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Phenolic glycosides as inhibitors of inducible nitric oxide synthase from *Populus davidiana* in LPS-activated RAW 264.7 murine macrophages

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Received December 29, 2011, accepted January 27, 2012

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Pharmazie 67: 870–873 (2012) *doi: 10.1691/ph.2012.1869*

Nitric oxide (NO) produced in large amounts by inducible nitric oxide synthase (i-NOS) is known to be responsible for the vasodilation and hypotension observed in septic shock and inflammation. Inhibitors of i-NOS, thus, may be useful candidates for the treatment of inflammatory diseases that accompany the overproduction of NO. Two phenolic glycosides, salicortin (**1**) and salicortin-6′-benzoate (**2**), were purified as active principles from the extracts of *Populus davidiana* by activity-guided purification procedures. They showed dose dependent inhibition of NO production in lipopolysaccharide (LPS)-activated RAW 264.7 cells. The IC_{50} values of salicortin (**1**) and salicortin-6′-benzoate (**2**) was 15 μM and over 50 μM, respectively. In immunoblot analysis, salicortin inhibited the expression of i-NOS. These new inhibitors of overproduction of NO may have potentials for the treatment of inflammation.

1. Introduction

Application of natural products for the treatment of human diseases has had a long history from ancient to modern drug development. Indeed, majority of present pharmaceuticals have their origin in natural products (Newman and Cragg 2004; Subramanian et al. 2006). We have been involved in searching for and discovering new anti-inflammatory agents from natural products. Inflammation is an important host defense mechanism against tissue injuries and invading pathogens. However, inflammation plays a central role in pathological processes such as septic shock, inflammatory bowel disease, cancer, rheumatoid arthritis, bronchitis, gastritis, multiple sclerosis and metabolic disorder (Kundu and Surh 2008). Macrophages are key players in inflammatory response and the production of pro-inflammatory mediators including nitric oxide (NO) (Lin and Karin 2007).

Nitric oxide (NO), a gaseous free radical, is produced through the oxidation of arginine by nitric oxide synthase (NOS) (Nathan and Xie 1994). The endothelial (e-NOS) and neuronal (n-NOS) isoforms are calcium-dependent and have important roles in the regulation of blood pressure and neurotransmission, whereas the inducible isoform (i-NOS) is calcium-independent and inducible by LPS and various cytokines such as IFN-α, IL-1β, and TNF-α. Low concentrations of NO produced by i-NOS exerts beneficial roles in antimicrobial activity of macrophages against pathogens, while the overproduction of NO has been suggested to be mutagenic *in vivo* and to provoke the pathogenesis of septic shock and various inflammatory processes (Forstermann et al. 1991). Furthermore, NO has also been shown to be carcinogenic (Hiraku et al. 2010). Therefore, the inhibition of NO production by i-NOS may present an useful strategy for the treatment of various inflammatory diseases. Previously, we reported the inhibitory activity of NO production by alcoholic extracts of woody plants in LPS-activated RAW 264.7 murine macrophages. In the course of screening experiments on potential inhibitors of i-NOS, we found that *Artemisia iwayomogi*, *Machilus thunbergii*, *Populus davidiana* and *Populus maximowiczii* showed potent inhibitory activity of NO production among 83 kinds of alcoholic extracts of woody plants (Ryu et al. 2003). In the current study, we report the activity-guided fractionation of *Populus davidiana* and the isolation of phenolic glycosides as active principles. The structures of isolated components were elucidated as salicortin (**1**) and salicortin-6′-benzoate (**2**) on the basis of spectroscopic analysis. The pure compounds salicortin (**1**) and salicortin-6′-benzoate (**2**) inhibited the LPS-induced NO production in murine macrophages in a dose dependent manner. In immunoblot analysis, salicortin (**1**) inhibited the expression of i-NOS. Our work suggests that salicortin (**1**) and salicortin-6′-benzoate (**2**) may have therapeutic potentials for the inflammatory diseases accompanied by the overproduction of NO.

2. Investigations and results

2.1. *Inhibitory activity of NO production by solvent fractions of Populus davidiana*

Previous reports showed that a methanolic extract of *Populus davidiana* inhibited the LPS-induced NO production in activated RAW 264.7 murine macrophages. For further study, we performed sequential solvent fractionation with hexane, EtOAc and BuOH. We evaluated the effect of solvent fractions of *Populus davidiana* on LPS induced NO production in RAW 264.7 cells. Cells were treated with 10, 30 and 50 μg/ml of solvent fractions in the presence of LPS (1 μg/ml) for 20 h. Each fraction suppressed the production of NO dose-dependently in LPS-stimulated macrophages (Table 1). As shown in Table 1,

Table 1: Effects of solvent fractions from *Populus davidiana* on LPS-induced NO production in RAW 264.7 macrophages

Solvent fractions	Inhibition (%)		
	10 μg/ml	30 μg/ml	50 μg/ml
Hexane	$13.7 \pm 0.7^{*}$	$38.3 \pm 1.1^{**}$	$41.6 \pm 0.6^{**}$
EtOAc	$46.0 \pm 2.1^{*}$	$86.0 \pm 2.7^{**}$	$86.4 \pm 3.5^{**}$
BuOH	$32.7 \pm 1.5^{*}$	$58.7 \pm 1.8^{**}$	$73.0 \pm 2.4^{**}$

Hexane: Hexane fraction; EtOAc: Ethylacetate fraction; BuOH: Butanol fraction. LPS-stimulated value differs significantly from control at a level of $p < 0.001$. Results differ significantly from the LPS-treated, $^{*}p < 0.01$ and $^{**}p < 0.001$, respectively.

the EtOAc fraction exerted the most potent inhibitory activity (86.4 ± 3.5 %) on LPS induced NO production at 50 μg/ml, while hexane and BuOH fractions inhibited NO production with 41.6 and 73.0% at 50 μg/ml, respectively. The EtOAc fraction was subjected to further activity-guided fractionation for the purification of active principle. The inhibitory activities of NO production by solvent fractions were calculated using the followed equation;

$\text{Inhibition (\%)} = 100 \times [OD_{LPS} - OD_{sample}] / [OD_{LPS} - OD_{media}]$

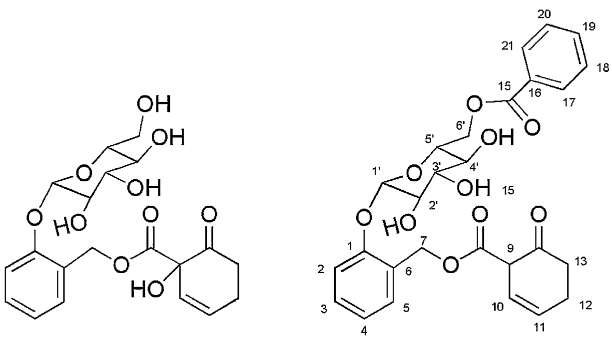
The values of OD were measured at 540 nm as described in the Experimental section for each treated groups.

2.2. *Inhibitory activity of NO production by phenolic glycosides*

Bioassay−guided fractionation of EtOAc soluble fraction from *Populus davidiana* led to the isolation of salicortin (**1**) and salicortin-6′-benzoate (**2**) as active compounds (Fig. 1). We evaluated the inhibitory effects of salicortin (**1**) and salicortin-6′-benzoate (**2**) on the production of NO in LPS-activated RAW 264.7. Salicortin (**1**) and salicortin-6′-benzoate (**2**) inhibited the NO production in a dose dependent manner (Table 2), whereas LPS treatment dramatically increased the concentrations of NO. The IC_{50} values of salicortin (**1**) and salicortin-6′-benzoate (**2**) were 15 μM and over 50 μM, respectively. The activity of salicortin (**1**) was greater than that of salicortin-6′-benzoate (**2**) that contains an extra benzoyl moiety in the salicortin skeleton. These result guided us to examine the effect of salicortin on the expression of i-NOS in LPS-activated macrophages.

2.3. *Effect of salicortin on i-NOS protein expression*

We determined the effect of salicortin (**1**) on the expression of the pro-inflammatory enzyme i-NOS which produce NO as a key mediator of inflammation by Western blot analysis. As shown



1 **2**

Fig. 1: Chemical structures of salicortin (**1**) and salicortin-6′-benzoate (**2**) purified from *Populus davidiana*

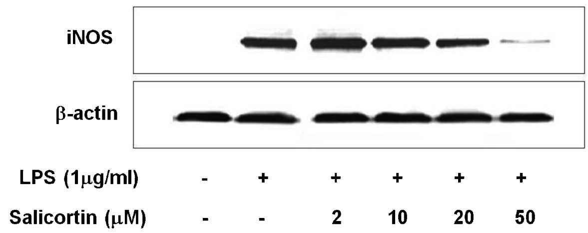


Fig. 2: Effect of salicortin (**1**) on LPS-induced i-NOS protein expression in RAW 264.7 macrophages. Whole cell lysates were subjected to SDS-PAGE, and the protein levels of i-NOS were determined by Western blot analysis. RAW 264.7 macrophages were treated with LPS (1 μg/ml) in the presence or absence of salicortin (**1**) for 20 h. Images are the representative of three independent experiments that shows similar results

in Fig. 2, i-NOS protein levels were markedly up-regulated by LPS treatment. Salicortin (**1**) attenuated the expression of i-NOS protein levels in a concentration-dependent manner, whereas the housekeeping gene β-actin expression was not changed. These results are consistent with the profile of the suppressive activity of salicortin (**1**) on LPS induced overproduction of NO. These results suggest that salicortin (**1**) controls the LPS-induced expression of i-NOS protein in murine macrophages.

3. Discussion

In the course of searching for naturally occurring anti-inflammatory agents, we found that the methanol extracts of *Populus davidiana* showed the inhibitory activity of LPS-induced NO production. *Populus davidiana* (Salicaceae) has been used in folk medicine as an antipyretic, analgesic, anti-inflammatory treatment in Korea. The flavonoids from *Populus davidiana* showed inhibitory activity against cyclooxygenase 1 and cyclooxygenase 2 (Zhang et al. 2006a). Zhang et al. (2006 b) reported the anti-oxidant activities of phenolic glycosides from the stem bark of *Populus davidiana*. The methanolic extracts of *Populus davidiana* were subjected to solvent fractionation with hexane, EtOAc and BuOH for further study (Table 1). Subsequent activity-guided fractionation of EtOAc extracts led to the isolation of two phenolic glycosides (salicortin and salicortin-6′-benzoate) as active compounds (Fig. 1).

Chronic inflammatory diseases such as multiple sclerosis, bronchitis and colon cancer lead to the up-regulation of pro-inflammatory enzymes and the production of inflammatory mediators in affected tissues and cells. These pro-inflammatory enzymes include the inducible nitric oxide synthase which is responsible for the overproduction of NO (Appleton et al. 1996). Nitric oxide (NO), generated from L-arginine by nitric oxide synthase (NOS), is an important regulatory molecule in a range of physiological and pathological processes (Garcia and Stein 2006). The calcium-regulated constitutive isoforms (e-NOS, endothelial NOS; n-NOS, neuronal NOS) play crucial roles in smooth muscle relaxation, platelet inhibition, vasodilation and neurotransmission by releasing the nanomolar range of NO (Palmer et al. 1988; Nathan 1992; Bredt 1999). In stimulated macrophages, the micromolar range of NO can be produced by the inducible form of nitric oxide synthase (i-NOS), that has been implicated in a number of pathogenic conditions including inflammatory diseases (Moncada and Higgs 1995; Kubes and McCafferty 2000). Thus, the inhibition of NO production by blocking i-NOS expression may be a useful strategy for the treatment of various inflammatory diseases. As shown in Table 2, salicortin (**1**) and salicortin-6′-benzoate (**2**) showed significant and dose-dependent suppression of LPS-stimulated NO production in RAW 264.7 macrophages. Salicortin (**1**) inhibited the overexpression of i-NOS protein in LPS-stimulated macrophage model (Fig. 2). These results demonstrate that the

Table 2: Effects of compounds 1 and 2 from *Populus davidiana* on LPS-induced NO production in RAW 264.7 macrophages

Compounds	Inhibition (%)						
	5 μM	10 μM	20 μM	30 μM	50 μM	100 μM	150 μM
1	$27.2 \pm 0.2^*$	$28.1 \pm 0.4^*$	$60.2 \pm 2.0^{**}$	$72.6 \pm 5.3^{**}$	$79.7 \pm 3.1^{**}$	–	–
2	–	–	–	–	$28.8 \pm 0.9^*$	$60.0 \pm 4.6^{**}$	$76.6 \pm 2.8^{**}$

LPS-stimulated value differs significantly from control at a level of $p < 0.001$. Results differ significantly from the LPS-treated, $^*p < 0.01$ and $^{**}p < 0.001$, respectively.

EtOAc soluble fraction of *Populus davidiana* and their active compounds (salicortin and salicortin-6′-benzoate) may be useful for the treatment of inflammatory diseases accompanying the overproduction of NO.

4. Experimental

4.1. Reagents and materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Laboratories (Detroit, MI) and LPS (*Escherichia coli*, 0127:B8), bovine serum albumin, sodium nitrite, and *N*-(1-naphthyl) ethylenediamine were obtained from Sigma Chemical Co. (St. Louis, MO).

4.2. Plant material

Populus davidiana (Salicaceae) was collected from Pochun-Gun, the northern part of Kyung-Gi Province, Korea. Voucher was deposited in the laboratory of forest chemistry, department of forest products, Kookmin University. Verification of voucher was performed by Sungsik Kim, a researcher of Kwangnung Arboretum, Forest Research Institute, Korea.

4.3. Activitiy-guided fractionation, purification and structure elucidation

The methanolic extract of *Populus davidiana* (200 g) was dispersed in water and extracted with hexane to give a hexane soluble fraction. The remaining water layer was extracted again with EtOAc and n-BuOH, sequentially to yield EtOAc and BuOH soluble fractions. The EtOAc fraction (18 g) was applied to a silica gel column and eluted in a stepwise gradient mode with CH_2Cl_2 and MeOH. Four fractions were obtained and each fraction was tested for the inhibitory effect on LPS induced NO production. The third fraction was further subjected to bioactivity-guided fractionation resulting the isolation of compound **1** (20 mg) and compound **2** (15 mg) as active compounds. The chemical structures of compounds **1** and **2** were verified by ^1H and ^{13}C-NMR spectra. The overall spectral patterns of **1** and **2** were quite similar except that compound **2** contained the benzoly moiety. Chemical shift values of compounds **1** and **2** are well matched with those reported for the respective phenolic glycosides, salicortin and salicortin-6′-benzoate (Dommisse et al. 1986; Nichols-Orians et al. 1992). The treated concentrations of materials showed no toxicity in RAW 264.7 murine macrophages.

Salicortin (1) $C_{20}H_{24}O_{10}$, ^1H NMR (300 MHz, CD_3OD) δ: 6.96–7.40 (4H, *m*, H-2 ∼ H-5), 6.12 (1 H, *dt*, *J* = 7.0, 4.0 Hz, H-11), 5.96 (1H, *d*, *J* = 7.0 Hz, H-10), 5.36 (1H, *d*, *J* = 11.0 Hz, H-7a), 5.21–5.45 (2 H, *m*, H-1′ and H-2′), 5.21 (1H, *d*, *J* = 11.0 Hz, H-7b), 3.43–3.95 (4 H, *m*, H-3′ ∼ H-6′), 2.45–2.61 (2H, *m*, H_2–12), 2.61–2.85 (2H, *m*, H_2–13). ^{13}C NMR (100 MHz, CD_3OD) δ: 207.4 (C-14), 171.5 (C-8), 156.9 (C-1), 133.4 (C-10), 131.0 (C-3), 130.6 (C-11), 129.3 (C-5), 126.0 (C-2), 123.4 (C-4), 116.6 (C-6), 102.4 (C-1′), 79.2 (C-9), 78.1 (C-3′), 77.9 (C-5′), 74.9 (C-2′), 71.2 (C-4′), 64.4 (C-7), 62.5 (C-6′), 36.8 (C-13), 27.2 (C-12).

Salicortin-6′-benzoate (2) $C_{27}H_{28}O_{11}$, ^1H NMR (300 MHz, CD_3OD) δ: 8.12 (2H, *dd*, *J* = 7.5, 1.2 Hz, H-17 and H-21), 7.53 (1H, *t*, *J* = 7.3 Hz, H-19), 7.42 (2H, *t*, *J* = 7.6 Hz, H-18 and H-20), 7.02–7.26 (4H, *m*, H-2 ∼ H-5), 6.16 (1 H, *dt*, *J* = 9.8, 3.7 Hz, H-11), 5.73 (1H, *d*, *J* = 9.8 Hz, H-10), 5.21–5.45 (2 H, *m*, H-1′ and H-2′), 5.06 (1H, *d*, *J* = 12.5 Hz, H-7a), 4.97 (1H, *d*, *J* = 12.5 Hz, H-7b), 3.60–3.98 (4 H, *m*, H-3′ ∼ H-6′), 2.45–2.61 (2H, *m*, H_2–12), 2.61–2.85 (2H, *m*, H_2–13). ^{13}C NMR (100 MHz, CD_3OD) δ: 207.3 (C-14), 171.1 (C-8), 167.1 (C-15), 156.4 (C-1), 134.4 (C-19), 133.3 (C-10), 131.1 (C-3), 130.9 (C-11), 130.8 (C-16), 130.1 (C-17. and C-21), 1129.6 (C-18. and C-20), 129.2 (C-5), 125.8 (C-2), 123.7 (C-4), 116.6 (C-6), 100.7 (C-1′), 79.1 (C-9), 78.4 (C-3′), 75.8 (C-5′), 75.5 (C-2′), 71.5 (C-4′), 63.9 (C-7), 62.4 (C-6′), 36.8 (C-13), 27.2 (C-12).

4.4. Cell culture

The murine macrophage cell line (RAW 264.7) was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM containing 10% fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, penicillin (100 U/ml) and streptomycin (10 μg/ml). Cells were grown at 37 °C, 5% CO_2 in fully humidified air, and were split twice a week. RAW 264.7 cells were seeded at 8×10^5 cells/ml in 24 well plates and were activated by incubation in medium containing LPS (1 μg/ml) and various concentrations of test materials dissolved in DMSO. The supernatants were collected as the sources of secreted NO.

4.5. Nitrite assay

The amounts of NO released from macrophages was assessed by the determination of NO_2^- concentration in culture supernatant. Samples (100 μl) of culture media were incubated with 150 μl of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine in 2.5% phosphoric acid solution) at room temperature for 10 min in 96-well microplate (Green et al. 1982). Absorbance at 540 nm was read using a microplate reader. Standard calibration curves were prepared using sodium nitrite as standard.

4.6. Western blot analysis of i-NOS

To prepare whole-cell lysate, RAW 264.7 cells were treated with 1 μg/ml of LPS in the presence or absence of various concentrations of salicortin. Following 20 h treatment, cells were harvested, washed twice with ice-cold PBS (pH 7.4), and gently lysed with cell lysis buffer (Cell Signaling Technology, Danvers, MA). Cell lysates were then centrifuged at 10,000 × *g* for 20 min at 4 °C. The protein concentrations of the collected supernatants were determined by Bradford method. Thirty μg protein of cell lysates was applied on 8% SDS-polyacrylamide gels and transferred to PVDF membrane by the standard method. Antibodies against i-NOS (BD Biosciences, Franklin Lakes, NJ) and β-actin (Sigma Chemical Co., St. Louis, MO) were used for the immunoblot analysis. The Western blot was visualized using an enhanced chemiluminescence detection kit (Amersham Bioscience, Piscataway, NJ) according to the manufacturer's instruction.

4.7. Statistical analysis

Data were presented as mean ± S.D. and *P* values were determined using the unpaired Student's *t*-test. The difference was considered to be statistically significant when *P* values are less than 0.05.

Acknowledgements: This work was supported by Basic Science Research Program (2010 - 0009582) and MRC program (2011- 0030699) through the National Research Foundation of Korea (NRF) funded by Korea Government (MEST).

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