

## Inhibitory Principles from *Magnolia officinalis* on Tumor Necrosis Factor- $\alpha$ Production in Lipopolysaccharide-Activated RAW264.7 cells

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**Abstract** – In the course of a search for tumor necrosis factor (TNF)- $\alpha$  inhibitory compounds from medicinal plants, we identified neolignans, honokiol and magnolol, from the alcoholic extract of *Magnolia officinalis* as active inhibitory principles. These compounds dose-dependently inhibited TNF- $\alpha$  production without displaying cytotoxicity and their inhibitory activities measured by IC<sub>50</sub> values were 53.7 and 61.4  $\mu$ M, respectively.

**Key words** – *Magnolia officinalis*, honokiol, magnolol, TNF- $\alpha$  production.

### Introduction

*Magnolia officinalis* is a well known plant, widely distributed in China, Korea and Japan. The plant has been used for the relief of fever, headache, anxiety, diarrhea, stroke, allergy, and inflammation. The major constituents of the plant that have been identified include neolignans such as honokiol and magnolol.

These compounds were known to have many different biological activities tested in *in vitro* and *in vivo* including anti-platelet-aggregation effect (Teng *et al.*, 1988), anti-microbial effect (Chang *et al.*, 1998), anti-peroxidative effect (Fujita and Taira 1994; Haraguchi *et al.*, 1997), inhibitory effect of skin tumor promotion (Konoshima *et al.*, 1991), inhibitory effect of acetyl-Co A : cholesterol acyltransferase (Kwon *et al.*, 1997), anxiolytic effect (Maruyama *et al.*, 1998), anti-neutrophil aggregation effect (Wang *et al.*, 1998) and anti-inflammatory effect (Wang *et al.*, 1992; 1993; 1995).

In our previous screening study, a potent inhibitory activity on TNF- $\alpha$  production was found in total ethanol extract of *Magnolia officinalis*. TNF- $\alpha$  has been known to be involved in several inflammatory and allergic diseases such as asthma, rheumatoid arthritis, acute inflammation, endotoxin shock, allergic skin diseases, and transplanted organ rejection (Manogue

*et al.*, 1992; Firestein, 1994). This paper describes identification and inhibitory effect of major active principles, honokiol and magnolol, from *Magnolia officinalis*, monitored by TNF- $\alpha$  production assay.

### Materials and Methods

**Animals** – Eight-week-old C57BL/6 male mice were purchased from B & K Universal (Fremont, CA, USA). The mice were maintained in plastic cages

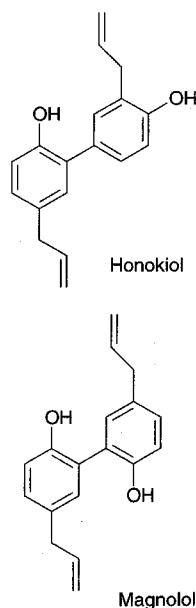


Fig. 1. The chemical structures of magnolol and honokiol isolated from *Magnolia officinalis*.

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under conventional conditions. Water and pelleted diets (Samyang, Taejeon, Korea) were supplied *ad libitum*.

**Materials** – *Magnolia officinalis* (cortex) was supplied from a drug store (Dongyang Yakup Co.) in Korea. The voucher specimen is deposited in our laboratory. Honokiol and magnolol (Figure 1) as a standard compound were purchased from Wako Pure Chemical (Japan). Prednisole, pentoxifylline, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and LPS (*E. coli* 0111 : B4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS), penicillin, streptomycin and RPMI1640 were obtained from GIBCO (Grand Island, NY, USA). RAW264.7 cell, a murine macrophage cell line, was purchased from ATCC (Rockville, MD, USA). All other chemicals were of reagent grade.

**Extraction** – Air-dried *Magnolia officinalis* was pulverized using milling machine and extracted at 95 with 70% aq. EtOH ( $\times 3$ ). The extract was filtered, and the filtrate was concentrated *in vacuo* and lyophilized. The lyophilized crude extract (2.4 g) of *Magnolia officinalis* (cortex, 20 g) was suspended in distilled water and extracted with *n*-hexane (470 mg), methylene chloride ( $\text{CH}_2\text{Cl}_2$ , 695 mg), ethyl acetate (EtOAc, 386 mg) and butanol (*n*-BuOH, 580 mg) sequentially to yield respective solvent extracts. The hexane extract was separated by prep-TLC using *n*-hexane : EtOAc (1 : 1) to obtain three sub-fractions.

**TNF- $\alpha$  production *in vitro*** – The inhibitory effect of testing fractions or compounds on TNF- $\alpha$  production was determined as previously described (Cho *et al.*, 1998c). Briefly, RAW 264.7 cells were maintained in RPMI1640 supplemented with 100 U/ml of penicillin and 100  $\mu\text{g}/\text{ml}$  of streptomycin, and 5% FBS. Cells were grown at 37°C and 5%  $\text{CO}_2$  in humidified air. The isolated compounds and fractions solubilized with vehicle (89.9% propylene glycol, 10% ethanol and 0.1% dimethyl sulfoxide (DMSO)) were diluted with RPMI1640. The final concentration of vehicle never exceeded 0.1% in the culture medium. In these conditions, none of the solubilization solvents altered TNF- $\alpha$  production in RAW264.7 cells. Before stimulation with LPS (1  $\mu\text{g}/\text{ml}$ ) and testing samples, RAW264.7 cells (1  $\times 10^6$  cells/ml) were incubated for 18 h in 24 well plates with the same conditions. Stimuli and the various concentrations of testing samples were then added to the wells for 4 h incubation that was found to be optimal for TNF- $\alpha$  production in our conditions. Supernatants were then

collected and assayed for TNF- $\alpha$  content using mouse TNF- $\alpha$  ELISA kit (Amersham, Little Chalfont, Buckinghamshire, UK). Viability of the cells was assessed by Trypan blue dye exclusion and was always more than 95%.

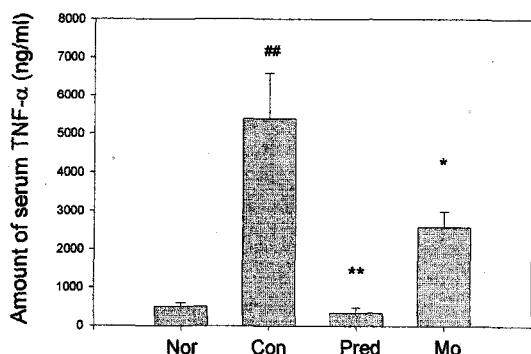
**TNF- $\alpha$  production *in vivo*** – The methods of Novgrodiski *et al.*, (1994) was used for LPS induction of TNF- $\alpha$  *in vivo*. Fasted mice were orally administered with the test fraction (200 mg/kg) suspended in 0.5% sodium carboxymethylcellulose and standard drug (prednisolone, 10 mg/kg) 2 h before challenge with intraperitoneal injection of LPS. After 90 min, blood was collected and serum samples were used to measure TNF- $\alpha$  levels by ELISA kit.

**Cytotoxicity assay** – Cytotoxicity of honokiol and magnolol in TNF- $\alpha$  production assay was evaluated by MTT assay with minor modification as reported previously (Cho *et al.*, 1998b). The cell suspension having concentration of 1  $\times 10^6$  cells/ml was plated in 96 well plate. After 18 h culture, varying concentrations of testing compounds and stimuli were added to each well and cultured for another 4 h. Finally, 10  $\mu\text{l}$  of MTT solution (10 mg/ml in phosphate buffered saline, pH 7.4) was added to each well and incubated for another 3 h. Culture was stopped by addition of 15% sodium dodecyl sulfate into each well for solubilization of formazan and the optical density (OD) at 570 nm ( $\text{OD}_{570-630}$ ) was measured by a microplate Spectramax 250 microplate reader. The mean value of OD content of 4 wells was used for calculating the viability (% of control).

**Statistical analysis** – All values expressed as mean  $\pm$  SEM were obtained from 4 or 6 observations. The Student's *t*-test for unpaired observation between control and experimental samples was carried out for statistical evaluation of a difference; *p* values of 0.05 or less were considered as statistically significant.

## Results and Discussion

**Inhibitory effect of *Magnolia officinalis* on TNF- $\alpha$  production** – In the sequential fractionation of ethanol extract, the inhibitory activity on TNF- $\alpha$  production was assayed in order to trace the active principle. The highest inhibitory activity of sequential fraction from crude extract (67.1%) was obtained with the *n*-hexane (98.0%) and  $\text{CH}_2\text{Cl}_2$  extract (60.0%) as compared with the other solvents including EtOAc (42.3%), *n*-BuOH (18.3%) and  $\text{H}_2\text{O}$  (3.1%) at 50  $\mu\text{g}/\text{ml}$  as a final concentration (Table 1). In addition to *in*



**Fig. 2.** The inhibitory effect of ethanol extract from *Magnolia officinalis* on TNF- $\alpha$  production in LPS-treated mice (n = 6).

Fasted mice were administered with the test fraction and standard drug (Nor. normal; Con. control; Pred. prednisolone; Mo. *Magnolia officinalis*) 2 h before challenge with intraperitoneal injection of LPS (1.5 mg/mouse). After 90 min, blood was collected and serum samples were used to measure TNF- $\alpha$  levels by ELISA kit. Values are expressed as the mean  $\pm$  SEM of 6 animals. \*\*p<0.01, \*p<0.05 represent significant difference compared to control (LPS-treated group) and #: p<0.01 represents significant difference compared to normal group.

*in vitro* effect, the crude extract also significantly suppressed serum TNF- $\alpha$  production in LPS-treated mice (Fig. 2) at the dose of 200 mg/kg with inhibitory effect of  $52.1 \pm 8.6\%$ . The *n*-hexane extract was separated by preparative TLC using *n*-hexane/EtOAc (1 : 1) solvent mixture to obtain three fractions. Among them, the highest inhibitory activity was shown in *n*-hexane-2 fraction (74.1%) (Table 1).

To identify the active principles, we compared  $R_f$  values of the commercially available honokiol and magnolol which are known to be the main compounds from *Magnolia officinalis* with that of major spot from the active *n*-hexane-2 fraction in the TLC analysis using *n*-hexane/EtOAc (1 : 2) solvent mixture. As expected, the  $R_f$  value of major spot from the fraction was in agreement with those of standard honokiol and magnolol showing the same  $R_f$  value on the TLC plate (Data not shown). Furthermore, we also found that the major spot of the fraction was composed of two compounds, honokiol and magnolol, compared to the peaks of standard honokiol and magnolol at HPLC analysis (Data not shown). Therefore, we concluded that the major spot from *n*-hexane-2 fraction was the mixture of honokiol and magnolol, and these compounds are the possible

**Table 1.** The inhibitory effect of solvent fractions from *Magnolia officinalis* on TNF- $\alpha$  production in RAW264.7 stimulated by 1  $\mu$ g/ml of LPS for 4 h

Fraction	Concentration ( $\mu$ g/ml)	% of inhibition
Total ethanol extract	100	67.1 $\pm$ 2.3
Solvent fraction		
<i>n</i> -Hexane	50	98.0 $\pm$ 2.1
<i>n</i> -Hexane-1	25	2.8 $\pm$ 5.4
<i>n</i> -Hexane-2	25	74.1 $\pm$ 2.4
<i>n</i> -Hexane-3	25	27.2 $\pm$ 4.3
Methylene chloride	50	60.0 $\pm$ 1.8
Ethyl acetate	50	42.3 $\pm$ 6.1
<i>n</i> -Butanol	50	18.3 $\pm$ 6.4
Water	50	3.1 $\pm$ 1.7

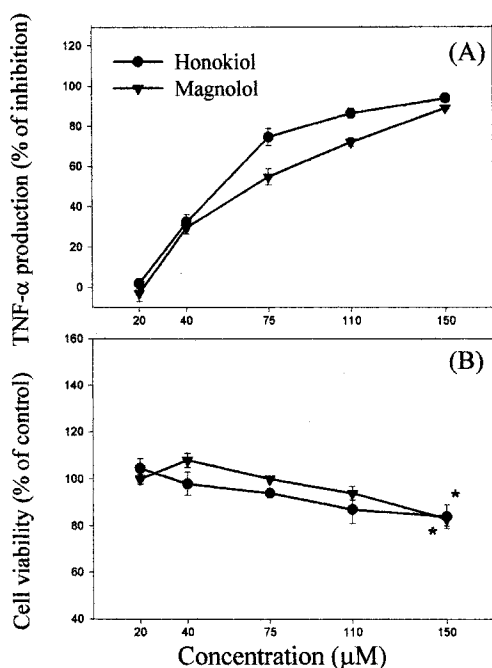
RAW264.7 cells ( $1 \times 10^6$  cells/ml) were stimulated by 1  $\mu$ g/ml of LPS with each concentration of fractions. Supernatants were collected after 4 h and assayed by ELISA. Data represent mean  $\pm$  SEM of 4 observations. Basal and stimulated levels of TNF- $\alpha$  were 0.5–1.0 ng/ml and 40–50 ng/ml, respectively.

active principles from *Magnolia officinalis* on TNF- $\alpha$  production in LPS-activated RAW264.7 cells.

**Honokiol and magnolol inhibited TNF- $\alpha$  production** – As previously mentioned, culture supernatant of RAW264.7 cells cultured for 6 h contained 0.5 to 1 ng/ml of TNF- $\alpha$ . When RAW264.7 cells were stimulated with 1  $\mu$ g/ml of LPS for 6 h, the cells released TNF- $\alpha$  into culture medium until about 50 to 60 ng/ml, more than 60–100-fold amount than the basal level. However, to exclude the cytotoxic effect by testing drugs and solvent extracts we minimized the incubation time of the drug. Consequently, 4 h incubation was applied to analyze suppressive effect on TNF- $\alpha$  production by these compounds and positive control drugs, prednisolone and pentoxifylline.

In agreement with the subfraction results, honokiol and magnolol significantly inhibited TNF- $\alpha$  production in a dose-dependent manner with  $IC_{50}$  values of 53.7 and 61.4  $\mu$ M, respectively. The compounds were not cytotoxic at tested concentration and 4 h of incubation. Hence, it was suggested that their inhibitory effects on TNF- $\alpha$  production were due to the interference of the biosynthetic pathway of TNF- $\alpha$  but not non-specific reaction (Fig. 3 and Table 2).

In terms of TNF- $\alpha$  inhibitory potency compared to natural products, their activities were comparable to that of previously reported lignan compounds of furofuran type (pinoresinol and eudesmin :  $IC_{50} = 50\sim$



**Fig. 3.** The inhibitory effect of honokiol and magnolol on TNF- $\alpha$  production (A) in LPS-stimulated RAW 264.7 cells for 4 h and the effect of these compounds on cell viability of RAW264.7 cells at TNF- $\alpha$  production conditions (B).

RAW264.7 cells ( $1 \times 10^6$  cells/ml) were stimulated by 1  $\mu$ g/ml of LPS with various concentrations of honokiol and magnolol. Supernatants were collected after 4 h and assayed by ELISA. Cytotoxicity was assayed by conventional MTT method at the same condition with TNF- $\alpha$  production assay. Data represent mean  $\pm$  SEM of 4 observations. Basal and stimulated levels of TNF- $\alpha$  were 0.5–1.0 ng/ml and 40–50 ng/ml, respectively.

\* $p < 0.05$  represents significant difference compared to control.

100  $\mu$ M) (Chae *et al.*, 1998; Cho *et al.*, 1998b) and coumarin type (osthol and oxypeucedanin : IC<sub>50</sub> = 50–70  $\mu$ M) (Cho *et al.*, 1998a), but was lower than that of sesquiterpene lactones (cynaropicrin and parthenolide : IC<sub>50</sub> = 0.5–10  $\mu$ M) under the same conditions (Hwang *et al.*, 1996; Cho *et al.*, 1998c), and bisbenzylisoquinoline (fangchinoline and isotetrandrine : IC<sub>50</sub> = 2.2–18.3  $\mu$ M) (Onai *et al.*, 1995).

In respect to the possible inhibitory mechanism, major pathway by honokiol and magnolol appears to be due to the antioxidative effect as the activities were proportional to their scavenging effects on hydroxyl radicals (Fujita and Taira, 1994; Haraguchi *et al.*, 1997). In general, it was known that radical scavengers such as *N*-acetyl-L-cysteine, pyrrolidine dithio-

**Table 2.** The molar concentrations of honokiol and magnolol, and standard compounds (prednisolone and pentoxifylline) producing 50% inhibition (IC<sub>50</sub>) of TNF- $\alpha$  production in RAW264.7 cells stimulated by LPS.

Compound	IC <sub>50</sub> ( $\mu$ M)
Honokiol	53.7 $\pm$ 1.8
Magnolol	61.4 $\pm$ 3.6
Pentoxifylline	247.2 $\pm$ 14.3
Prednisolone	32.3 $\pm$ 4.7

RAW264.7 cells ( $1 \times 10^6$  cells/ml) were stimulated by 1  $\mu$ g/ml of LPS for TNF- $\alpha$  assay with various concentrations of testing compounds. TNF- $\alpha$  was assayed as described under Materials and Methods. Data represent mean  $\pm$  SEM of 4 observations.

carbamate, acetylsalicylic acid and curcumin inhibit the activation of NF- $\kappa$ B, transcription factor, resulting in inhibition of various kinds of inflammatory mediators such as NO, TNF- $\alpha$ , and IL-1 (Peristeris *et al.*, 1992; Vulcano *et al.*, 1998). In view of these, it is suggested that honokiol and magnolol as radical scavengers may inhibit the other inflammatory mediators.

In conclusion, we have shown not only that ethanol extract from *Magnolia officinalis* significantly inhibited *in vitro* and *in vivo* TNF- $\alpha$  production, but also that honokiol and magnolol were major active inhibitory principles from the extract. The molecular mechanism of action or other immunomodulatory effects of the compounds remains unclear, and to be solved with further studies.

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