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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.

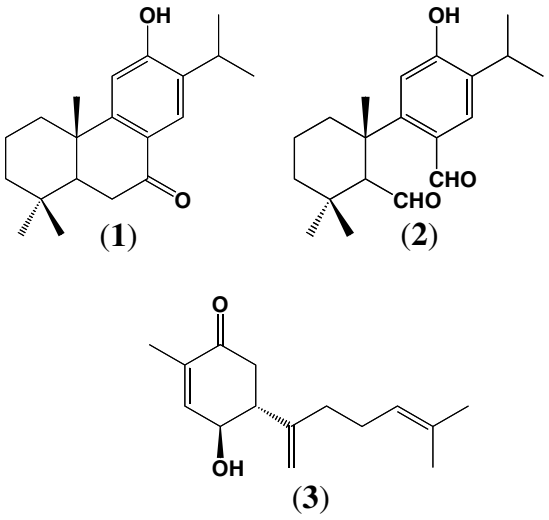

Fig. 1. Chemical structures of the three major bioactive terpenoids, sugiol (**1**), 12-hydroxy-6,7-secoabieta-8,11,13-triene-6,7-dial (**2**), and (1S,6R)-2,7(14),10-bisabolatrien-1-ol-4-one (**3**), identified from the heartwood extract of *C. japonica*.

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-