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Protective Constituents Against Sepsis in Mice from the Root Cortex of *Paeonia suffruticosa*

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The bioassay-guided fractionation of protective agents against sepsis-induced lethality from the root cortex of *Paeonia suffruticosa* ANDREWS (Ranunculaceae) led to the isolation of eight known compounds: paeonol (**1**), 2,5-dihydroxy-4-methoxyacetophenone (**2**), acetovanillone (**3**), paeonoside (**4**), paeoniflorin (**5**), oxypaeoniflorin (**6**), apiopaeonoside (**7**), and methyl 3-hydroxy-4-methoxybenzoate (**8**). Among them, **3** showed the highest survival rate (100% with a dose of 30 mg/kg *versus* 17% for the control experiment) and reduced alanine aminotransferase level to be a half of the control value on the sepsis model induced by lipopolysaccharide/D-galactosamine.

Key words: *Paeonia suffruticosa*, Sepsis, LPS, D-GalN, ALT

INTRODUCTION

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 macrophage, to release the endogenous mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, interleukin-6, interleukin-10, prostanoids, leukotrienes and nitric oxide (Wakabayashi *et al.*, 1991; Zhang *et al.*, 1997). These mediators are frequently resulted from excessive stimulation of host immune system through complex signal transductions, and result in hypotension and multi-organ dysfunction, with a high mortality rate (Wakabayashi *et al.*, 1991; Zhang *et al.*, 1997). There have been reported that synthetic compounds: IRFI 042 (Altavilla *et al.*, 2002), tyrphostin AG 126 (Novogrodsky *et al.*, 1994) and SR 27388 (Herbert *et al.*, 1993) showed significant protection against lethality due to septic shock, and lysophosphatidylcholine (LPC) can effectively prevent and treat sepsis and microbial infections (Yan *et al.*, 2004). Recently, protective compounds against sepsis have been reported from medicinal plants (Lee *et al.*, 2003; Kawaguchi *et al.*, 2004). In our previous studies, protective activity against sepsis-induced lethality of methanol extracts of about one hundred Korean medicinal plants have been screened based on the sepsis model induced by LPS/D-galactosamine (D-GalN), from which the root cortex of *Paeonia suffruticosa* (Moutan Cortex) was chosen as one of the active plants.

The root cortex of *P. suffruticosa* is used as an analgesic, a sedative, an anti-inflammatory agent, and a remedy for female diseases in Korean and Chinese traditional medicine (Chang *et al.*, 1986). Biological activities of the compounds from this plant, such as hyaluronidase inhibitory activity (Jeong *et al.*, 1998), antimicrobial activity (Kwon *et al.*, 1999), melanin biosynthesis inhibition in cultured B-16 mouse melanoma cells (Lee *et al.*, 1998), inhibition of blood platelet aggregation and antifibrinolytic activity (Arichi *et al.*, 1979; Kubo *et al.*, 1982), oxygen radical scavenging activity (Kubo *et al.*, 1982), and inhibition of phenylhydroquinone-induced oxidative DNA cleavage (Okubo *et al.*, 2000) have been reported. In this paper, isolation of eight known compounds (**1**-**8**) by bioassay-guided fractionation of the methylene chloride

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extract of the root cortex of *P. suffruticosa* and their anti-sepsis activities are reported.

MATERIALS AND METHODS

General experimental procedures

Optical rotations were measured using a JASCO DIP-1000 (Tokyo, Japan) automatic digital polarimeter. The NMR spectra were recorded on a Bruker 250 MHz (DMX 250) spectrometer using Bruker's standard pulse program. Samples were dissolved in $CDCl_3$ or CD_3OD and chemical shifts were reported in ppm downfield from TMS. FABMS spectra were measured by VG TRIO 2A mass spectrometer Stationary phases for column chromatography (Silica gel 60, 70-230 and 270-400 mesh and Lichroprep RP-18 gel, 40-63 μm, Merck) and thin layer chromatography plates (Silica-gel 60 F_{254} and RP-18 $F_{254,}$ Merck) were purchased from Merck KGaA (Darmstadt, Germany). Spots were detected under UV radiation and by spraying with 10% H_2SO_4, followed by heating. Dexamethasone was purchased from Sigma Chemicals (St. Louis, MO, USA). All other chemicals and solvents were analytical grade and used without further purification.

Plant materials

The root cortex of *P. suffruticosa* was purchased in March 1999 from a folk medicine market, "Yak-ryong-si" in Daegu and the material was confirmed taxonomically by Professor Ki-Hwan Bae, of Chungnam National University in Daejeon, Republic of Korea. A voucher specimen (YNS-99-01) is preserved at the College of Pharmacy, Yeungnam University.

Extraction and isolation

The dried root cortex of *P. suffruticosa* (9.6 kg) were extracted twice with 70% MeOH (20 L) under reflux for 12 h. The MeOH solution was evaporated to dryness (2.3 kg) and the residue partitioned between H_2O (1 L) and CH_2Cl_2 (3×1 L). The resulting H_2O layer was extracted with EtOAc (3×1 L) successively. The CH_2Cl_2 extract (80.3 g) was chromatographed on a silica gel column (230-400 mesh, 70×6.5 cm) with CH_2Cl_2-MeOH-H_2O in a stepwise gradient mode. The fractions (250 mL in each flask) were combined on the basis of silica gel TLC and 16 fractions (F1-F16) were obtained. The fractions F3 (51.1 g) and F11 were recrystallized and left in the refrigerator for 12 h to give paeonol (**1**, 36.4 g, white crystals) (Kitagawa *et al.*, 1979) and paeoniflorin (**5**, 4.9 g, white powder) (Kitagawa *et al.*, 1979). The fractions F5 (600 mg) and F10 (800 mg) were separately rechromatographed over a reverse-phase column (4063 mm, 75×2.0 cm) with CH_3OH-H_2O in a stepwise gradient mode to give 2,5-dihydroxy-4-methoxy-acetophenone (**2**, 19.9 mg) (Kwon *et al.*, 1998) and methyl 3-hydroxy-4-methoxybenzoate (**8**, 11.5 mg, white power) (Quideau *et al.*, 2001) from F5 and paeonoside (**4**, 24.8 mg, white power) (Yu *et al.*, 1986) from F-10, respectively. The fractions F7 (2.8 g) and F 13 (2.0 g) were also separately rechromatographed over a reverse-phase column (4063 mm, 75×4.0 cm) with CH_3OH-H_2O in a stepwise gradient mode to give acetovanillone (**3**, 11.5 mg, white power) (Crestini and DAuria, 1997), oxypaeoniflorin (**6**, 630 mg, white power) (Kitagawa *et al.*, 1979) and apiopaeonoside (**7**, 594 mg, white power) (Yu *et al.*, 1986), respectively.

Animals and reagents

Five male ICR mice weighing 23-28 g were housed in a cage at a room temperature between 22±1°C with an alternating 12 h light-dark cycle. Food and water were available *ad libitum*. LPS (*Escherichia coli* 055:B5, Sigma, USA) was dissolved in phosphate-buffer saline (PBS) at 1 μg/μL and stored at 80°C until use. D-GalN (ICN, USA) was dissolved in PBS at 0.16 g/mL and added to 7.2 μL LPS. The LPS/GalN mixture was used immediately. Each mouse received LPS/D-GalN (LPS 36 μg/kg, D-GalN 0.8 g/kg) intra-peritoneally at volume of 1 mL/100 g of body weight. Crude extracts and purified compounds of this plant were dissolved in 10% DMSO.

LPS/D-GalN-induced lethality measurements

Mice were pretreated with crude solvent extracts intra-gastrically or with purified compounds of this plant intra-peritoneally or with vehicle at 30 min before i.p. injection of LPS/D-GalN. Survival rate was observed once daily for up to 2 days.

Alanine aminotransferase (ALT) analysis

Liver damage was evaluated by measuring plasma ALT using ALT kit (Yeongdong Pharmaceutical Corp., Seoul, Korea) according to Reitman-Frankel method. Plasma samples were diluted to 1:20 prior to ALT measurement. Assays were performed exactly as described by manu-facturers.

Statistical analysis

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

RESULTS AND DISCUSSION

The MeOH extract of the root cortex of *P. suffruticosa* was partitioned with methylene chloride, EtOAc and H_2O, successively, which were then dried. When each of those

solvent fractions were pretreated to LPS/D-GalN-induced lethality model (500 mg/kg), the group of mice, pretreated with the methylene chloride fraction, showed increased survival rate of 4 out of 5 mice comparing to the control experiment in which all 5 mice were dead after 48 h (Table I). The methylene chloride fraction was further chromatographed on a silica gel column, reverse-phase column and two major column fractions were recrystallized, which afford paeonol (**1**), 2,5-dihydroxy-4-methoxyacetophenone (**2**), acetovanillone (**3**), paeonoside (**4**), paeoniflorin (**5**), oxypaeoniflorin (**6**), apiopaeonoside (**7**), and methyl 3-hydroxy-4-methoxybenzoate (**8**), respectively. Their structures were identified by comparison of ^{1}H-NMR, ^{13}C-NMR, DEPT, MS data, and optical rotation with the reported ones of the corresponding compounds (Kwon *et al.*, 1998; Kitagawa *et al.*, 1979; Quideau *et al.*, 2001; Crestini and DAuria, 1997; Yu *et al.*, 1986).

Among these purified compounds, **3** showed the strongest protective effect against sepsis induced by LPS/D-GalN and pretreatments of mice with this compound at doses of 30 mg/kg increased both survival rates to 80% comparing to the control experiment in which all 5 mice were dead after 24 and 48 h (Table II). Compounds **1**, **2**, and **4** were less active than **3**, and **5**, **6**, **7**, and **8** were not active at all.

When 3, 10, and 30 mg/kg of **3** were pretreated, survival rates were 50%, 50% and 100%, respectively (Table III). In order to investigate the effect of **3** on liver damage due to LPS/D-GalN treatment in mice, ALT values were measured, which showed about 50% reduction of ALT activity in plasma samples of mice pretreated with **3** (30 mg/kg) comparing to those of control LPS/D-GalN-treated group (Fig. 1). However, **3** exhibited less protective activity against sepsis than the positive control, dexamethasone

Table I. Effects of solvent fractions on LPS/D-GalN induced lethality in mice

	Control	CH_2Cl_2	EtOAc	H_2O
Survival rate* (after 24 h)	0/5	4/5	2/5	1/5
Survival rate* (after 48 h)	0/5	4/5	0/5	1/5

* Number of live mice/number of total mice

Mice were administered intragastrically with a dose of 500 mg/kg of solvent extracts of the plant or vehicle 30 min before i.p. injection of LPS/ D-GalN. Survival rates were observed after 24 h and 48 h.

Table II. Effects of the isolated compounds on LPS/D-GalN induced lethality in mice

Compounds	Survival rate[a]		Control
	after 24 h	after 48 h	
1	2/5	2/5	1/5
2	3/5	3/5	0/5
3	4/5	4/5	0/5
4	2/5	2/5	1/5
5	1/5	1/5	1/5
6	1/5	1/5	1/5
7	1/5	1/5	1/5
8	0/5	0/5	0/5
Dexamethasone[b]	5/5	5/5	1/5

[a] Number of survival mice/number of total mice. [b] positive control.

Mice were i.p. injected with 30 mg/kg of the isolated compounds from the plant, 10 mg/kg of dexamethathone for positive control or vehicle for control experiments at 30 min before i.p. injection of LPS/D-GalN. Survival rates were observed after 24 h and 48 h.

Table III. Effect of pretreatment (i.p.) with 3 on LPS/D-GalN-induced lethality and plasma ALT levels in mice

Dose (mg/kg)		Survival rate[1]		ALT (U/L)
		after 24 h	after 48 h	
Normal (untreated control)	0			29.21.3 (N^2=4)
Control	0	1/6	1/6	24,3372,289 (N=5)
3	3	3/6	3/6	12,0191,093** (N=5)
	10	3/6	3/6	
	30	6/6	6/6	
Dexamethasone[3]	3	4/6	4/6	2,245216** (N=5)
	10	6/6	6/6	
	30	6/6	6/6	

[1] Number of live mice/number of total mice.

[2] N=number of mice used to obtain plasma ALT levels. Plasma ALT activities were measured at 90 min after injection of LPS. Data are means±SEM. **P<0.01 significant difference from control group.

[3] Dexamethasone: positive control.

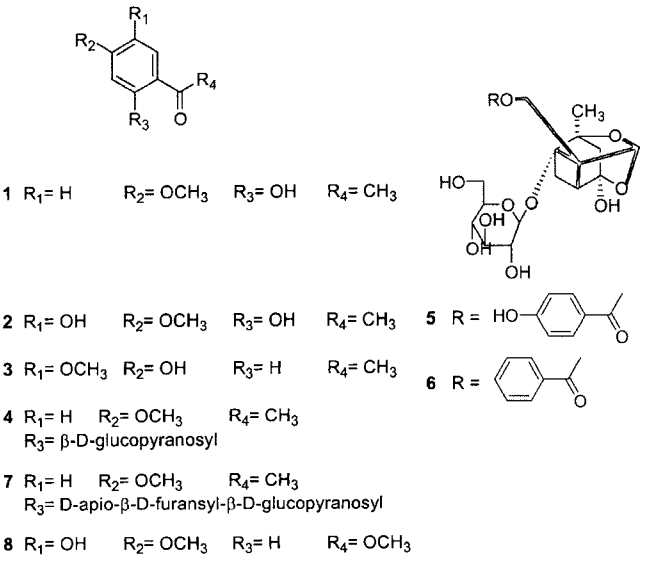

Fig. 1. Structures of compounds **1-8** isolated from the root cortex *of Paeonia suffruticosa*

when both survival rates and ALT activities were compared between two.

In structure and activity relationship among them, while di- or tri-substitutited methylcarbonyl benzene derivatives **1**, **2**, **3**, and **4** exhibited activity, diterpene glycoside **5** and **6** showed no activity. Di-substituted methyl benzoate **8** and methylcarbonyl benzene glycosides **4** and **7** showed either least or no activity. Therefore, it is speculated that methyl carbonyl benzene moiety is essential for the activity, and introduction of either sugar moiety or hydroxyl group on C-2 position on the benzene ring reduce the activity.

In conclusion, acetovanillone (**3**) from the root cortex of *Paeonia suffruticosa* was isolated as a major compound which exhibited the protective effect against sepsis induced by LPS/D-GalN in mice and it reduced ALT level in the same sepsis model. Other biochemical mechanistic studies of these compounds are under progress now.

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