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Phenolic Compounds from *Caesalpinia sappan* and Their Inhibitory Effects on LPS-induced NO Production in RAW264.7 Cells

Byung Sun Min[1,2,*] and To Dao Cuong[1]

[1]*College of Pharmacy, Catholic University of Daegu, Gyeongsan, Gyeongbuk 712-702, Korea*
[2]*Advanced Medical Research Center, Catholic University of Daegu, Gyeongsan, Gyeongbuk 712-702, Korea*

Abstract – Thirteen phenolic compounds, 1,4-dimethoxybenzene (**1**), 3,4-dihydroxybenzaldehyde (**2**), (2*E*)-3-(4-hydroxy-3,5-dimethoxyphenyl)acrylaldehyde (**3**), 3,7-dihydroxy-4*H*-chromen-4-one (**4**), 2,3-dihydroxy-1-(3,4-dihydroxyphenyl)propan-1-one (**5**), 4-hydroxy-3-methoxybenzoic acid (**6**), 4-hydroxy-3,5-dimethoxybenzoic acid (**7**), methyl 3,4-dihydroxybenzoate (**8**), 4-hydroxy-3,5-dimethoxybenzaldehyde (**9**), 3,4-dihydroxybenzoic acid (**10**), 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one (**11**), 2,4,6-trihydroxybenzaldehyde (**12**) and benzene-1,2,4-triol (**13**) were isolated from the heartwood of *Caesalpinia sappan*. Their anti-inflammatory activity was evaluated against LPS-induced NO production in macrophage RAW264.7 cells. Among them, compounds **3** and **8** showed strong inhibitory activities toward the LPS-induced NO production in macrophage RAW264.7 cells with IC_{50} values of 14.5 and 21.5 μM, respectively.
Keywords – *Caesalpinia sappan,* Leguminosae, Phenolic, Anti-inflammatory activity

Introduction

Inflammation is the normal physiological and immune response to tissue injury. Increased blood supply, enhanced vascular permeability and migration of immune cells occur at damaged sites. The inflammatory process is a protective response that occurs in response to trauma, infection, tissue injury or noxious stimuli (Zedler *et al*., 2006). In this process, activated inflammatory cells (neutrophils, eosinophils, mononuclear phagocytes and macrophages) secrete increased amounts of nitric oxide (NO), prostaglandin E_2 (PGE_2) and cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α. These substances not only induce cell and tissue damage, but also activate macrophages in rheumatoid arthritis, chronic hepatitis, etc (Vane *et al*., 1994). In particular, a growing body of evidence suggests chronic inflammation can lead to cancer (Schottenfild *et al*., 2006). NO is a major product and its production is controlled by the nitric oxide synthases (NOS), which include iNOS, eNOS and nNOS. Most importantly, iNOS is highly expressed in macrophages; its activation leads to organ destruction in some inflammatory and autoimmune diseases. PGE_2 is another important inflammatory mediator and is produced from arachidonic acid metabolites by the catalysis of cyclooxygenase-2 (COX-2) (Murakami *et al*., 2007). During inflammation, macrophages play a central role in managing many different immune-pathological phenomena, including the overproduction of pro-inflammatory cytokines and inflammatory mediators such as IL-1β, IL-6, NO, iNOS, COX-2 and TNF-α. Indeed, a number of inflammatory stimuli, such as LPS and pro-inflammatory cytokines, activate immune cells to up-regulate such inflammatory states. Therefore, NO and PGE_2 production induced by LPS thought iNOS and COX-2, respectively, can reflect the degree of inflammation, and the change in NO and PGE_2 level thought inhibition of iNOS and COX-2 enzyme activity provides a means of assessing the effect of agents on the inflammatory process.

Caesalpinia sappan L (Leguminosae) is distributed in Southeast Asia, and its heartwood, Sappan Lignum, is famous as a red dyestuff. Sappan Lignum is also used as an herbal medicine for inflammation and to improve blood circulation (Nagai *et al*., 1984; Fuke *et al*., 1985), as well as for its anti-influenza (Liu *et al*., 2009), anti-allergic (Yodsaoue *et al*., 2009), and neuroprotective activities (Shen *et al*., 2007). Many reports have shown that the main compounds in Sappan Lignum are phenolics, and they are divided into four structural sub-types: *i.e.* brazilin, chalcone, protosappanin, and

*Author for correspondence
Byung Sun Min, College of Pharmacy, Catholic University of Daegu, Gyeongsan 712-702, Korea
Tel: +82-53-850-3613; E-mail: bsmin@cu.ac.kr

homoisoflavonoids. Among the protosappanin derivatives, such as protosappanin B and isoprotosappanin B, 10-*O*-methy-protosappanin B and 10-*O*-methylisoprotosappanin B, as well as protosappanin E1 and protosappanin E2 occur as pairs of epimers. The homoisoflavonoid epimers, sappanol and episappanol, 4-*O*-methylsappanol and 4-*O*-methylepisappanol, and 3'-*O*-methylsappanol and 3'-*O*-methylepisappanol have been successively isolated (Shimokawa *et al*., 1985; Saitoh *et al*., 1986). Many other sappanchalcone skeleton compounds show anti-inflammatory (Cuong *et al*., 2012; Min *et al*., 2012; Washiyama *et al*., 2009), anti-bacterial and anti-influenza activities (Hu *et al*., 2003; Oh *et al*., 1998). From the continuing investigation on the discovery of the inhibitory effect on LPS-induced NO production in macrophage RAW264.7 cells, further fractionation of the EtOAc-soluble fraction resulted in the isolation of thirteen phenolic compounds (**1** - **13**). This study describes the isolation and structural elucidation of these isolates, as well as the evaluation of their inhibitory effects on LPS-induced NO production in macrophage RAW264.7 cells.

Experimental

General experimental procedures – Optical rotations were measured with a JASCO DIP 370 digital polarimeter. UV spectra were taken in MeOH using a Thermo spectrometer, and IR spectra were obtained on a JASCO FT/IR - 4100 spectrometer. The nuclear magnetic resonance (NMR) spectra were obtained on Varian Unity Inova 400 MHz spectrometer. Silica gel (Merck, 63 - 200 μm particle size), RP-18 (Merck, 75 μm particle size) were used for column chromatography. TLC was carried out using Merck silica gel 60 F_{254} and RP-18 F_{254} plates. HPLC was carried out using a Water system with a UV detector and an YMC Pak ODS-A column (20 × 250 mm, 5 μm particle size, YMC Co., Ltd., Japan) and HPLC solvents were from Burdick & Jackson, USA.

Plant material – The *C. sappan* heartwood was purchased from a folk medicine market "Yak-ryoung-si" in Daegu, Korea, in May 2010. Botanical identification was performed by Prof. Byung Sun Min, and the voucher specimen CUD-3174 was deposited at the Herbarium of the College of Pharmacy, Catholic University of Daegu, Korea.

Extraction and isolation – The *C. sappan* heartwood (10 kg) was extracted three times (3 h × 3 L) with refluxing methanol. After the solvent was removed under reduced pressure, the residue was suspended in H_2O and then partitioned with *n*-hexane, EtOAc, and *n*-BuOH, successively. By the guided-fractionation-activity, the EtOAc-soluble fraction (960 g) was chromatographic on a silica gel column using a stepwise gradient of $CHCl_3$/MeOH (50 : 1 to 0 : 1, each 10 L) to yield fourteen fractions (Fr.1 - Fr.14) according to their TLC profiles. Fraction 3 (2.8 g) was subjected to reversed phase (ODS-A) column chromatography and eluted with MeOH/H_2O (1 : 1 to 1 : 0, 2 L for each step) to afford ten sub-fractions (Fr.3-1 to Fr.3-10). Further purification of Fr.3-1 (250 mg) by semi-preparative Waters HPLC systems [using an isocratic solvent system of 40% MeOH in H_2O + 0.1% trifluoroacetic acid (flow rate 5 mL/min) over 90 min; UV detection at 210 nm; YMC Pak ODS-A column (20 × 250 mm, 5 μm particle size] resulted in the isolation of compounds **1** (5.8 mg, t_R = 38.8 min), **3** (12.6 mg, t_R = 42.4 min), and **4** (52.1 mg, t_R = 45.9 min). Fraction 3-4 (228 mg) was purified by semi-preparative HPLC [using an isocratic solvent system of 35% MeOH in H_2O + 0.1% trifluoroacetic acid (flow rate 5 mL/min) over 90 min; UV detection at 210 nm; YMC Pak ODS-A column (20 × 250 mm, 5 μm particle size] resulted in the isolation of compounds **2** (6.4 mg, t_R = 34.2 min), **5** (11.2 mg, t_R = 36.8 min), and **8** (15.1 mg, t_R = 40.9 min), respectively. Further purification of Fr.3-5 (410 mg) by semi-preparative Waters HPLC systems [using an isocratic solvent system of 35% MeOH in H_2O + 0.1% trifluoroacetic acid (flow rate 5 mL/min) over 90 min; UV detection at 210 nm; YMC Pak ODS-A column (20 × 250 mm, 5 μm particle size] resulted in the isolation of compounds **6** (5.4 mg, t_R = 42.2 min), **7** (11.4 mg, t_R = 46.1 min), and **10** (8.3 mg, t_R = 49.2 min). Sub-fraction F.3-7 (154 mg) was further purified by semi preparative HPLC [using an isocratic solvent system of 30% MeOH in H_2O + 0.1% trifluoroacetic acid (flow rate 5 mL/min) over 90 min; UV detection at 210 nm; YMC Pak ODS-A column (20 × 250 mm, 5 μm particle size] resulted in the isolation of compounds **9** (9.8 mg, t_R = 40.9 min) and **11** (10.7 mg, t_R = 44.2 min), respectively. Fraction 3-9 (220 mg) was purified by semi-preparative HPLC [using an isocratic solvent system of 30% MeOH in H_2O + 0.1% trifluoroacetic acid (flow rate 5 mL/min) over 90 min; UV detection at 210 nm; YMC Pak ODS-A column (20 × 250 mm, 5 μm particle size] resulted in the isolation of compounds **12** (8.4 mg, t_R = 39.9 min) and **13** (8.2 mg, t_R = 43.8 min), respectively.

1,4-Dimethoxybenzene (1) – Colorless needles; mp 54 - 56°C; ESI-MS *m*/*z* 139.0 $[M+H]^+$.

3,4-Dihydroxybenzaldehyde (2) – White amorphous powder; mp 154 - 156 °C; ESI-MS *m*/*z* 139.0 $[M + H]^+$.

(2*E*)-3-(4-Hydroxy-3,5-dimethoxyphenyl)acrylaldehyde

(3) – Colorless plates; mp 104 - 106 ºC; ESI-MS *m/z* 209.0 $[M + H]^+$.

3,7-Dihydroxy-4*H*-chromen-4-one (4) – Yellow plates; ESI-MS *m/z* 179.0 $[M + H]^+$.

2,3-Dihydroxy-1-(3,4-dihydroxyphenyl)propan-1-one (5) – Yellow amorphous powder; α_D^{25} : +267 (*c* 0.15, MeOH); ESI-MS *m/z* 199.0 $[M + H]^+$.

4-Hydroxy-3-methoxybenzoic acid (6) – Colorless needles; mp 210 - 213 ºC; ESI-MS *m/z* 169.0 $[M + H]^+$.

4-Hydroxy-3,5-dimethoxybenzoic acid (7) – Colorless plates; mp 206 - 208 ºC; ESI-MS *m/z* 199.0 $[M + H]^+$.

Methyl 3,4-dihydroxybenzoate (8) – Colorless needles; mp 134 - 135 ºC; ESI-MS *m/z* 169.0 $[M + H]^+$.

4-Hydroxy-3,5-dimethoxybenzaldehyde (9) – Colorless plates; mp 110 - 112 ºC; ESI-MS *m/z* 183.0 $[M + H]^+$.

3,4-Dihydroxybenzoic acid (10) – Colorless needles; mp 198 - 200 ºC; ESI-MS *m/z* 155.0 $[M + H]^+$.

3-Hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one (11) – White amorphous powder; ESI-MS *m/z* 197.0 $[M + H]^+$.

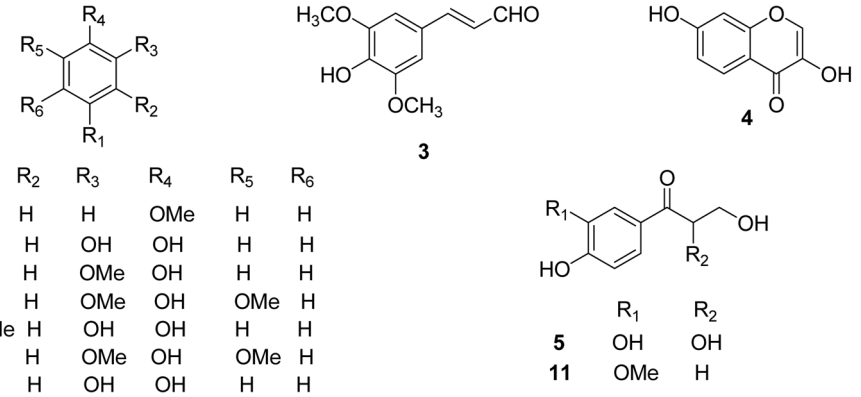
2,4,6-Trihydroxybenzaldehyde (12) – Yellowish needles; mp 223 - 225 ºC; ESI-MS *m/z* 155.0 $[M + H]^+$.

Benzene-1,2,4-triol (13) – gray powder; mp 139 - 141 ºC; ESIMS *m/z* 127.0 $[M + H]^+$.

Determination of NO Production and the Cell Viability Assay – The level of NO production was determined by measuring the amount of nitrite present in cell culture supernatants as described previously (Ahn *et al*., 2005). Briefly, the RAW264.7 cells (1×10^5 cells/well) were stimulated with or without 1 μg/mL of LPS (Sigma Chemical Co., St. Louis, MO) for 24 h in the presence or absence of the test compounds (3 - 100 μM). The cell culture supernatant (100 μL) was then reacted with 100 μL of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphtylethylenediamine dihydrochloride in distilled H_2O). The absorbance at 540 nm was determined with a microplate reader (Molecular Devices, Emax, Sunnyvale, CA, USA), and the absorption coefficient was calibrated using a $NaNO_2$ solution standard. The amount of TNF-α in the culture supernatant was measured using the ELISA kit (R&D systems, Minneapolis, MN, USA). Cell viability was measured with MTT-based colorimetric assay. For this experiment, celastrol was used as a positive control.

Results and Discussion

The MeOH extract of the heartwood of *C. sappan* was partitioned into hexane-, EtOAc-, and water-soluble fractions. Chromatographic purification of the EtOAc-soluble fraction led to the isolation of thirteen compounds (**1 - 13**) (Fig. 1). The structures of these compounds were determined as 1,4-dimethoxybenzene (**1**), 3,4-dihydroxy benzaldehyde (**2**), (2*E*)-3-(4-hydroxy-3,5-dimethoxyphenyl) acrylaldehyde (**3**), 3,7-dihydroxy-4*H*-chromen-4-one (**4**), 2,3-dihydroxy-1-(3,4-dihydroxyphenyl)propan-1-one (**5**), 4-hydroxy-3-methoxybenzoic acid (**6**), 4-hydroxy-3,5-dimethoxybenzoic acid (**7**), methyl 3,4-dihydroxybenzoate (**8**), 4-hydroxy-3,5-dimethoxybenzaldehyde (**9**), 3,4-dihydroxybenzoic acid (**10**), 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one (**11**), 2,4,6-trihydroxyben zaldehyde (**12**), and benzene-1,2,4-triol (**13**) by comparing their physical and spectroscopic data with previous reported papers (Stephane *et al.,* 2001; Qu *et al*, 2012; Ana *et al*., 2008; Lai *et al.,* 2011; Liya and Navindra., 2001; Stalin and Rajendiran., 2006; Bitzer *et al*., 2004., Li *et al*., 2009; Stalin and Rajendiran., 2005; Ma *et al*., 2012; Peng *et al*., 2011; Friedman *et al*., 2003; Jan *et al*., 2009).



	R_1	R_2	R_3	R_4	R_5	R_6
1	OMe	H	H	OMe	H	H
2	CHO	H	OH	OH	H	H
6	COOH	H	OMe	OH	H	H
7	COOH	H	OMe	OH	OMe	H
8	COOMe	H	OH	OH	H	H
9	CHO	H	OMe	OH	OMe	H
10	COOH	H	OH	OH	H	H
12	CHO	OH	H	OH	H	OH
13	CHO	H	OH	OH	H	OH

	R_1	R_2
5	OH	OH
11	OMe	H

Fig. 1. Chemical structures of isolated compounds **1 - 13**.

Table 1. Inhibition of NO production in macrophage RAW264.7 cells by compounds **1** - **13**

Compound	IC_{50} value (μM)[a]
1	> 100
2	85.0 ± 7.0
3	14.5 ± 3.0
4	> 100
5	> 100
6	> 100
7	> 100
8	21.5 ± 0.1
9	> 100
10	> 100
11	65.0 ± 5.0
12	> 100
13	> 100
Celastrol[b]	1.0 ± 0.1

[a]The inhibitory effects are represented as the molar concentration (μM) giving 50% inhibition (IC_{50}) relative to the vehicle control. These data represent the average values of three repeated experiments (mean ± S.D.).
[b] Positive control for NO production.

The cytotoxic effects of the isolated compounds (**1** - **13**) were evaluated in the presence or absence of LPS by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. These compounds did not affect the cell viabilities of RAW 264.7 cells in either the presence or absence of LPS, even at a dose of 50 μM after a period of 24 h (data not shown). As shown in Table 1, compounds **3** and **8** showed inhibitory effects with IC_{50} values of 14.5 and 21.4 μM, respectively. Compounds **2** and **11** displayed weak effects with IC_{50} values of 85.0 and 65.0 μM, respectively, but the others were inactive. Several studies of evidence have previously demonstrated the anti-inflammatory potential of *C. sappan* extracts and their isolated compounds. In LPS-stimulated RAW264.7 mouse macrophages, Hu and coworkers found that brazilin, the main constituent of *C. sappan*, suppressed the release of IL-1β, TNF-α, NO, and PGE2 and they suggested that these effects are mediated by heme oxygenase-1 (Hu *et al*., 2009). Washiyama *et al*. compared seven compounds isolated from a methanol extract of Sappan Lignum in mouse macrophage-like J774.1 cells and demonstrated inhibitory effects on the expression of inflammatory mediators (Washiyama *et al*., 2009). More importantly, *in vivo* data from mouse and rat models further support our findings on the anti-inflammatory activity of *C. sappan* isolates (Washiyama *et al*., 2009; Shen *et al*., 2007). Recently, a 70% ethanol extract of *C. sappan* has been evaluated in a human cell model of TNF-α-stimulated umbilical vein endothelial cells (Lee *et al*., 2010). One of the biological messengers associated with inflammation is NO. NO is biosynthesized endogenously from L-arginine by nitric oxide synthases and can activate transcription factors or protein kinases in a cGMP-dependent manner. Although NO might also regulate physiological processes in chondrocytes, including collagen type X expression and alkaline phosphatase activity, several reports have shown that osteoarthritis (OA) chondrocytes overexpress iNOS and that the resulting excess NO production by OA chondrocytes is a contributing factor to OA cartilage degradative processes (Studer *et al*., 1999). Experiments in other cell types have indicated that the anti-inflammatory activity of *C. sappan* involves the reduction of NO synthesis via inhibition of iNOS mRNA expression (Washiyama *et al*., 2009; Bae *et al*., 2005). In this study, we demonstrated that compounds **3** ((2*E*)-3-(4-hydroxy-3,5-dimethoxyphenyl)acrylaldehyde) and **8** (Methyl 3,4-dihydroxybenzoate) inhibit the production of NO in LPS-stimulated macrophages, suggesting that these compounds maybe able the additional source that useful in the treatment of inflammation diseases.

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