Agents Protecting against Sepsis from the Roots of *Angelica dahurica*

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In the course of isolating agents preventing sepsis from the EtOAc extract of the roots of *Angelica dahurica*, four known furanocoumarins, isoimperatorin (1), oxypeucedanin (2), (±)-byakangelicin (3), and (+)-oxypeucedanin hydrate (4), were isolated as active compounds based on the *in vivo* assay model of sepsis induced by lipopolysaccharide (LPS) and D-galactosamine (D-GalN). Among them, 3 showed the highest survival rate (100% with a dose of 30 mg/kg versus 20% for the control experiment) and decreased the plasma levels of tumor necrosis factor- α and alanine aminotransferase in mice administered LPS/D-GalN.

Key words *Angelica dahurica*; sepsis; lipopolysaccharide; furanocoumarin; tumor necrosis factor- α ; alanine aminotransferase

Sepsis is a clinical syndrome frequently induced by lipopolysaccharide (LPS), which is one of the major cell wall components of gram-negative bacteria and stimulates immunocytes, mainly macrophages, to release endogenous mediators such as tumor necrosis factor (TNF)- α , interleukin (IL)-1, IL-6, IL-10, prostanooids, leukotrienes, and nitric oxide.^{1,2} These mediators frequently result from excessive stimulation of the host immune system through complex signal transductions and lead to hypotension and multiorgan dysfunction with a high mortality rate.^{1,2} In patients with sepsis, TNF- α plays a major role in the associated systemic toxicity.³ Loss of hepatic function is one of the hallmarks of the development of multiple-organ failure associated with severe sepsis.^{4,5} There have been reports that the synthetic compounds IRFI 042,⁶ tyrphostin AG 126,⁷ and SR 27388⁸ show significant protection against lethality due to septic shock, and lysophosphatidylcholine (LPC) can effectively prevent and treat sepsis and microbial infections.⁹ Recently, protective compounds against sepsis have been reported from medicinal plants.^{10,11}

The protective activity against sepsis-induced lethality of the methanol extracts of about 100 Korean medicinal plants was determined based on the sepsis model induced by lipopolysaccharide/D-galactosamine, from which *Angelica dahurica* (Umbelliferae) was chosen as one of the active plants. The roots of *A. dahurica* have been used in Korean folk medicine as an antipyretic and analgesic for colds.¹² Biological activities of the components from this plant, such as hepatoprotective activity against tacrine-induced cytotoxicity in Hep G2 cells,¹³ inhibition of compound 48/80-induced histamine release in the mouse peritoneal cavity,¹⁴ antimicrobial activity,¹⁵ and affinity to brain benzodiazepine receptors *in vitro*,¹⁶ have been reported. In this paper, the isolation and activity of four known furanocoumarins as anti-septic shock agents from the roots of this plant are described.

MATERIALS AND METHODS

General Procedures Melting points were measured using a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were measured using a JASCO DIP1000 (Tokyo, Japan) automatic digital polarimeter. The

NMR spectra were recorded on Bruker 250-MHz (DMX 250), and Bruker 300-MHz (ARX 300) spectrometers. Samples were dissolved in either chloroform-*d*₁ or CD₃OD-*d*₄, and chemical shifts are reported in ppm downfield from TMS. The two-dimensional NMR spectra were recorded using Bruker's standard pulse program. The FAB-MS spectra were measured with a VG TRIO 2A mass spectrometer. Silica gel 60 (70–230 and 270–400 mesh), reverse-phase support (LiChroprep RP-18) and TLC plates (Si-gel 60F₂₅₄) were purchased from Merck KGaA (Darmstadt, Germany). Spots were detected under UV radiation and by spraying with 10% H₂SO₄, followed by heating. Dexamethasone was purchased from Sigma Chemical (St. Louis, MO, U.S.A.). All other chemicals solvents were of analytical grade and used without further purification.

Plant Material Dried roots of *A. dahurica* BENTHAM *et* HOOKER were purchased in November 1997 from Yak-ryong-si the folk medicine market in Daegu, and the material was confirmed taxonomically by Professor Ki-Hwan Bae, Chungnam National University, Daejeon, Republic of Korea. A voucher specimen (YNS-97-01) is deposited at the College of Pharmacy, Yeungnam University.

Extraction and Isolation The dried roots of *A. dahurica* (10 kg) were extracted twice with 70% MeOH (20 l) under reflux for 12 h. The MeOH solution was evaporated to dryness (3 kg) and the residue partitioned between H₂O (1 l) and hexane (3 × 1 l). The resulting H₂O layer was extracted with EtOAc (3 × 1 l) and *n*-BuOH (3 × 1 l) successively. The resulting solutions were evaporated to dryness to give *n*-hexane, EtOAc, *n*-BuOH, and H₂O extracts (711 g, 36 g, 128.9 g, and 1800 g, respectively). The EtOAc extract (35.8 g) was chromatographed on a silica gel column (230–400 mesh, 6.5 × 45 cm) to give 16 fractions (Fr. 1–Fr. 16) with hexane–EtOAc (gradient from 2 : 8 to 100% EtOAc) and EtOAc–MeOH (gradient from 100% EtOAc to 100% MeOH). Fr. 1 (543 mg) was recrystallized from hexane–EtOAc (6 : 4) to give isoimperatorin (1, 380 mg, pale yellow crystal, mp 104–106 °C).¹⁷ Fr. 4 (600 mg), Fr. 10 (1.97 g), and Fr. 11 (3 g) were separately recrystallized from EtOAc to give (±)-oxypeucedanin [2, 436 mg, white amorphous powder, mp 138–140 °C, $[\alpha]_D^{20} +1.5^\circ$ (c, 0.225 in CHCl₃)],^{13,18,19} (±)-byakangelicin [3, 900 mg, pale yellow amorphous powder,

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Fig. 1. Compounds Purified from the Roots of *Angelica dahurica*

der, mp 122–124 °C, $[\alpha]_D^{20} +4.4^\circ$ (*c*, 0.45 in MeOH),^{13,20} and (+)-oxypeucedanin hydrate [4, 920 mg, yellow amorphous powder, mp 131–133 °C; $[\alpha]_D^{20} +7.9^\circ$ (*c*, 0.375 in acetone)],^{17,20,21} respectively. The structures of the four compounds are shown in Fig. 1.

Animals and Reagents Male ICR mice weighing 23–28 g (Myung-Jin, Inc., Seoul, South Korea) were housed 5 per cage in a room maintained at $22 \pm 1^\circ\text{C}$ with an alternating 12-h light–dark cycle. Food and water were available *ad libitum*. LPS (*Escherichia coli* 055: B5, Sigma) was dissolved in phosphate-buffered saline (PBS, pH 7.2) 1 $\mu\text{g}/\mu\text{l}$ and stored at -80°C until use. D-GalN (ICN, U.S.A.) was dissolved in PBS 0.16 g/ml and added to 7.2 μl of LPS solution. The LPS/D-GalN mixture was used immediately. Each mouse received LPS/D-GalN (LPS 36 $\mu\text{g}/\text{kg}$, D-GalN 0.8 g/kg) intraperitoneally at volume of 1 ml/100 g body weight. Solvent extracts and purified compounds of the plant were dissolved in 10% DMSO. Dexamethasone (3, 5, 10 mg/kg) was used for the positive control experiment.

LPS/D-GalN-Induced Lethality and Cytokine Measurements Mice were injected intraperitoneally with crude solvent extracts or purified compounds of the plant or vehicle 30 min before intraperitoneal injection of LPS/D-GalN. The survival rate was recorded once daily for up to 3 d. For cytokine measurements, blood was collected at various time points (0, 1.5, 3, 6 h) after LPS/D-GalN administration from the retroorbital venous plexus and centrifuged at 4000 rpm $\times$ 15 min at 4 °C. Plasma samples were stored at -20°C until assayed. Plasma levels of TNF- α , IL-6, and IL-10 were measured with an enzyme-linked immunoassay kit (Genzyme, U.S.A.). The sensitivity limits of TNF- α , IL-6, and IL-10 were 3 pg/ml, 3 pg/ml and 13 pg/ml, respectively. Assays were performed exactly as described by the manufacturer.

Alanine Aminotransferase Analysis Liver damage was evaluated by measuring plasma alanine aminotransferase activity using an ALT kit (Yeongdong Pharmaceutical Corp., Seoul, Korea) according to the Reitman-Frankel method. Mice were pretreated with crude extracts intragastrically or with purified compounds of this plant intraperitoneally 30 min before injection of D-galN (800 mg/kg) and LPS (36 $\mu\text{g}/\text{kg}$). Blood (400 μl) was collected 6 h after injection of D-galN and LPS by puncturing the retroorbital venous plexus. Plasma was separated by centrifuging at 400 rpm. Plasma samples were diluted to 1:20 with saline prior to

Table 1. Effects of Solvent Extracts on LPS/D-GalN-Induced Lethality in Mice

	Control	Hexane	EtOAc	<i>n</i> -BuOH	H ₂ O
Survival rate ^{a)}	0/5	0/5	3/5	3/5	1/5

a) Number of live mice/number of total mice. Mice were injected intraperitoneally with a dose of 100 mg/kg of solvent extracts of the plant or vehicle 30 min before intraperitoneal injection of LPS/D-GalN. The survival rate was recorded once daily for up to 3 d.

Table 2. Effects of the Purified Compounds on LPS/D-GalN-Induced Lethality in Mice

Dose (mg/kg)	1	2	3	4	Dexamethasone ^{b)}
Control	1/5 ^{a)}	1/5	1/5	0/5	1/5
3	1/5	1/5	2/5	1/5	3/5
10	2/5	2/5	4/5	1/5	5/5
30	1/5	2/5	5/5	2/5	5/5

a) Number of live mice/number of total mice. b) Dexamethasone: positive control. Mice were injected intraperitoneally with various doses of the purified compounds from the plant or vehicle 30 min before intraperitoneal injection of LPS/D-GalN. The survival rate was recorded once daily for up to 3 d.

ALT measurement. Assays were performed exactly as described by the manufacturer.

Statistical Analysis Statistical analysis was carried out using one-way analysis of variance (ANOVA). Bonferroni and Newman–Keuls tests were used for *post-hoc* comparisons. Probability (*p*) values of less than 0.05 were considered to indicate statistical significance.

RESULTS AND DISCUSSION

The MeOH extract of the roots of *A. dahurica* was partitioned successively with hexane, EtOAc, *n*-BuOH, and H₂O and then dried. When each of these solvent extracts (100 mg/kg) was pretreated with the LPS/D-GalN-induced lethality model, the groups of mice pretreated with the EtOAc and *n*-BuOH extracts showed increased survival rates (3 of 5 mice) compared with the control experiment in which all 5 mice died (Table 1). The EtOAc extract was further chromatographed on a silica gel column and major column fractions were recrystallized, which afforded isoimperatorin (1), oxypeucedanin (2), (±)-byakangelicin (3), and (+)-oxypeucedanin hydrate (4).

Their structures were identified by comparison of optical rotations, ¹H- and ¹³C-NMR, DEPT, and MS data with the reported ones of the corresponding compounds.^{13,17–21} Among the purified compounds, 3 showed the strongest protective effect against lethality induced by LPS/D-GalN (Table 2). Pretreatment of mice with 3 at doses of 3, 10, and 30 mg/kg increased survival rates to 40%, 80%, and 100%, respectively, compared with 20% in the control experiment, while pretreatment with dexamethasone as a positive control at doses of 3, 10, and 30 mg/kg increased survival rates to 60%, 100%, and 100%, respectively. As an initial mechanistic study, levels of TNF- α , IL-6, and IL-10 in plasma samples from mice treated with 3 (30 mg/kg) were measured and compared with those of the control LPS/D-GalN group (Fig. 2). One and one-half hours after treatment with LPS/D-GalN, the level of TNF- α in plasma samples of mice pretreated with 3 (30 mg/kg) was 5-fold lower than that in the control

Fig. 2. Effect of **3** (30 mg/kg) on Plasma TNF- α (A), IL-6 (B), and IL-10 (C) Levels at Various Time Points after Intraperitoneally Administration of LPS (36 μ g/kg)/D-GalN (0.8 g/kg)

Data are mean $\pm$ S.E.M. of 5 to 7 mice. *** p < 0.001 significant difference from LPS/D-GalN-treated group at the time point.

Fig. 3. Effects of Pretreatment with **3** (30 mg/kg) on the Increase in Plasma ALT Levels 6 h after Intraperitoneal Administration of LPS/D-GalN

Data are mean $\pm$ S.E.M. of 5 to 7. ** p < 0.01 significant difference from LPS/D-GalN-treated group. Dexamethasone (10 mg/kg), positive control.

LPS/D-GalN-treated group. However, no significant changes in levels of IL-6 and IL-10 after pretreatment with **3** were observed. To investigate the effects of **3** on liver damage due to LPS/D-GalN administration in mice, ALT values were measured. The effects were almost the same as those of dexamethasone with more than 70% reduction of ALT activity in plasma samples of mice pretreated with **3** (30 mg/kg) compared with that of the control LPS/D-GalN-treated group (Fig. 3).

In conclusion, **3** at a dose of 30 mg/kg completely pro-

tected mice from LPS/D-GalN induced death. In the initial mechanistic study, **3** showed inhibitory activity against LPS/D-GalN induced plasma levels of TNF- α but did not affect IL-6 and IL-10 levels and exhibited hepatoprotective activity. These results suggest that the protective effect of **3** against LPS/D-GalN induced lethality may occur through downregulation of TNF- α and hepatoprotection. The purification of other active compounds from the *n*-BuOH extract of this plant is in progress.

Acknowledgments This research was financially supported by a Korean Research Foundation Grant (KRF-2002-005-E00019).

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