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Effects of *Chamaecyparis formosensis* Matasumura extractives on lipopolysaccharide-induced release of nitric oxide

Yu-Hsin Hsieh[a], Pei-Min Kuo[b], Shih-Chang Chien[a], Lie-Fen Shyur[a], Sheng-Yang Wang[b,*]

[a]*Institute of BioAgricultural Sciences, Academia Sinica, Taipei 115, Taiwan*
[b]*Department of Forestry, National Chung-Hsing University, Taichung 402, Taiwan*

Abstract

Chamaecyparis formaosensis, commonly known as Taiwan red cypress, is native to Taiwan and grows at elevations of 1500–2150 m in Taiwan's central mountains. Many compounds have been identified from different pasts of *C. formosensis*, but up until now, little research has been done on the link between the constituents of *C. formosensis* and its bioactivities. In this study, we found that an ethyl acetate fraction (EA) of methonal extract of *C. formosecsis*, strongly inhibited LPS-mediated nitric oxide (NO) production in Raw 264.7 cells. The EA was further divided into 25 subfractions (EA1–EA25) by column chromatography. EA12 possessed the strongest NO production inhibition activity (IC_{50} was 4.1 μg/mL). At a dosage of 20 μg/mL, EA12 completely inhibited NO production and the mRNA expression of inducible nitric oxide synthase (iNOS) in LPS-stimulated macrophage RAW264.7 cells. Bioactivity-guided chromatographic fractionation and metabolite profiling coupled with spectroscopic analyses, including ^{1}H-NMR, ^{13}C-NMR analyses, identified six compounds: vanillin (**1**), 4-hydroxybenzaldehyde (**2**), *trans*-hinokiresinol (**3**), taiwanin E (**4**), 4α-hydroxyeudesm- 11-en-12-al (**5**), savinin (**6**). All of these six compounds were the first identified and reported from this tree species. Compounds (**1**), (**3**) and (**5**) demonstrated significant NO inhibition effect through reduction of NO production in activated RAW 264.7 cells due to the suppression of iNOS gene expression: compounds that can selectively inhibit undesirable expression of iNOS are important as they may serve as potential cancer chemopreventatives. This study suggests that *C. formosensis* may have potential for use as a natural resource for human health care.

Keywords: *Chamaecyparis formosensis*; iNOS; Vanillin; *Trans*-hinokiresinol; 4α-hydroxyeudesm-11-en-12-al

Introduction

Plants have formed the basis for sophisticated traditional medicine systems that have been in existence for thousands of years in several countries. These plant-based medicinal systems continue to play an essential role in health care today, and it has been estimated by the World Health Organization that approximately 80% of the world's inhabitants rely mainly on traditional medicines for primary health care. Owing to its unique ecosystem, Taiwan is famous for the richness and diversity of its flora, with over 6500 species classified to date. Over the past few years, we have systematically evaluated and characterized selected plant species for their putative bioactivities or potential medicinal applications. *Chamaecyparis formosensis* (Cupressaceae), commonly known as the Taiwan red cypress is an endemic tree that grows at elevations of 1500–2150 m in Taiwan's central mountains (Liu et al., 1988). *C. formosensis* is

*Corresponding author. Tel.: +886 4 22840345x138.
E-mail address: taiwanfir@dragon.nchu.edu.tw (S.-Y. Wang).

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renowned for its excellent wood quality, beautiful texture and attractive fragrance. The fragrance of wood is mainly attributed to its essential oils, which are primarily composed of terpenes and their oxygenated derivatives. Besides their use in perfumes, these essential oils and their constituents have a long history of use as traditional medicinal agents, i.e. antibacterial and antifungal agents. More than one hundred compounds have been identified from different parts of *C. formosensis* as a result of phytochemistry studies (Kafuka and Ichikawa, 1931; Nozoe et al., 1966; Fang et al., 1986a, b; Hsu et al., 1995). However, little research has been done on the link between constituents of *C. formosensis* and their bioactivities. Recently, we analyzed the wood volatile constituents of *C. formosensis* by GC–MS. The relative quantities of its 34 main essential oil constituents were identified. Results obtained from antifungal assays demonstrated that the wood oil exhibits excellent antifungal properties. It is plausible that volatile constituents of the wood oil contribute to the excellent resistance to decay seen in *C. formosensis* (Wang et al., 2005). Systematic investigations of the bioactivities of specific volatile or nonvolatile compounds of *C. formosensis* are in progress by us.

According to the results of our current study, Lipopolysaccharide (LPS)-induced nitric oxide (NO) production in Raw 264.7 cells was strongly inhibited by the ethyl acetate fraction (EA) of methanol extracts from *C. formosensis*. Nitrogen oxides, such as NO and its metabolite, peroxynitrite, are considered mutagenic because they can cause deamination of DNA and inactivation of DNA repair enzymes (Keefer and Wink, 1996; Tamir and Tannenbaum, 1996), and are predominant effectors in neurodegeneration (Chabrier et al., 1999). Inducible nitric oxide synthase (iNOS) catalyzes the oxidative deamination of *L*-arginine to produce NO. Therefore, aberrant or improper up-regulation of iNOS is often implicated in the oncogenesis and pathogenesis of cancer. Compounds that can selectively inhibit undesirable expression of iNOS may serve as potential cancer chemopreventive candidates. Chemotherapy has long been a cornerstone of cancer therapy. Dietary supplements with anti-oxidant properties are considered to be able to enhance the anticancer effects of chemotherapy, reducing or preventing certain chemotherapy-induced side effects (Wang et al., 2003). The evidence from this study suggests that *C. formosensis* may have a potential to use as a natural resource for human health care.

Materials and methods

Plant materials and chemicals

Eighty-year-old *C. formosensis* logs were collected from the Experimental Forest of National Taiwan University. *C. formosensis* samples were identified by Prof. Yen-Hsueh Tseng (Department of Forestry, National Chung-Hsing University). The voucher specimens were deposited as CF001-4 in the herbarium of Department of Forestry, National Chung-Hsing University. Agarose was obtained from Bio-Rad (California, USA), and Dulbecco's Modified Essential Medium and RPMI 1640 from Gibco BRL (USA). All other chemicals and solvents used in this study were of the reagent or HPLC grade.

Compound isolation

Heartwood chips were prepared from a green cut tree. Fifteen kilograms of wood chips were exhaustively extracted with methanol (MeOH). The extracts were condensed to 904 g, followed by extraction with ethyl acetate (EtOAc). After removing solvents from the extracts, an EA soluble fraction was obtained (575 g). The EA fraction (80 g) was divided into 25 subfractions (EA1–EA25) by using a silica-gel column eluted with EtOAc /*n*-hexane by 0/100–100/0 gradient elution. The EA12 (600 mg) was further separated by semi-preparative high performance liquid chromatography (HPLC) using a C-18 column (Phenomenex Luna 5 μm, 250 mm × 10 mm), eluting with a acetonitrile (ACN)/H_2O gradient solvents systems. The gradient elution profile was as follows: 0–27 min, 33% ACN to 88% ACN (linear gradient); 27–30 min, 88% ACN to 100% ACN (linear gradient); the flow rate was 2.5 mL/min and the detector wavelength was set at 280 nm.

Cell lines, cell culture and nitric oxide inhibitory assay

Nitric oxide (NO) inhibition activities of methanolic extracts of *C. formosensis* were performed according to the method reported by Hwang et al. (2002) with minor modifications. RAW264.7 cells, a murine macrophage cell line, were obtained from American Type Culture Collection (ATCC) and cultured at 37 °C in Dulbecco's Modified Essential Medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 incubator as recommended by ATCC. Briefly, RAW264.7 cells grown on a 75 cm^2 culture dish were seeded in 96 well plates at a density of 2×10^5 cells/well. Adhered cells were then incubated for 24 h with or without 1 μg/mL of lipopolysaccharide (LPS), in the absence or presence of *C. formosensis* extracts. Nitrite concentration was measured using the supernatant of RAW264.7 cells by the Griess reaction (Schmidt and Kelm, 1996). Hundred microliters cell culture supernatants were reacted with 100 μL of Griess reagent [1:1 mixure of 0.1% *N*-(1-naphthyl) ethylenediamine in H_2O

and 1% sulfanilamide in 5% phosphoric acid] in a 96 well plate and the absorbance was recorded using an ELISA reader at 540 nm (Labsystems Multiskan MS, USA). $NaNO_2$ was used as an external calibration. Data were expressed at the total μM nitrite produced by 2×10^5 RAW 264.7 cells for 24 h, and reduced to NO inhibition percentage as shown.

RT-PCR analysis

RAW264.7 cells were seeded in 6-well plates at a density of 3×10^6 cells/well. The cells were treated with test compounds in various concentrations for 1 h, and then incubated for 6 h with or without 1 μg/mL of LPS. The cells were harvested and total RNA was isolated by a kit according to the manufacturer's instruction (TRI_{ZOL} Reagent, Invitrogen). For each RT-PCR reaction, 4 μg total RNA was used to synthesze 1st strand cDNA by the SuperScript™ II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Equal amounts of each RT product were amplified by PCR with *Taq* polymerase (GeneTeks BioScience Inc.). For amplification of the iNOS gene by the following primers: sense strand, 5′-CAG AAG CAG AAT GTG ACC ATC-3′; antisence strand, 5′-CTT CTG GTC GAT GTC ATG AGC-3′. The cDNA sequence of glyceraldelyde-3-phosphate dehydrogenase (GA3PDH) was also amplified as a control by the following primers : sense strand, 5′-CCA TCA ATG ACC CCT TCA TTG ACC-3′; antisence strand, 5′-GAA GGC CAT GCC AGT GAG CTT CC-3′. The PCR amplification condition was as follows: 94 °C for 2 min, and then 30 cycles consisting of 94 °C for 1 min, 55 °C for 30 s and 72 °C for 1 min. The PCR products were analyzed with 0.8% agarose gel and visualized by staining with ethidium bromide.

Protein extraction and Western analysis

Raw264.7 cells were incubated for 18 h with or without various concentrations of *C. formosensis* compound and 1 μg/mL of LPS. The cells were washed with ice-cold PBS (calcium and magnesium-free phosphate buffered saline) and homogenized in 0.3 mL ice-cold lysis buffer [20 mM Tris-HCl pH7.9, 10% glyserol, 1% Triton X-100, 137 mM NaCl, 1 mM EGTA, 5 mM EDTA, 100 mM *β*-glycerophosphate, 10 mM NaF, 1 mM Na_3VO_4, 1 mM sodium pyrophosphate, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin]. The protein concentration was determined by the Bradford method (Bradford, 1976). Twenty nanograms per lane of protein were loaded in 5–20% gradient sodium dodecyl sulfate-polyacrylamide gels to detect iNOS expression. After running at 100 V for 2 h, the size-separated proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore, Bedford, MA) at 100 V for 1 h. The membranes were incubated in blocking buffer (3% w/v skim milk in TBS buffer) for 30 min, and then incubated with anti-iNOS monoclonal antibody (Santa Cruz Biochemicals, Santa Cruz, CA), anti-actin monoclonal antibody (Oncogene Science, Cambridge, UK). After washing two times with 0.1% TPBS (PBS containing 0.1% Tween 20), the membranes were incubated with the antimouse secondary antibodies conjugated with horseradish peroxidase and detected by the enhanced chemiluminescence reagents (ECL, Amersham).

Results and discussion

Nitric oxide is a well-known signal in physical and pathological reactions, especially in acute inflammatory response (Surh et al., 2001). In organisms, nitric oxide is derived from the oxidation of *L*-arginin through nitric oxide synthase. There are three kinds of nitric oxide synthase, including endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). iNOS mainly exists in macrophage, it expresses by stimulated of endotoxins, tumor necrosis factors or LPS. It is known that activation of iNOS by pro-inflammatory agents such as LPS can significantly increase nitric oxide (NO) production in macrophages (Kojima et al., 2000; Park et al., 2000). In this study, a LPS-stimulated RAW264.7 cell assay was employed to evaluate the NO inhibition activity of *C. formosensis* extracts. The methanol extract of *C. formosensis* was further divided into EA and EA-insoluble fractions through liquid–liquid partition. EA was further divided into 25 subfractions (EA1–EA25) with open column chromatograph. Fig. 1 shows the inhibitory effect of EA1–EA25 (dosage = 20 μg/mL) on NO production in LPS-stimulated RAW264.7 cells. The NO levels in tested cells with and without LPS stimulation were 1.16 ± 0.08 and 25.98 ± 1.04 μM, respectively. After LPS-treated cells were co-incubated with *C. formosensis* extracts, NO production was inhibited; the inhibitory effects were from 15% (EA1) to 100% (EA12). The test cells were healthy and viable at the dosage of 20 μg/mL, as determined by MTT colorimetric assay, as described elsewhere (Mossmann, 1983) (data not shown).

These results indicated that the EA12 subfraction was most effective at inhibiting NO production. The IC_{50} (50% inhibition concentration) of EA12 against NO radical was 4.1 μg/mL (Fig. 2), and there was no cytotoxic effect presented within the dosage (1–20 μg/mL) used in this assay. To investigate the phytochemical characteristics of EA12, it was further separated by HPLC. Six compounds (**1**–**6**) were isolated and characterized from EA12. According to the mass and NMR analyses, compounds **1**–**6** were identified as vanillin (**1**)

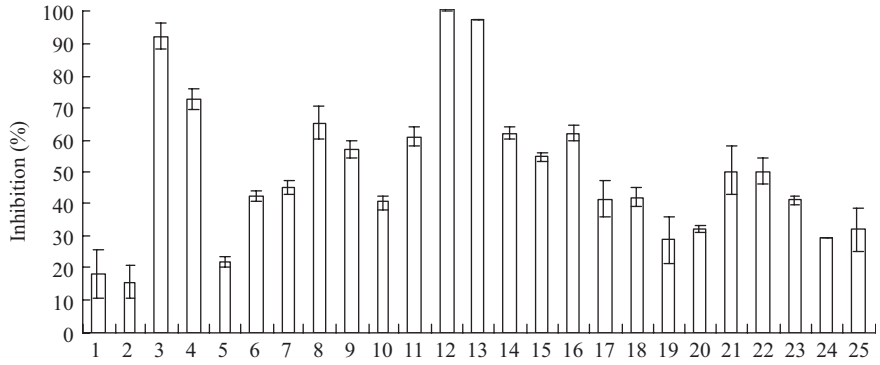

Fig. 1. Inhibitory effects of subfractions (EA1–EA25) derived from *C. formosensis* at 20 μg/mL on nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells.

(Lee et al., 2004), 4-hydroxybenzaldehyde (**2**) (Lee et al., 2004), *trans*-hinokiresinol (**3**) (Minami et al., 2000), taiwanin E (**4**) (Chang et al., 1999a), 4α-hydroxyeudesm-11-en-12-al (**5**) (Bohlmann et al., 1983) and savinin (**6**) (Chang et al., 1999b) (Fig. 3). Moreover, the NO inhibitory effectiveness of compounds **1**–**6** were evaluated by NO production in LPS-stimulated RAW264.7 cells test. According to the results summarized in Table 1, the quercetin was used as a reference in this study. The IC_{50} values of quercetin was 8.6 μM; in the mean time, compounds isolated from EA12 revealed good NO inhibition activity. Compound (**5**) shown the most significant activity against the NO production ($IC_{50} = 0.99 \pm 0.03$ μM). Compounds (**1**), (**2**) and (**3**) also showed significant activity to against NO production in a LPS-stimulated RAW264.7 cell system, and the IC_{50} were lower than 25 μM. Based on the results of the NO inhibition assay, the strong NO inhibition activity of EA12 may contribute by compounds (**5**), (**1**), (**2**) and (**3**).

It is known that iNOS is involved in the synthesis of nitric oxide. In order to evaluate potential mechanisms for the inhibition of NO production, we further investigated the effect of compounds isolated from *C. formosensis* on the expression of iNOS mRNA in LPS-stimulated RAW264.7 cells by using RT-PCR analysis. After RAW264.7 cells were stimulated with 1 μg/mL LPS for 6 h in the presence or absence of various concentrations of compounds (**1**), (**3**) and (**5**), the total RNAs of RAW264.7 cells were prepared and then analyzed by RT-PCR as performed. Fig. 4a–c show that RAW264.7 cells not stimulated with LPS expressed very low or undetectable levels of iNOS mRNA; on the other hand, when cells were treated with LPS only, iNOS mRNA was strongly expressed. All of the test compounds, including compounds (**1**) and (**3**), treated with 0.5–20 μg/mL and effectively suppressed LPS-induced iNOS mRNA expression in a dose-dependent manner as shown in Fig. 4. Compounds (**1**) and (**3**) could inhibit ~80% and ~74% of LPS-induced iNOS expression at

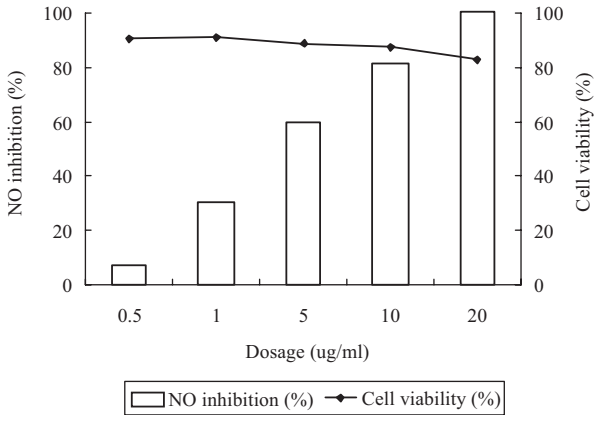

Fig. 2. Inhibitory effects of EA12 derived from *C. formosensis* at 20 μg/mL on nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells and cell viability determined by MTT colorimetric assay. (Quercetin was used as a reference compound in NO inhibitory assay, the IC_{50} of quercetin was 8.6.)

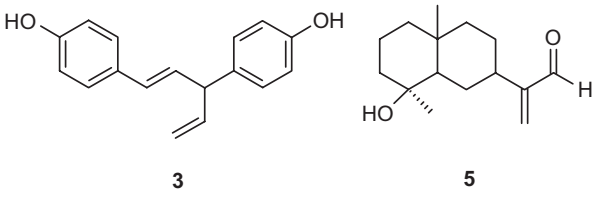

Fig. 3. Structures of compounds **3** and **5**.

the dosage of 10 μg/mL, respectively. As regard to the cytotoxicity of 4α- hydroxyeudesm-11-en-12-al (**5**), the dosage used in RT-PCR analysis was 0.1–5 μg/mL and also shown a dose-dependent inhibition activity, it can inhibit ~80% expression at 1 μg/mL. Since compounds (**1**), (**3**) and (**5**) could suppress the LPS-induced iNOS mRNA expression, we further confirmed the effect of these compounds on LPS-induced iNOS protein expression with immunoblot analysis. After RAW264.7 cells were stimulated for 18 h with 1 μg/mL of LPS in the

Table 1. NO inhibition activity and cytotoxicity of compounds isolated from *C. formosensis*[a]

	NO inhibition activity IC_{50} (μM)	Cytotoxicity IC_{50} (μM)
Vanillin	15.12±1.05	>131.54
4-hydroxybenzaldehyde	21.29±0.26	>163.77
Hinokiresinol	9.12±0.52	>79.27
Taiwanin E	84.62±0.89	53.31±1.99
4α-hydroxyeudesm-11-en-12-al	0.99±0.03	6.86±0.91
Savinin	51.94±0.62	>56.76
Quercetin[b]	8.6±0.02	>50

[a]Each assay was performed in triplicate.
[b]Quercetin was used as reference compounds in this experiment.

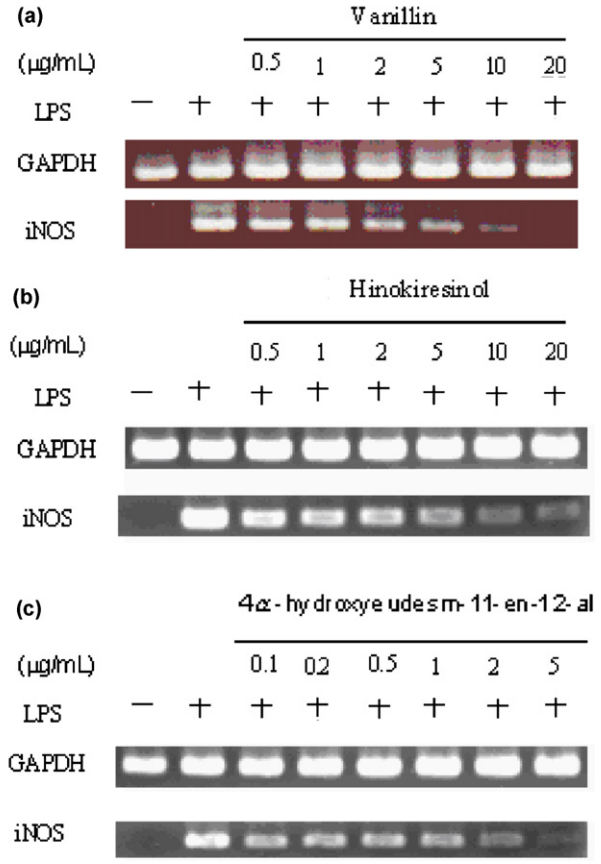

Fig. 4. Effect of vanillin (**a**), *trans*-hinokiresinol (**b**), and 4α-hydroxyeudesm-11-en- 12-al (**c**) on the expression of iNOS mRNA in LPS-stimulated RAW 264.7. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for the experiment.

presence of various concentrations of compounds (**1**), (**3**) and (**5**), total lysates and the immunoblot analysis was as performed. The results are shown in Fig. 5; all three of the compounds showed inhibition ability in LPS-induced iNOS protein expression (130 kDa) in a

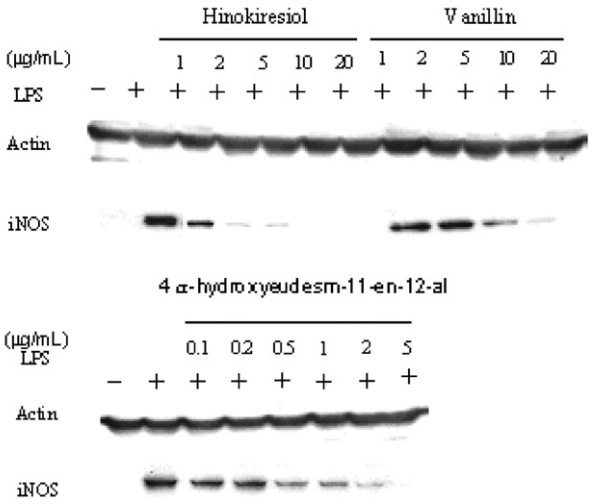

Fig. 5. Effect of vanillin, *trans*-hinokiresinol, and 4α-hydroxyeudesm-11-en-12-al on the expression of iNOS protein in LPS-stimulated RAW 264.7. Actin was used as an internal control for the experiment.

dose-dependent manner. The results imply that compounds decreased the protein levels of iNOS by reducing the expression of iNOS mRNA. As mentioned above, iNOS is one of the important enzyme mediators that mediate inflammatory processes. Improper expression of iNOS has been associated with the pathophysiology of certain types of human cancers as well as inflammatory disorders. The abilities of compounds (**1**), (**3**) and (**5**) to inhibit NO production and iNOS expression were in good consistency, indicating the NO production was inhibited through the inhibition of iNOS expression. The modulation of NO production by inhibiting iNOS expression may be of therapeutic value in relation to inflammation and septic shock (Surh et al., 2001). Therefore, the similar inhibition actions on NO and iNOS observed for *C. formosensis* extracts may be correlatable to the anti-inflammatory of this *C. formosensis*. Further epidemiological studies on this plant, for nutraceutical or pharmaceutical applications, are warranted.

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