

Short Communication

Bioactivity assay of extracts from *Calocedrus macrolepis* var. *formosana* bark

Louis Kuoping Chao^a, Kuo-Feng Hua^b, Hsien-Yeh Hsu^b,
Yu-Chang Su^c, Shang-Tzen Chang^{d,*}

^a Department of Biological Science and Technology, Chung Hwa College of Medical Technology, Tainan 717, Taiwan

^b Faculty of Biotechnology and Laboratory Science in Medicine, Institute of Biotechnology in Medicine, National Yang-Ming University, Taipei 112, Taiwan

^c Division of Wood Cellulose, Taiwan Forestry Research Institute, Taipei 100, Taiwan

^d School of Forestry and Resource Conservation, National Taiwan University, Taipei 106, Taiwan

Received 21 June 2005; received in revised form 17 October 2005; accepted 23 October 2005

Available online 15 December 2005

Abstract

Alcoholic extracts from bark of *Calocedrus macrolepis* var. *formosana* Florin (Cupressaceae) were extracted successively using *n*-hexane, dichloromethane, ethyl acetate, 1-butanol and water, which gave 34.8%, 34.1%, 24.1%, 3.3% and 3.7% soluble fractions, respectively. Antioxidation activity of these fractions by DPPH assay and dissimilar IC₅₀ values of the DPPH showed that ethyl acetate fraction had the best antioxidant activity; its IC₅₀ was 2.6 µg/ml. Analyses of the composition and anti-inflammatory activity of the subfractions from *n*-C₆H₁₄ fraction showed that the T3 and H5_{ppt} had the best anti-inflammatory activity in LPS-stimulated murine macrophage J774A. 1 cells, respectively; moreover, their major constituent was sugiol (T3 37.1%, H5_{ppt} 81.1%), which at dosages of 10 µg/ml inhibited proIL-1β protein production completely. Furthermore, the T1 also exhibited anti-inflammatory activity, and its major constituent was ferruginol (above 85.6%).

© 2005 Elsevier Ltd. All rights reserved.

Keywords: *Calocedrus macrolepis* var. *formosana*; Extracts; Antioxidant activity; Anti-inflammatory capacity

1. Introduction

Lipopolysaccharide (LPS) is the major wall component of Gram-negative bacteria (Raetz and Whitfield, 2002), which is able to activate monocytes/macrophages to secrete various inflammatory cytokines. Interleukin-1 beta (IL-1β) is one of the major mediators of inflammatory response mainly secreted from LPS-stimulated macrophages (Hsu and Wen, 2002; Wang et al., 2003). The *Calocedrus macrolepis* var. *formosana* Florin (Cupressaceae) is a native tree that grows at elevations of 800–1500 m in Taiwan's central mountains, whose bark is always discarded in the forestry industry. In addition, the bark of *C. macrolepis* var. *formo-*

sana has been the prevailing folklore medication in Taiwan's countryside in the past. Our recent research demonstrated that alcoholic extracts from *C. macrolepis* var. *formosana* exhibited a significant inhibitory activity against the DPPH radical (Wang et al., 2004). To further explore its potential as a source of natural drugs, the purpose of this study was to investigate the effective composition and anti-inflammatory capacity of the alcoholic extracts from barks of *C. macrolepis* var. *formosana* by using a murine macrophage model (J774A. 1 cell).

2. Methods

2.1. Extraction and fractionation

The samples of *C. macrolepis* var. *formosana* were collected in September 2003 from the Lien Hua-Chin

* Corresponding author. Tel.: +886 2 33664626; fax: +886 2 23654520.
E-mail address: peter@ntu.edu.tw (S.-T. Chang).

Research Center located in Nantou County in central Taiwan. The species was identified and voucher specimens were deposited at the laboratory of wood chemistry (School of Forestry and Resource Conservation, National Taiwan University). Barks of *C. macrolepis* var. *formosana* (10 kg d.w.) were extracted with ethanol (95% v/v, 10 d repeated 3 times) at room temperature. The extracts were concentrated to get alcoholic extracts (AE) approximately 410 g. AE (205 g) was successively extracted with *n*-hexane ($n\text{-C}_6\text{H}_{14}$), dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), 1-butanol (BuOH), and water (H_2O). The *n*-hexane fraction was applied on the top of a 160 g silica gel column. The column was eluted with $n\text{-C}_6\text{H}_{14}$ /EtOAc from 95/5 to 0/100, followed by eluting with EtOAc/alcohol from 100/0 to 0/100. Each eluted volume of subfraction was 1000 ml, except that H1 subfraction was 4000 ml. The collected subfractions were then dried. The water-soluble alcoholic extracts (WAE) fraction was extracted directly from AE using only H_2O .

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

The scavenging action of DPPH free radicals using different fractions of *C. macrolepis* var. *formosana* alcoholic

extracts was measured according to the method of Wang et al. (2004). The inhibition ratio (%) was calculated using the following equation: % inhibition = [(absorbance of control – absorbance of test sample)/absorbance of control] × 100.

2.3. Gas chromatography–mass spectrometry (GC–MS) analysis

All of the different samples from extracts and separated fractions were performed by GC–MS (HP 6890N, with MS detector model HP 5973) and equipped with a DB-5HT column (15 m in length, 0.25 mm i.d., 0.1 μm). The oven temperature was increased from 85 to 300 °C at a rate of 10 °C/min. The injection temperature was 300 °C, detector temperature was 280 °C and helium was used as a carrier gas at a split ratio of 20:1. The compounds were identified by comparison of their mass spectrometric fragmentation patterns with those of authentic standards.

2.4. Bioassay

Murine macrophage J774A.1 cell was obtained from ATCC (Rockville, MD), propagated in RPMI 1640

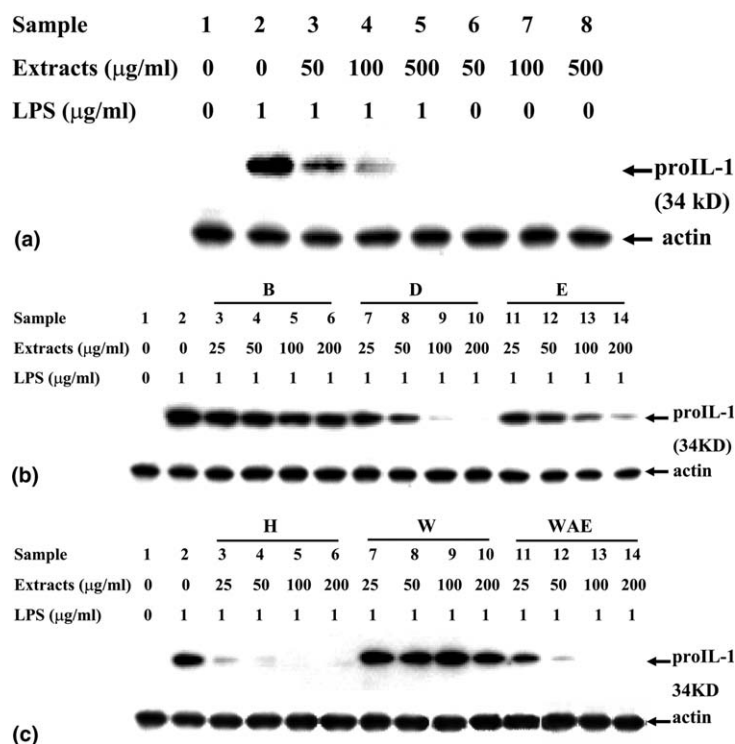


Fig. 1. Effects of alcoholic extracts from barks of *C. macrolepis* var. *formosana* on proIL-1 β protein expression in LPS-stimulated J774A.1 cells. Whole cell lysates were analyzed by Western blot using anti-IL-1 β and anti-actin antibody. The proIL-1 β and actin (as an internal control) are indicated as arrows on the right side. These experiments were repeated three times and a representative result is shown in the figure. (a) Cells were pretreated with indicated concentration of alcoholic extracts from barks of *C. macrolepis* var. *formosana* for 30 min, followed by LPS (1 $\mu\text{g/ml}$) or medium treatment for additional 6 h. Sample 1 was the control and sample 2 was only treated with LPS. (b) Cells were pretreated with indicated concentration of extracts from different fractions (B, BuOH fraction; D, CH_2Cl_2 fraction; E, EtOAc fraction) for 30 min, followed by LPS (1 $\mu\text{g/ml}$) treatment for additional 6 h. Sample 1 was the control and sample 2 was only treated with LPS. (c) Cells were pretreated with indicated concentration of extracts from different fractions (H, $n\text{-C}_6\text{H}_{14}$ fraction; W, H_2O fraction; WAE, WAE fraction) for 30 min, followed by LPS (1 $\mu\text{g/ml}$) treatment for additional 6 h. Sample 1 was the control and sample 2 was only treated with LPS.

medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Co., Logan, UT) and 2 mM L-glutamine (Life Technologies, Inc., MD) and cultured in a 37 °C, 5% CO₂ incubator, unless otherwise indicated. J774A. 1 cells were pretreated with extracts (dissolved in DMSO) for 30 min, followed by LPS (1 µg/ml) treatment for additional 6 h. ProIL-1β protein expression level was analyzed by Western blot using anti-IL-1β antibody as described by Hsu et al. (2001).

2.5. 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 ± SD.

3. Results and discussion

3.1. Yields of fractions and antioxidant activity

The *n*-C₆H₁₄ soluble fraction showed the highest yields (about 34.8%) and the next were the CH₂Cl₂ fraction 34.1%, EtOAc fraction 24.3%, H₂O fraction 3.7% and *n*-BuOH fraction 3.1%. The results of DPPH assay revealed EtOAc fraction had the best inhibitory activity against the DPPH radical, with an IC₅₀ value of 2.6 µg/ml. This value was 9-fold higher than the 25 µg/ml of alcoholic extracts of *C. formosana* in IC₅₀ (Wang et al., 2004). The secondary IC₅₀ value was 6.8 µg/ml from WAE fraction, and except

for the H₂O fraction, which showed the worst inhibitory activity at approximately 81.5 µg/ml, the others were between 15 and 17 µg/ml.

3.2. Anti-inflammatory activity of different fractions

Fig. 1a shows that a dosage of 50 µg/ml of the alcoholic extract (AE) from *C. macrolepis* var. *formosana* markedly inhibited proIL-1β protein expression in LPS-stimulated J774A. 1 cells (sample 3). At 100 µg/ml dose, approximately 95% of proIL-1β protein expression was inhibited (sample 4). AE did not stimulate the inflammatory cytokine proIL-1β in J774A. 1 cells (samples 6–8). Fig. 1b showed that the anti-inflammatory bioactivity of BuOH fraction (samples 3–6) and CH₂Cl₂ fraction (samples 7–10) were worse at different dosages for the J774A. 1 cells. Interestingly, the EtOAc fraction had the best antioxidant activity against DPPH in this study, but the anti-inflammatory bioactivity was not primarily effective. The *n*-C₆H₁₄ fraction (Fig. 1c, samples 3–6) at a low dosage of 25 µg/ml restrained 95% of proIL-1β protein expression (Fig. 1c). Both the anti-inflammatory ability and the antioxidant activity of the H₂O fraction were low (samples 7–10). At a dosage of 50 µg/ml, the anti-inflammatory ability of the WAE fraction could inhibit 98% of proIL-1β protein expression (Fig. 1c, samples 11–14), indicating that its efficacy was only slightly lower than that of the *n*-C₆H₁₄ fraction.

Fig. 2a demonstrated that T3 (the mixture of subfractions H5 and H6) and H5_{ppt} (the precipitant from subfraction H5 when it was concentrated) had the best anti-inflammatory capacity, respectively. At a dosage of 10 µg/ml, they inhibited

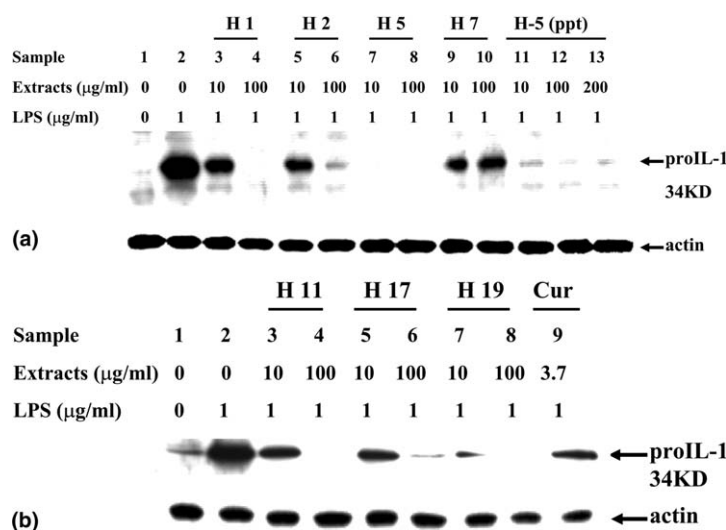


Fig. 2. Effects of subfractions from the *n*-C₆H₁₄ fraction on intracellular pro-IL-1β in LPS-stimulated J774A. 1 cells. The cells were pretreated with 10 and 100 µg/ml of different subfractions of hexane fraction for 30 min, respectively, followed by LPS (1 µg/ml) treatment for an additional 6 h. (a) Sample 1 was the control and sample 2 was only treated with LPS. The T1 sample contained only H1 subfraction, T2 was the mixture of subfractions H2, H3 and H4. T3 was the mixture of subfractions H5 and H6. T4 was the mixture of subfractions H7, H8, H9 and H10. The H5_{ppt} was the precipitant from subfraction H5 when it was concentrated. (b) Sample 1 was the control and sample 2 was only treated with LPS. T5 was the mixture of subfractions H11, H12, H13, H14, H15 and H16. T6 was the mixture of subfractions H17 and H18. T7 was the mixture of subfractions H19, H20, H21 and H22. Curcumin (3.7 µg/ml) was used as a positive control. Whole cell lysates were analyzed by Western blot using anti-IL-1β and anti-actin antibody. The proIL-1β and actin (as an internal control) are indicated as arrows on the right side.

all proIL-1 β protein expression (Fig. 2a, samples 7 and 8, and 11–13). Next, T7 and T1 separately, at a dosage of 100 μ g/ml completely inhibited proIL-1 β protein expression (Fig. 2b, samples 7 and 8).

3.3. Chemical composition of bioactive subfractions

By using GC–MS, it was found that the major constituent of T3 was sugiol (37.1%). Moreover, H5_{ppt} contained 81.1% sugiol. Similar result was obtained in the WAE fraction, which had 39.3% sugiol, and it was also accompanied by a good inhibitory effect on LPS-induced proIL-1 β protein expression. The major constituent of T1 was ferruginol (85.6%), which had fine anti-inflammatory activity.

4. Conclusion

Results demonstrated that a low dosage of 10 μ g/ml of H5_{ppt} from *C. macrolepis* var. *formosana* completely inhibited proIL-1 β protein expression in LPS-stimulated macrophages. Its major constituent was sugiol (contains 81.1%). This is the first report to demonstrate that alcoholic extracts of *C. macrolepis* var. *formosana* bark have an anti-inflammatory activity in macrophages. However, the efficacy and safety of these compounds needs to be further

investigated, if it is to be used as a source of natural drugs in the future.

Acknowledgements

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

References

- Hsu, H.Y., Wen, M.H., 2002. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. *J. Biol. Chem.* 277, 22131–22139.
- Hsu, H.Y., Chiu, S.L., Wen, M.H., Chen, K.Y., Hua, K.F., 2001. Ligands of macrophage scavenger receptor induce cytokine expression via differential modulation of protein kinase signaling pathways. *J. Biol. Chem.* 276, 28719–28730.
- Raetz, C.R.H., Whitfield, C., 2002. Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* 71, 635–700.
- Wang, J.H., Majella Doyle, M., Manning, B.J., Blankson, S., Wu, Q.D., Power, C., Cahill, R., Redmond, H.P., 2003. Cutting Edge: Bacterial lipoprotein induces endotoxin-independent tolerance to septic shock. *J. Immunol.* 170, 14–18.
- Wang, S.Y., Wu, J.H., Cheng, S.S., Lo, C.P., Chang, H.N., Shyur, L.F., Chang, S.T., 2004. Antioxidant activity of extracts from *Calocedrus macrolepis* var. *formosana* leaf, bark and heartwood. *J. Wood Sci.* 50, 422–426.