliverdin, free iron, and carbon monoxide. HO-1, one of the HO isoforms, is an inducible stress-responsive protein with important cytoprotective effects (e.g., hepatoprotection) against oxidative stress and inflammation (Yao et al., 2007; Lee and Chau, 2002).

Plants are a good source of useful hepatoprotective agents that can modulate the activities of free radicals,

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iNOS, COX-2, and/or HO-1 (Yao et al., 2007). Silymarin, a mixture of flavonolignanes from milk thistle (*Silybum marianum* L.), is a hepatoprotective herbal medicine worth $180 million dollars of business annually in Germany alone (Pradhan and Girish, 2006), with potent antioxidative, anti-inflammatory, and immunomodulatory activities against liver disease in various animal models (Crocenzi and Roma, 2006; Hoofnagle, 2005). The continuing search for novel hepatoprotective phytocompounds, especially from plants with historical or anecdotal pharmacological properties, holds exciting nutraceutical or pharmaceutical promise.

Cryptomeria japonica D. Don (Taxodiaceae), a widely distributed conifer known as "sugi" in Japanese, is an important plantation tree species in Taiwan (Cheng et al., 2005). *C. japonica* has been used as a building material for Japanese-style houses and also used for ceiling board, wall paneling, etc. In addition to its industrial and agricultural importance, terpenoids and essential oils isolated from different tissues of *C. japonica* have proven to possess antibacterial, antifungal, and termiticidal properties (Cheng et al., 2005, 2007). Some sesquiterpenes or diterpenes isolated from *C. japonica* possess anti-cancer and immunomodulatory properties *in vitro* (Yoshikawa et al., 2006; Takei et al., 2005, 2006). *Cis*-communic acid was the first compound from *C. japonica* leaf extracts reportedly exhibiting anti-inflammatory activity against carrageenan-induced paw edema in rats (Shimizu et al., 1988). This is the first study to demonstrate that the phytocompounds (**1**–**3**) (Fig. 1) from wood extracts of *C. japonica* posses potent anti-inflammatory and hepatoprotective activities as investigated using *in vitro* cell- and gene-based assays in macrophages or cancer cells and *in vivo* in a mitogen-induced mouse skin inflammation system and in a CCl_4-induced mouse liver injury model.

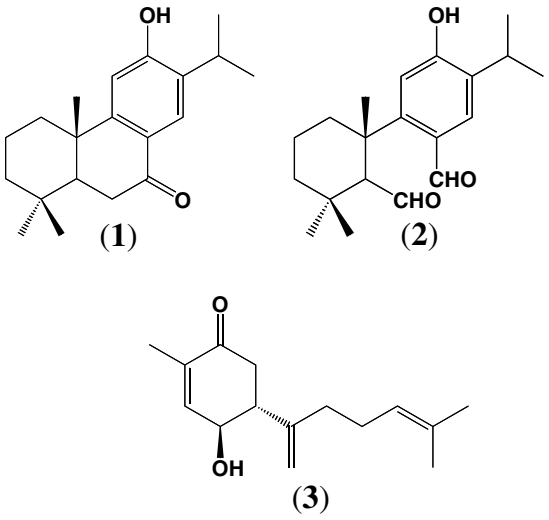

Fig. 1. Chemical structures of the three major bioactive terpenoids, sugiol (**1**), 12-hydroxy-6,7-secoabieta-8,11,13-triene-6,7-dial (**2**), and (1S,6R)-2,7(14),10-bisabolatrien-1-ol-4-one (**3**), identified from the heartwood extract of *C. japonica*.

2. Results and discussion

2.1. Bioactivity-guided fractionation of C. japonica extracts

Activation of macrophages is critical to inflammatory processes by their release of a variety of inflammatory mediators, such as NO (Zhuang et al., 1998). NO is important in inflammatory processes of the liver, such as septic shock, hepatocarcinoma, and autoimmune diseases. iNOS is an important enzyme mediator of inflammatory processes associated with the pathophysiology of many diseases and inflammatory disorders (Tipoe et al., 2006; Surh et al., 2001). In the present study, the LPS-stimulated murine macrophage assay system was employed to evaluate the effects of *C. japonica* extracts on NO radical production. In order to identify novel bioactive constituents from *C. japonica* wood extracts, the fractionation strategy shown in Fig. 2 was employed, and inhibition of NO production by various fractions was assayed in LPS-stimulated RAW 264.7 cells. Among the four fractions of total EtOH–H_2O (70:30, v/v) extract from *C. japonica* wood, the hexane fraction (CJH) was the most effective. The IC_{50} values (μg/mL) of the tested extracts were in the following order: Hex (15) > EA (40) > EtOH (50) > *n*-BuOH (>150) and water (>150) (data not shown). The IC_{50} of reference control curcumin from *Curcuma longa* L. (Zingiberaceae) was 6.5 μg/mL. CJH was thus further divided into CJH1–CJH9 subfractions by silica gel column chromatography (Fig. 2), CJH7 possessed the most potent activity, with an IC_{50} value of approximately 15 μg/mL, in NO production induced by LPS in RAW 264.7 cells. No cytotoxicity of CJH7 on RAW 264.7 cells at this concentration was observed by MTT assay (data not shown). The further enriched bioactive subfraction (e.g., CJH7-2) and phytocompounds **1**,**2**, and **3** (Fig. 1) derived from CJH7 were then identified and characterized as guided by bioactivity assays described below.

2.2. Anti-inflammatory properties of CJH7 fraction in vitro

To investigate whether the inhibition of NO production by CJH7 was due to suppression of iNOS expression, Western blotting was employed to examine the levels of iNOS protein in LPS-stimulated macrophages treated with CJH7 (Fig. 3a). CJH7 drastically reduced iNOS protein levels by 70–100% at concentrations 5–25 μg/mL, implying a translational down-regulation of iNOS. Moreover, CJH7 also induced HO-1 protein expression in a dose-dependent manner in LPS-stimulated RAW264.7 cells (Fig. 3a), with an approximately 4.9-fold increase with 25 μg/mL CJH7.

COX-2 is known to involve in the inflammatory process in response to a wide variety of external stimuli including TPA and LPS in macrophages, mouse skin or rat liver (Chiang et al., 2005; Chun et al., 2004). Western blotting showed a significant inhibition (approximately 50%) of LPS-stimulated COX-2 levels in CJH7-treated (10–25 μg/mL) macrophages (Fig. 3a). The CJH7 fraction also inhib-

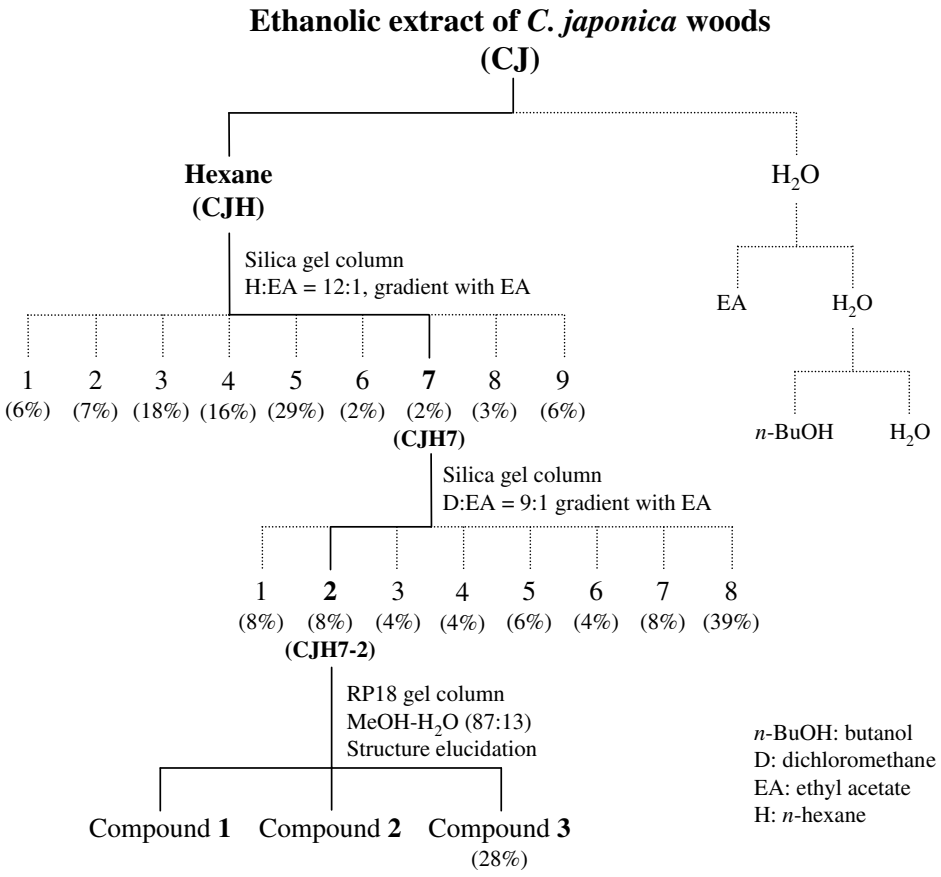

Fig. 2. The strategy for bioactivity-guided fractionation of the total ethanolic extract from *C. japonica* wood (CJ). The anti-inflammatory properties of CJ subfractions or derived phytocompounds were evaluated using *in vitro* or *in vivo* assays as described in the text.

ited TPA-induced *COX-2* transcriptional activity. Fig. 3b shows that TPA-induced enhanced transcription of *COX-2* in MCF-7 cells 5-fold, while the addition of 5, 25, 50, and 100 μg/mL CJH7 resulted in significant decreases of COX-2 activities by 34%, 41%, 44%, and 72% (black bars), respectively ($P < 0.05$). Indomethacin (3.5 μg/mL), a well-known NSAID used as reference control, caused a similar inhibition of *COX-2* activity in MCF-7 cells to 5–50 μg/mL CJH7.

2.3. Bioactivity characterization of CJH7 derived fractions

We further purified a more enriched and bioactive subfraction of CJH7 by chromatography on a silica gel column. Eight resultant subfractions CJH7-1 to CJH7-8 were assayed for their effect on NO inhibition. As shown in Fig. 4a, the nitrite level (equivalent to the NO level) in the culture supernatants of LPS-treated macrophages was greatly increased from 2.5 ± 0.1 μM (vehicle control) to 47 ± 1 μM, CJH7-2, CJH7-4 and CJH7-7 blocked approximately 50% of NO production in LPS-stimulated cells at 5 μg/mL and more than 87% at 25 μg/mL (Fig. 4a), with no or little detectable cytotoxicity (treated cells were >90% viable). The more abundant fractions CJH7-2 and CJH7-7 (both 8% of total CJH7) were then assayed for anti-inflammatory effects *in vivo* by examining COX-2 expression in TPA-treated mouse skin. The effect of CJH7-2, CJH7-7 and dexamethasone were compared. A small amount of COX-2 immunostaining was observed in the dermal sebaceous glands of acetone (vehicle) treated mouse skin (Fig. 4b), and COX-2 expression increased dramatically across the epidermal layer following TPA treatment (10 nmol/200 μL). TPA-induced COX-2 expression was inhibited by CJH7-2, but not by CJH7-7 at the same concentrations. The inhibition of COX-2 protein expression by 1 mg/200 μL/site CJH7-2 was comparable to that of dexamethasone (5 mg/200 μL/site) (Fig. 4b). Similar results were obtained in three independent repeated experiments. On the basis of these *in vitro* and *in vivo* results, CJH7-2 was chosen for further systematic investigation.

2.4. Anti-inflammatory and hepatoprotective properties of CJH7-2 fraction

Fig. 5a shows that CJH7-2 significantly inhibited LPS-induced iNOS protein expression in macrophages by 76% at 5 μg/mL and only minimal iNOS expression remained with 10–25 μg/mL CJH7-2. CJH7-2 only slight inhibited (17%) LPS-induced COX-2 expression at the higher dose of 25 μg/mL. In contrast, HO-1 levels increased to 152% of control in LPS-stimulated macrophages treated with CJH7-2 (Fig. 5a). We further investigated whether CJH7-2 can also play a role as antioxidant. DPPH and NBT assays

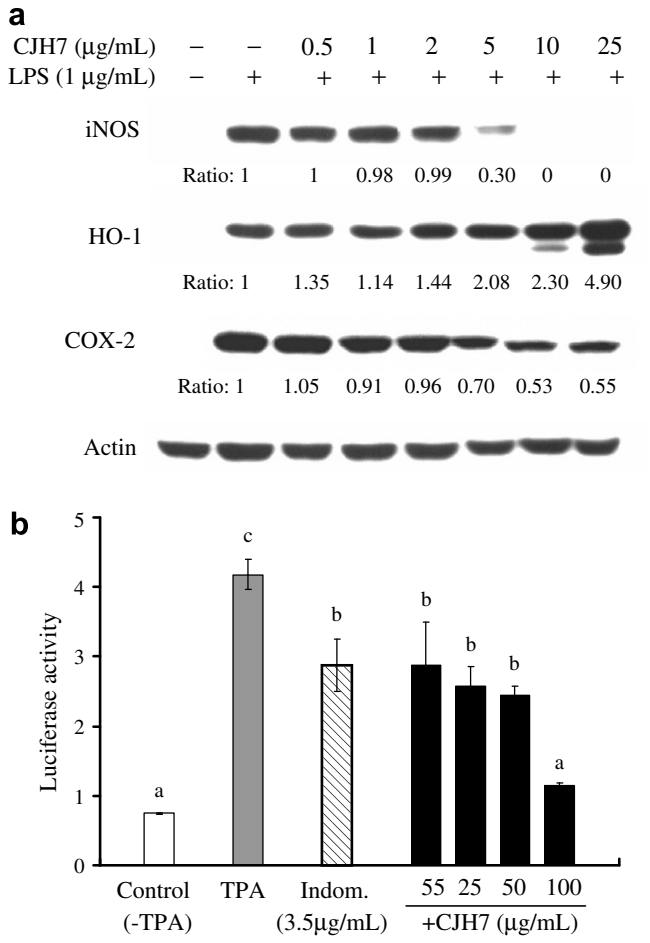

Fig. 3. Anti-inflammatory properties of CJH7 fraction. (a) RAW 264.7 cells were treated with the indicated concentration of CJH7 for 1 h followed by LPS (1 μg/mL) for 18 h. Total cellular proteins (20 μg) were resolved by SDS–PAGE, then immunoblotted with specific antibody. iNOS, HO-1, and COX-2 proteins were quantified using densitometry, and normalized to actin levels. Relative ratio to the control group (LPS only) is presented. (b) *COX-2* promoter activity in MCF-7 cells co-transfected with the full-length pCOX-2-Luc and internal control *Renilla* pRL-TK-Luc plasmid, treated with vehicle control (0.1% ethanol), 50 ng/mL TPA, or TPA mixed with CJH7 for 6 h. The data are presented as mean ± SD of three experiments. Different letter superscripts indicate significant difference ($P < 0.05$, one-way ANOVA).

were used for determining scavenging activity of free radicals and superoxide anion, and flow cytometric analysis was used to demonstrate the possible suppressive effect on oxidative stress induced by H_2O_2 in human promyelocytic leukemia HL-60 cells. CJH7-2 at concentrations up to 500 μg/mL neither inhibited free-radical or superoxide activity nor suppressed oxidative stress in HL-60 cells (data not shown).

Exogenous administrations of either anti-inflammatory compounds or antioxidants (e.g., *S*-adenosylmethionine) are effective hepatoprotectants through modulation of NO production in rats or in hepatocytes (Majano et al., 2001). As CJH7-2 inhibited NO and iNOS, we therefore investigated the hepatoprotective action of CJH7-2 in CCl_4-induced acute liver injury, a widely used model for testing hepatoprotection in natural compounds. Administration of CCl_4 led to 2.9-fold and 7.8-fold increases, respectively, in serum AST and ALT activities, compared to the vehicle control group ($P < 0.0001$), while treatment with CJH7-2 only did not alter either enzyme activity in serum (Fig. 5b). Pretreatment of mice with CJH7-2 before CCl_4 challenge, however, significantly attenuated the CCl_4-induced increase in AST and ALT activities from 2.9-fold to 1.9-fold ($P < 0.0001$) and 7.8-fold to 5-fold ($P < 0.0001$), respectively. This hepatoprotection by CJH7-2 was also observed in CCl_4-induced histopathological changes of mouse liver (Fig. 5c). Panlobular focal hepatocellular necrosis and neutrophil leukocyte infiltration were noted after CCl_4 intoxication for 6 h in CCl_4 group, whereas CJH7-2 pretreated mice (CJH7-2 + CCl_4 group) had much less severe hepatocellular necrosis. Administration of CJH7-2 only had no deleterious effect on hepatic tissues (CJH7-2 group).

CCl_4-induced hepatotoxicity is also known to be associated with oxidative stress and lipid peroxidation (Chen et al., 2004; Tipoe et al., 2006; Ohta et al., 2006). Hepatic antioxidant and myeloperoxidase enzyme activities and lipid peroxide levels in test mice liver tissues were also examined. In CCl_4-treated mice, hepatic SOD activity decreased by 80%, and MPO activity increased by 50% (both significant, $P < 0.05$), with little or no effect on GPX and CAT activities (Table 1). CJH7-2 + CCl_4 group showed no significant change in SOD, CAT, and MPO activities when compared either with CJH7-2 or CCl_4 alone groups (Table 1), however, GPX activity in CJH7-2 + CCl_4 group increased by 50% compared to control. Administration of CCl_4 caused a 3-fold increase in TBARS in liver tissues ($P < 0.05$) that was effectively suppressed in CJH7-2 + CCl_4 group (Table 1). It was reported by Hiroi et al. (1995) that the essential oil and its major terpene components (e.g., cadinene) from *C. japonica* or other woody plants can have significant activities on aniline hydroxylation or inducing the levels of specific isoforms of cytochrome P450 such as 2β, 6β, 16α, and 16β. A significant increase in protein level of forms 2β, 1, and 3A2 was also observed *in vivo* in rat liver when treated with essential oil of *C. japonica*. It may be worthy to further investigate whether the bioactive CJH7-2 extract identified from the same plant species can also modulate the activity of P450s, enzymes playing an important role in metabolism of drugs, carcinogens, steroids, etc.

Accumulating evidence has indicated that the induction of HO-1 expression negatively regulates iNOS and COX-2 expression in macrophages (Lin et al., 2003). HO-1 has attracted particular interest as it is upregulated in response to stresses, and it generates metabolites (such as bilirubin and CO) with important biological activities. Previous studies indicated that the inhibition of iNOS-dependent NO production by HO-1 appears to be mediated by bilirubin and CO (Wang et al., 2004). The hepatoprotective effect of quercetin was suggested to be associated with HO-1 induction (Yao et al., 2007). In this study, the potent stimulation of HO-1 expression in LPS-induced macrophages by CJH7-2 subfraction may in turn contribute to the reduc-

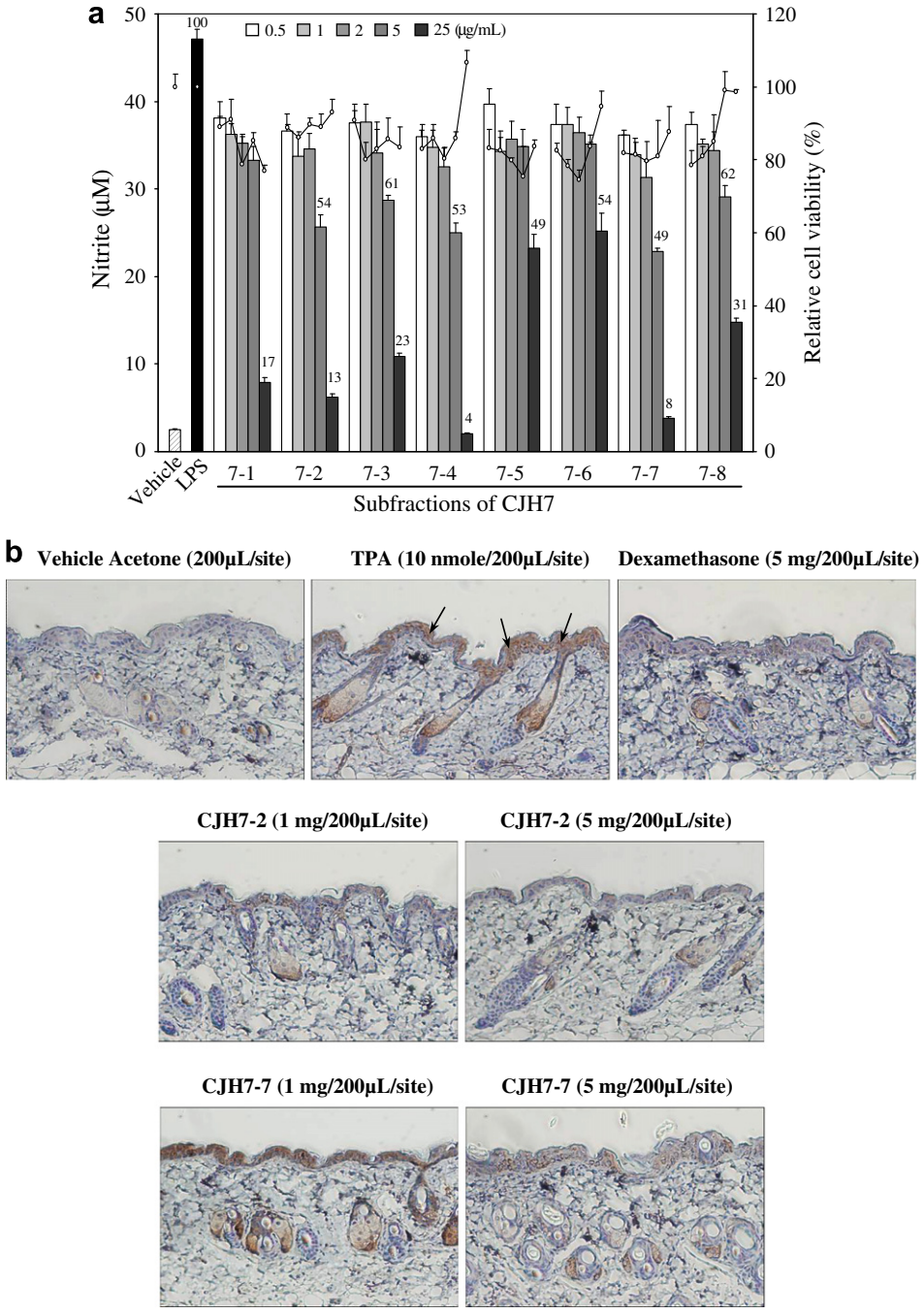

Fig. 4. Anti-inflammatory activities of CJH7 subfractions *in vitro* and *in vivo*. (a) Inhibition of NO production in LPS-stimulated RAW264.7 cells. Cells were treated with subfractions CJH7-1 to CJH7-8 or vehicle control for 1 h, then LPS (1 μg/mL) for 24 h. The bar graph represents the NO inhibition; the line graph represents the relative cell viability (%) by MTT assay. The data are presented as mean ± SEM. Significant differences between treatments are indicated by *, with $P < 0.0001$. (b) Immunohistochemical study of the inhibitory effect of CJH7-2 and CJH7-7 fractions on COX-2 expression in TPA-treated skin. Dorsal skin of female ICR mice was treated topically with acetone or TPA only (10 nmol) for 4 h, or treated with CJH7-2, CJH7-7 or dexamethasone for 30 min followed by TPA for 4 h. Paraffin-embedded skin tissues were immunostained for specific COX-2 protein and counter-stained with hematoxylin. Immunohistographs were taken with an Olympus DP-70 camera on a Nikon Eclipse E800 microscope (magnification: ×200).

tion of inflammatory responses by inhibiting iNOS and COX-2 expression in macrophages and also contribute to the hepatoprotective effect in mice. Taken together, our *in vitro* and *in vivo* results show that CJH7-2 is hepatoprotective in inflammatory liver, mediated at least in part through the inhibition of proinflammatory mediators NO, iNOS, and COX-2.

2.5. Metabolite and bioactivity profiling of the major chemical constituents in CJH7-2 fraction

Reversed-phase (RP-18) silica gel chromatography was employed to purify chemical constituents from CJH7-2 of *C. japonica*. Three major compound peaks were collected, and MS and NMR analyses together with other

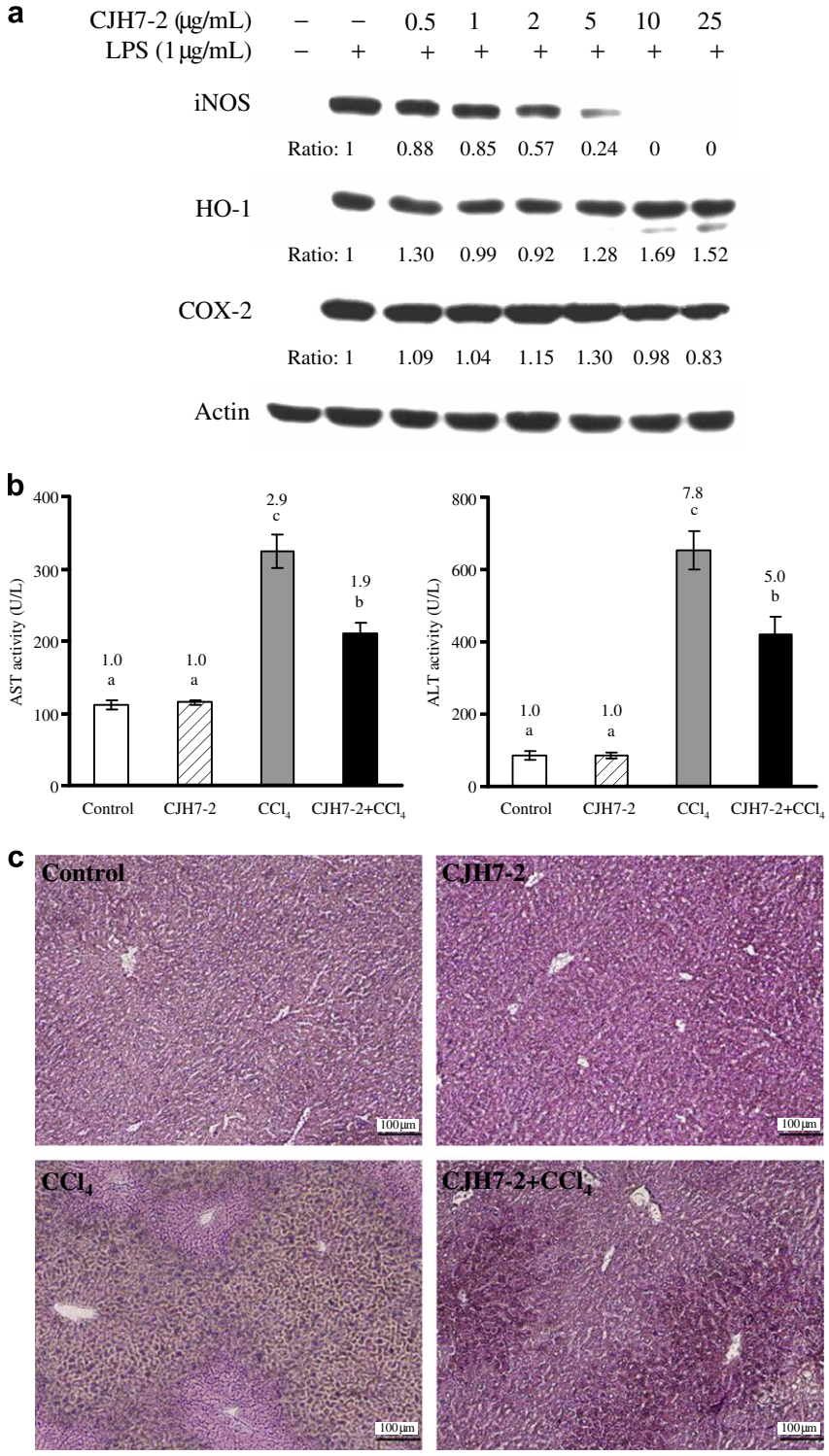

Fig. 5. Anti-inflammatory and hepatoprotective properties of CJH7-2 fraction. (a) Effects of CJH7-2 on iNOS, HO-1, and COX-2 expression in LPS-treated RAW264.7 cells. Cells were treated with CJH7-2 for 1 h followed by LPS (1 μg/mL) for 18 h. Total cellular proteins (20 μg) were resolved by SDS–PAGE and immunoblotted against specific antibody. Levels of iNOS, HO-1, and COX-2 proteins were normalized to actin. Relative ratio to the control group (LPS only) is presented. (b) Mice were divided into four groups. Control group: DMSO + olive oil; CJH7-2 group: CJH7-2 + olive oil; CCl_4 group: vehicle + 20% CCl_4 in olive oil; CJH7-2 + CCl_4 group: CJH7-2 + CCl_4 in olive oil. Data are presented as mean ± SEM ($n = 8$). Different letters are significantly different from each other ($P < 0.0001$, ANOVA). (c) Histopathological study. Specimens from control and CJH7-2 groups show normal liver architecture. Panlobular focal hepatocellular necrosis was found 6 h after CCl_4 intoxication in CCl_4 group. Section of CJH7-2 + CCl_4 shows a marked decrease in the severity of hepatocellular necrosis. Specimens were photographed with an Olympus DP-70 camera on a Nikon Eclipse E800 microscope (H&E stain, magnification: ×100).

Table 1
Effects of CJH7-2 on hepatic antioxidant enzymes and myeloperoxidase activities and lipid peroxide levels in CCl_4-induced acute liver injury[A]

	GPX	SOD	CAT	MPO	TBARS
	(U/g)				(MDA, μM)
Control	1041 ± 112^{a}	31 ± 2^{b}	2197 ± 240	292 ± 8^{a}	14 ± 2^{a}
CJH7-2	1311 ± 49^{ab}	30 ± 1^{b}	2205 ± 273	303 ± 6^{a}	13 ± 2^{a}
CCl_4	1268 ± 94^{ab}	24 ± 1^{a}	2967 ± 292	436 ± 34^{b}	41 ± 6^{b}
CJH7-2 + CCl_4	1554 ± 149^{b}	28 ± 3^{ab}	2596 ± 277	377 ± 43^{ab}	24 ± 7^{a}

Note: GPX: glutathione peroxidase; SOD: superoxide dismutase; CAT: catalase; MPO: myeloperoxidase; TBARS: thiobarbituric acid reactive substances.
[A] Data are mean ± SEM ($n = 3$). Values in the same column with different letters are significantly different from each other ($P < 0.05$) by one-way ANOVA with Fischer's test.

spectroscopic data led to the identification of sugiol (**1**), 12-hydroxy-6,7-secoabieta-8,11,13-triene-6,7-dial (**2**), and (1S,6R)-2,7(14),10-bisabolatrien-1-ol-4-one (**3**) (Fig. 6a). Compounds **1** and **2** are diterpenes, and compound **3** is a peculiar sesquiterpenol. The characteristics of compounds **1** (EIMS: *m/z* 300 $[M]^{+}$; ^{1}H and ^{13}C NMR data ($CDCl_3$)), **2** (EIMS: *m/z* 316 $[M]^{+}$; ^{1}H and ^{13}C NMR data ($CDCl_3$)) and **3** (EIMS: *m/z* 234 $[M]^{+}$; ^{1}H and ^{13}C NMR data ($CDCl_3$)) were in good agreement with previously reported results (Su et al., 1996; Chen et al., 2001). Compound **3** was quantitatively determined by HPLC to comprise 28% of the dry weight of CJH7-2. The metabolite profile and relative content of index compound **3** were adopted for routine quality control of the consistency of the CJH7-2 extract.

2.6. Comparative study of the anti-inflammatory activities of CJH7-2 and its major constituent compounds

A comparative bioactivity study was undertaken to determine which of the 3 compounds contributed to the anti-inflammatory effect of CJH7-2 (Fig. 6b). CJH7-2 and compounds **1**, **2**, and **3** all dose-dependently inhibited NO production in LPS-activated macrophages, with IC_{50} values of 7, 8, 8, and 4 μg/mL, respectively, calculated by linear regression. More than 90% of test cells were viable when treated with compounds **2** and **3** (1–25 μg/mL) for 24 h, whereas compound **1** exhibited mild cytotoxic or anti-cell proliferation activity at 25 μg/mL, with ca. 65% of test cells remaining viable. Furthermore, CJH7-2 and compounds **1**, **2**, and **3** all significantly inhibited COX-2 enzymatic activity with $IC_{50} = 5, 1, 1$, and 1 μg/mL, respectively ($P < 0.0001$) (Fig. 6c). These results indicate that compounds **1**–**3** play important roles in the observed anti-inflammatory effects of *C. japonica* extract.

3. Concluding remarks

This is the first study to demonstrate the bioactivity of *C. japonica* wood extracts and derived phytocompounds against various inflammatory responses in macrophages, mouse skin, or CCl_4-induced mouse liver injury. The potent *in vitro* anti-inflammatory activities of CJH7-2 fraction through down-regulation of proinflammatory mediators and up-regulation of HO-1 are likely responsible for the hepatoprotection in mice. The three major bioactive compounds (**1**–**3**) isolated from CJH7-2 are also potent anti-inflammatory agents, as the IC_{50} values for NO production and COX-2 enzymatic activity were between 5 and 10 μg/mL (<30 μM) and below 1 μg/mL (<5 μM), respectively. These phytocompounds from *C. japonica* extract may serve as novel lead compounds for future development of anti-inflammatory or hepatoprotective therapeutics.

4. Experimental

4.1. Preparation and fractionation of plant extracts

A thirty year old *C. japonica* D. Don (Taxodiaceae) was collected in July 2004 from the Experimental Forest of National Taiwan University, Taiwan. Voucher specimens of heartwood, sapwood, bark, and leaf (CJHO01, CJSO01, CJBO01, and CJLO01) were deposited at the Laboratory of Wood Chemistry in National Taiwan University.

The dried heartwood tissues of *C. japonica* (10 kg) were crushed and extracted with EtOH–H_2O (70:30, v/v) (100 L × 2) at room temperature for 7 days. One hundred gram of dried EtOH–H_2O wood extracts were resuspended in 400 mL MeOH–H_2O (20:80, v/v) and divided into hexane (H), EtOAc (EA), *n*-butanol (*n*-BuOH), and H_2O fractions by solvent partitioning (400 mL × 3 each) at room temperature. The hexane fraction was separated on silica gel using *n*-hexane/EA to give nine subfractions. Subfraction 7 (designated CJH7) was re-applied to a silica gel column using CH_2Cl_2/EA to give nine subfractions. The major bioactive subfraction 2 (designated CJH7-2) was then further purified via semi-preparative HPLC using a Phenomenex Luna C18 column (250 × 10 mm) to give compounds **1**, **2**, and **3**, (retention times of 34.0, 32.0, and 20.9 min), in MeOH–H_2O (87:13, v/v) at 1 mL/min. Their structures were determined by analyses of NMR (Bruker Advance-400 MHz FT-NMR) and mass spectra (Thermo-Finnigan/LCQ Advantage mass spectrometer). The amount of compound **3** in CJH7-2 fraction was calculated by comparison to a standard calibration curve of the compound **3**. All chemicals and solvents used in this study were of reagent or HPLC grade.

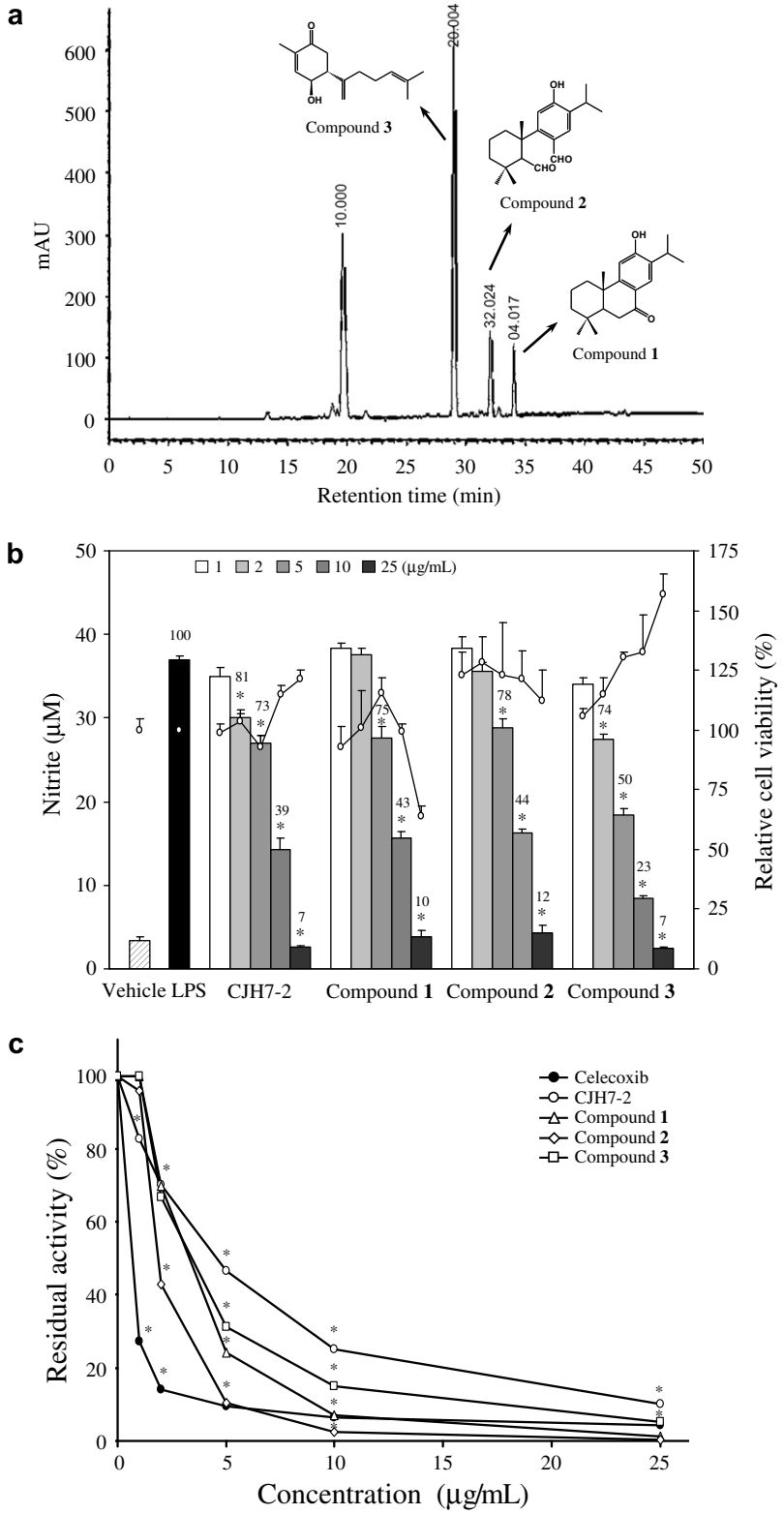

Fig. 6. (a) Metabolite profile analysis of the bioactive CJH7-2 fraction from *C. japonica* by HPLC at 254 nm. The chromatogram of the CJH7-2 fraction was obtained from a RP-18 silica gel column eluted with MeOH–H_2O (87:13, v/v). Three compounds were isolated: sugiol (**1**), 12-hydroxy-6,7-secoabieta-8,11,13-triene-6,7-dial (**2**), and (1 S,6R)-2,7(14), 10-bisabolatrien-1-ol-4-one (**3**). (b) Cells were treated with CJH7-2 or compounds **1**, **2**, and **3** or vehicle for 1 h, followed by LPS (1 μg/mL) for 24 h. The bar graph represents the NO inhibition, the line graph shows the relative cell viability (%) as evaluated by MTT assay. (c) Inhibition of COX-2 activity of CJH7-2 and compounds **1**, **2**, and **3** was measured using a chemiluminescent COX-2 enzyme inhibitor assay. Residual COX-2 activity (%) = $(RLU_{sample}/RLU_{control}) \times 100$. Data from three experiments are expressed as mean ± SEM. Significant inhibition is indicated by * with a P value <0.0001.

4.2. Cell Lines and culture conditions

RAW 264.7 and MCF-7 cells were obtained from the ATCC (Manassas, VA). Macrophages were grown in Dulbecco's Modified Eagle Medium (Gibco/BRL) and MCF-7 cells were grown at 37 °C in RPMI 1640 medium (Gibco/BRL), both supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, in a humidified 5% CO_2 incubator.

4.3. Animals

Male and female ICR mice (National Laboratory Animal Center, Taiwan) were given a standard laboratory diet and distilled water *ad libitum,* and kept on a 12 h light/dark cycle at 22 ± 2 °C.

4.4. Western blot analysis

Total cellular proteins were prepared according to Chiang et al. (2005). Protein content was measured by the Bradford method (Bio-Rad). Protein was resolved by 5–20% gradient SDS-PAGE and immunoblotted using enhanced chemiluminescence reagents (ECL, Amersham) and monoclonal antibodies against COX-2 (Cayman Chemical), iNOS, HO-1 and actin (Santa Cruz Biotechnology).

4.5. Reporter gene construct and luciferase assays

In order to identify novel anti-inflammatory agents, a high throughput luciferase gene reporter assay was developed for TPA-mediated induction of COX-2 promoter activity in human mammary cancer MCF-7 cells system. The chimeric luciferase reporter gene, pPGL3-Basic vector (Promega) containing the full length (1334/1) of human COX-2 gene promoter was constructed using a method described elsewhere (Chiang et al., 2005). Cells (1.2×10^5 cells/well) were seeded in 24-well plates and cotransfected with pCOX-2-Luc and pRL-TK-Luc (Promega) plasmids using the LipofectAMINE system (Invitrogen). Following transfection (after 4 h), the medium was replaced with fresh DMEM medium and cells were allowed to recover for 16 h. The transfected cells were then treated with vehicle (0.1% DMSO), 50 ng/mL 12-*O*-tetradecanoylphorbol-13-acetate (TPA) alone, or TPA mixed with test extracts for 6 h. Cell lysates were prepared using Passive Lysis Buffer (Promega) and then employed for luciferase activity assays. pPGL-3-Basic vector was used as a negative control in luciferase assays. COX-2 driven reporter activity in arbitrary units (AU) was normalized to that for the Renilla luciferase reporter.

4.6. Measurement of NO production and cell viability

RAW 264.7 cells were treated with test extracts or phytocompounds for 1 h and then incubated for 24 h with or without LPS. Nitrite levels in cell culture medium were determined using the Griess reaction (Green et al., 1982). Cell viability was examined using the MTT assay (Scudiero et al., 1988).

4.7. Cyclooxygenase-2 activity assays

COX-2 inhibition was measured with the chemiluminescent COX inhibitor screening assay kit (Cayman Chemical, MI). The COX-2 activity was evaluated by integrated chemiluminescent signal for the read time in relative luminescent units (RLU). Percent inhibition of COX-2 activity was calculated using the following formula: Residual activity $(\%) = (RLU_{sample}/RLU_{control}) \times 100$. IC_{50} represents the levels at which 50% of the enzymatic activity were inhibited by test samples. Celecoxib (CELEBREX™, Pharmacia), a well-known NSAID with potent COX-2 inhibitory activity, was used as reference control in this experiment.

4.8. Immunohistochemical study of COX-2 expression in mouse skin

Female ICR mice were topically treated on their shaven backs with vehicle (acetone) or TPA for 4 h, or with CJH7-2 at the indicated concentrations first for 30 min, and then treated with TPA for 4 h, before sacrifice by cervical dislocation. The formalin-fixed, paraffin-embedded skin tissues were stained according to Chiang et al. (2005). Mice were topically treated on their shaven back area of a 2 cm diameter (3.14 cm^2/site/mouse) with vehicle (acetone, 200 μL/site) or TPA (10 nmol/200 μL/site) for 4 h. For extract treatments, mice were treated with CJH7-2 at the indicated concentrations first for 30 min, then further treated with TPA for 4 h, and finally killed by cervical dislocation. Sections of formalin-fixed, paraffin embedded tissue were cut onto silanized glass slides and deparaffinized three times with xylene for 5 min each, prior to rehydration through a graded series of alcohol baths. For antigen retrieval, the deparaffinized sections were heated and boiled in 10 mM citrate buffer (pH 6.0) for 10 min and then rinsed with PBS containing 0.05% Tween-20 (PBST) buffer for 5 min. Each section was treated with 3% hydrogen peroxide in MeOH for 15 min to block endogenous peroxidase activity and diminish non-specific staining. The sections were then washed with blocking solution (PBS–1% BSA, PBA) for 30 min and then PBST twice for 5 min each. The slides were incubated with 2% normal goat serum in PBA for 30 min to minimize the non-specific binding to antibody, and then incubated with a 1:500 dilution of polyclonal COX-2 antibody (Cayman) at room temperature for 1–2 h. The slides were developed using anti-rabbit HRP EnVisionTM system (Dako), and the peroxidase-binding sites were detected by staining with 3,3′-diaminobenzidine tetrahydrochloride (Dako). Finally, sections were counterstained with Mayer's hematoxylin.

4.9. Induction of acute liver injury

For the hepatotoxicity study, CCl_4 was used to induce acute liver damage according to Chen et al. (2004) with slight modifications. Male ICR mice were randomly divided into four groups of three mice treated as follows: olive oil (vehicle group), CJH7-2 at 10 mg/kg body weight plus olive oil (CJH7-2 only group), vehicle plus CCl_4 (CCl_4 group), and CJH7-2 treatment plus CCl_4 in oil (CJH7-2 + CCl_4 group). The vehicle or CJH7-2 was administrated via intraperitoneal injection for three consecutive days before CCl_4 administration. Mice were treated with a single intraperitoneal administration of 20% CCl_4 solution in olive oil at a dose of 40 μL CCl_4/kg body weight to produce acute liver injury. The mice were killed after collecting blood by retro-orbital bleeding at 6 h after CCl_4 injection.

4.10. Measurement of serum AST and ALT activities

The enzymatic activities of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were used as biochemical markers for acute liver injury. Blood samples were centrifuged at 1400*g* at 4 °C for 15 min and the AST and ALT activities in serum supernatants were determined using a commercial kit from Randox Laboratories (UK).

4.11. Assays of hepatic components and enzymes

Liver tissues were homogenized in ice-cold buffer (0.25 M sucrose, 10 mM Tris–HCl, and 0.25 mM phenylmethylsulfonyl fluoride, pH 7.4), and a portion of the homogenate was measured immediately for thiobarbituric acid reactive substances (TBARS) using the method of Ohkawa et al. (1979) with minor modifications. The amounts of TBARS were expressed as malondialdehyde (MDA) equivalents using 1,1,3,3-tetramethoxypropane (TMP) and measured fluorometrically (excitation and emission wavelengths: 531 nm vs. 590 nm) using a Wallac Victor-2 1420 Multilabel Counter (Perkin–Elmer). Another portion of the homogenate was centrifuged at 10,000*g* for 20 min at 4 °C, and the protein content and hepatic glutathione peroxidase (GPX) and superoxide dismutase (SOD) activities in the supernatant were measured using a commercial kit (Randox). Hepatic catalase (CAT) activity was determined by the method of Beers and Sizer (1952). The pellets were washed twice in ice-cold 20 mM phosphate buffer (pH 6.0) and then sonicated in myeloperoxidase (MPO) buffer containing 0.5% hexadecyltrimethylammonium bromide, 10 mM EDTA and 50 mM phosphate (pH 6.0) at 25 °C for 1 min. The homogenates were then centrifuged at 17,000*g* at 4 °C for 15 min and MPO activity in the supernatant measured as previously described (Schierwagen et al., 1990). Total protein concentrations of samples were determined using a DC protein assay kit (Bio-Rad). Specific activity of test enzymes was expressed as U/g protein.

4.12. Histological examination of liver tissues

Fresh liver tissues were embedded in Tissue-Tek 4583 OCT compound (Sakura, Tokyo, Japan). Tissue sections of 6 μm were cryocut (HM550, Microm, Germany) and processed for histological examination with Hematoxylin and Eosin (H&E) stain and light microscopy.

4.13. Statistical analysis

Data are expressed as means ± SEM. Statistical significance of differences between treatments was determined by ANOVA with Fisher's *post hoc* test. $P < 0.05$ was considered to be statistically significant.

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