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Protective Effects of Luteolin against Apoptotic Liver Damage Induced by D-Galactosamine/Lipopolysaccharide in Mice

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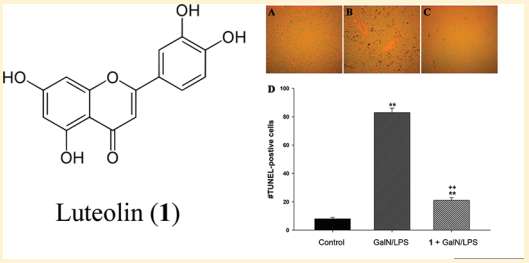
Supporting Information

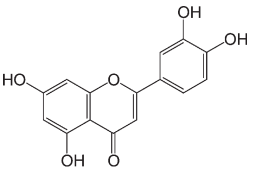
ABSTRACT: In this study, the protective effects of luteolin (**1**, a major component of *Cirsium japonicum*) were examined against D-galactosamine (GalN)/lipopolysaccharide (LPS)-induced fulminant hepatic failure. Mice received an intraperitoneal injection of **1** (25, 50, 100, and 200 mg·kg^{-1}) 1 h before treatment with GalN (700 mg·kg^{-1})/LPS (10 μg·kg^{-1}). Treatment with GalN/LPS resulted in increased mortality and serum aminotransferase activity. These increases were attenuated by pretreatment with **1**. Treatment with GalN/LPS induced an increase in the serum level of tumor necrosis factor-α (TNF-α) and protein expression of TNF-α receptor-associated death domain, and these increases were prevented by **1**. In addition, **1** attenuated apoptosis induced by GalN/LPS treatment, which was analyzed using a caspase-3 and -8 activity assay, as well as by proapoptotic BH3-only protein and cytochrome *c* protein expression, and by a terminal deoxynuleotidyl transferase-mediated dUTP nick end-labeling method. After GalN/LPS injection, nuclear phosphorylated c-Jun levels showed a significant increase, which were attenuated by **1**. The present findings suggest that luteolin ameliorates D-GalN/LPS-induced liver injury and that this protection is likely due to inhibition of the extrinsic and intrinsic apoptotic pathways.



Fulminant hepatic failure, characterized by hepatic encephalopathy, severe coagulopathy, jaundice, and hydroperitoneum, is associated with high patient mortality, for which there is still no available therapy, except for liver transplantation, which is limited by a chronic shortage of donor livers.[1] Galactosamine (GalN) and lipopolysaccharide (LPS)-induced liver failure in mice is a widely used animal model that resembles human liver failure.[2] In this model, liver injury is critically dependent on macrophage-derived pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and interferon (IFN)-γ. Current biochemical and pathological studies have suggested that hepatocyte apoptosis plays an important role in the development of fulminant hepatic failure.[3] Therefore, regulation of undesired hepatocyte apoptosis is an attractive strategy for the treatment of fulminant hepatic failure. Previous reports have suggested that TNF-α plays a pivotal role in the apoptotic response to GalN/LPS-induced acute liver injury and that TNF-α-R1 (TNFR-1) triggers apoptosis by binding to the adaptor protein TNFR-1-associated death domain (TRADD), which facilitates recruitment and activation of the aspartate specific cystein protease caspase-8 and subsequent apoptosis.[4]

In oriental folk medicine, *Cirsium japonicum* DC. (Asteraceae) has been prescribed in the treatment of hepatitis, liver cancer, and hemorrhage. Its methanol extract alleviates alcoholic toxicity by enhancement of ethanol oxidation, as well as inhibition of lipid peroxidation in the liver.[5] Recently, we screened the 70% ethanol extract of *C. japonicum* and its active components for hepatoprotective agents. Among them, luteolin and luteolin 7-*O*-glucoside inhibited GalN-induced hepatotoxicity in primary hepatocyte cultures (data not shown). Luteolin (**1**; 5,7,3′,4′-tetrahydroxyflavone) has shown various biological effects including anti-inflammatory and antioxidant properties, as well as its antiproliferative activities against various cancer cells. Recently, **1** was reported to inhibit LPS-induced cytokine production and lethal toxicity[6] and to suppress cisplatin-induced apoptosis in auditory cells through heme oxygenase-1 expression.[7] In contrast, **1**-induced apoptosis in a human hepatocellular carcinoma cell line, HepG2, through a mechanism involving mitochondrial translocation of Bax/Bak and activation of c-jun N-terminal kinase (JNK).[8] Little information is available on the in vivo hepatoprotective effect of **1**.

The present study was conducted to investigate the effect of luteolin (**1**) on GalN/LPS-induced liver injury and the specific molecular mechanisms of protection, particularly regarding the extent of apoptosis.



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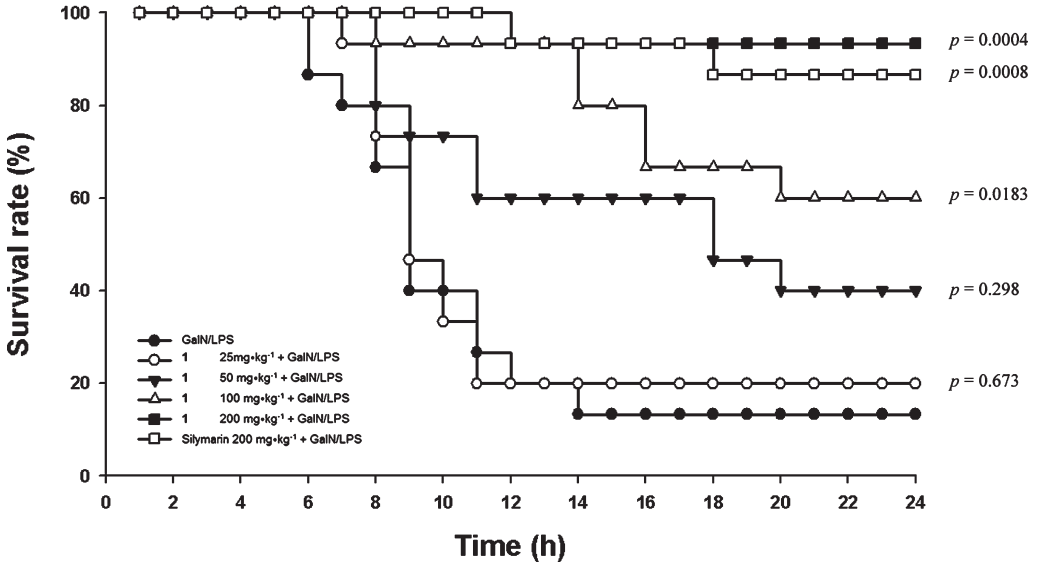

Figure 1. Effect of luteolin (**1**) on lethality induced by GalN/LPS. Each group consisted of 15 mice. Mice received intraperitoneal injection of vehicle, **1** (25, 50, 100, and 200 mg·kg^{-1}), or silymarin (200 mg·kg^{-1}) 1 h before GalN (700 mg·kg^{-1})/LPS (10 μg·kg^{-1}) treatment.

Table 1. Effect of Luteolin (1) on Serum Aminotransferase Activity, Secretion of TNF-α, and Caspase-3 and -8 Activities after GalN/LPS Treatment (means ± SEM, n = 8−10)[a]

group	ALT (U·L^{-1})	TNF-α (pg·mL^{-1})	caspase-3 activity (% of control)	caspase-8 activity (% of control)
control	59.9 ± 9.2	56.9 ± 2.4	100.0 ± 11.9	100.0 ± 10.2
GalN/LPS				
vehicle	6595.6 ± 361.3[b]	871.6 ± 63.7[b]	394.7 ± 40.3[b]	272.9 ± 23.8[b]
1	1059.0 ± 113.5[b,c]	574.2 ± 18.7[b,c]	251.7 ± 11.8[b,c]	176.8 ± 26.7[b,c]

[a] Compound **1** (200 mg·kg^{-1}) was administered intraperitoneally 1 h before GalN/LPS administration. Serum TNF-α levels were determined at 1 h after GalN/LPS treatment. Serum aminotransferase activity and caspase-3 and -8 activities were determined at 8 h after GalN/LPS treatment. [b] Denotes significant differences from the control group (p < 0.01). [c] Denotes significant differences from the GalN/LPS group (p < 0.01).

RESULTS AND DISCUSSION

Mortality and Serum Aminotransferase Activity. Administration of GalN with LPS induces liver damage that closely resembles human viral hepatitis in its morphological and functional features; therefore, this method is used widely as an experimental liver injury model for elucidating the mechanism of clinical liver dysfunction and for evaluation of the efficacy of hepatoprotective agents.[2]

In the GalN/LPS group, mice began to die 6 h after GalN/LPS injection. The survival rate was 86.7% at 6 h and stabilized at 13.3% 14 h after GalN/LPS injection. However, pretreatment with **1** (25, 50, 100, and 200 mg·kg^{-1}) before GalN/LPS resulted in markedly reduced mortality in a dose-dependent manner. In silymarin-treated mice (positive control), the mortality was 13.3% 24 h after GalN/LPS (Figure 1). In the control group, the serum level of alanine aminotransferase (ALT, #51) was 59.9 ± 9.2 U·L^{-1}. In the GalN/LPS-treated group, the serum level of ALT increased by approximately 110-fold over the control at 8 h after GalN/LPS injection. Recent studies have demonstrated that serum ALT level increases with hepatocyte apoptotic cell death, as well as hepatocyte necrosis.[9] The increase in ALT activity was attenuated by administration of **1** (Table 1).

Apoptotic Cells in Liver, TNF-α Level, and Caspase-3 and -8 Activities. According to the traditional view of fulminant hepatic failure, hepatocyte necrosis is the main feature. However, increasing evidence implicates a dominant role for hepatocyte apoptosis in its pathogenesis.[10] Abnormal apoptotic death of hepatocytes is thought to be a cause or contributing factor in many chronic as well as acute liver diseases.[11] Therefore, an analysis of apoptotic cells was performed by the terminal deoxynucleotidyl transferase-mediated dUTP nick-labeling (TUNEL) assay, which is based on direct, specific labeling of DNA breaks in the nuclei. The number of TUNEL-positive apoptotic cells was increased significantly in GalN/LPS-treated animals, and intraperioneal administration of **1** resulted in marked suppression of the increase in the number of apoptotic cells (Figure 2). The serum level of TNF-α showed a significant increase (57 ± 2 to 872 ± 63 pg·mL^{-1}) 1 h after GalN/LPS, and this elevation was attenuated by treatment with **1**. Caspase-3 and -8 activities in the cytosolic fraction of liver isolated 8 h after GalN/LPS injection increased to approximately 3.9 and 2.7 times those of the control group, respectively. Luteolin induced marked suppression of these elevated levels (Table 1).

Cytosolic Cytochrome *c* and TRADD Expression. The basic molecular framework underlying apoptosis is comprised of two fundamental signal transduction pathways that lead to activation of cell surface and intracellular death receptors (extrinsic pathway) and mitochondrial damage (intrinsic pathway).[12] Signaling of this

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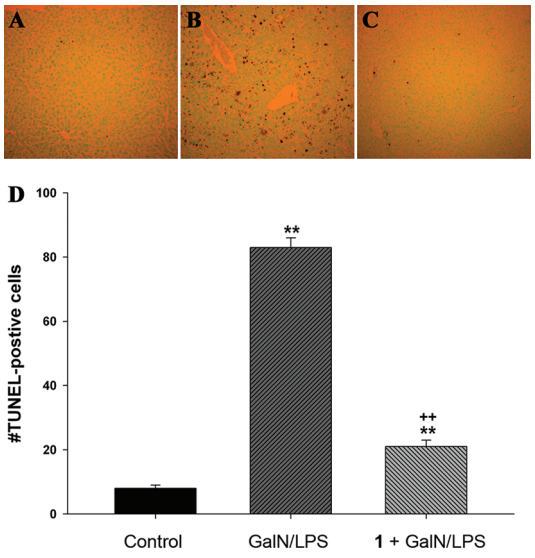

Figure 2. Detection of apoptotic hepatocytes in liver from mice after GalN/LPS injection (original magnification ×200). (A) Control group, (B) GalN/LPS group, (C) luteolin (**1**) (200 mg·kg^{-1}) and GalN/LPS-treated group, (D) TUNEL-positive hepatocytes. Values are presented as the means ± SEM of 8−10 mice per group. $^{**}p < 0.01$: denotes significant differences from the control group; $^{+2}p < 0.01$: denotes significant differences from the vehicle-treated GalN/LPS group.

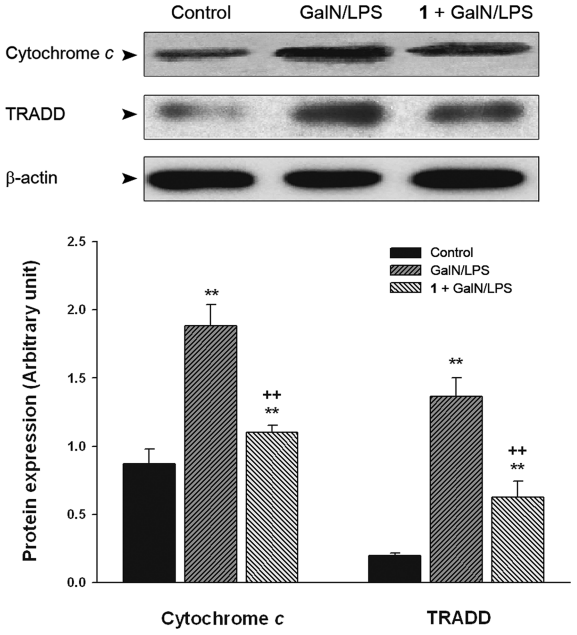

Figure 3. Effect of luteolin (**1**) (200 mg·kg^{-1}) on cytochrome *c* and TRADD protein expression. Values are presented as the means ± SEM of 8−10 mice per group. $^{**}p < 0.01$: denotes significant differences from the control group; $^{+2}p < 0.01$: denotes significant differences from the vehicle-treated GalN/LPS group.

extrinsic pathway occurs through cell surface death receptors,[13] including Fas, TNFR-1, and TNF-α-related-apoptosis-inducing-ligand receptors 1 and 2. TNFR-1 recruits the adaptor protein, TRADD, via homotypic interactions of the death domain. TRADD contains a death effector domain that recruits and cleaves procaspase-8, resulting in homodimerization and activation.[14] Caspase-8 then triggers activation of caspase-3, a downstream cysteine proteinase, through multiple apoptosis signaling pathways.[15]

As shown in Figure 3, the level of cytochrome *c* protein expression was barely detectable in liver cytosol fractions obtained from the control. In contrast, cytochrome *c* content showed a dramatic increase at 8 h after GalN/LPS injection. Luteolin suppressed the increase in the cytosolic cytochrome *c* content. The level of TRADD protein expression in the liver isolated after 3 h of GalN/LPS injection showed a considerable increase, by approximately 6.9-fold compared with the control. However, this elevation was attenuated by **1**.

Phosphorylation of JNK and Bcl-2 Protein Expression. The intrinsic pathway is based on damage or dysfunction of intracellular organelles, such as lysosomes, the endoplasmic reticulum, the nucleus, and mitochondria.[16] Activated caspase-8 initiates a proteolytic cascade that results in cleavage of the proapoptotic Bcl-2 family,[17] initiation of the mitochondrial death pathway with release of cytochrome *c*, and activation of caspase-3, which ultimately induces apoptosis.[18] Signaling through the JNK has been implicated in intrinsic apoptosis; however, information on death effectors acting downstream of JNK has been limited. Previous studies have demonstrated that GalN/LPS-induced phosphorylation of JNK directly activates Bid and Bim, and the combined loss of these proteins protects mice from fulminant hepatic failure.[19] These Bcl-2 family proteins are essential mediators for mitochondrial permeability transition and release of cytochrome *c*.

As shown in Figure 4, levels of phospho p-54 and phospho p-46 JNK protein expression in the liver isolated after 3 h of GalN/LPS injection increased by approximately 5.6-fold and 13.1-fold compared with the control, respectively. Bid and Bax protein expression increased approximately 13.1-fold and 3.2-fold, compared with the control, after 3 h of GalN/LPS injection. The ratio of p-Bim$_{\text{extra long}}$ (p-Bim$_{\text{EL}}$)/Bim$_{\text{EL}}$ and p-Bim$_{\text{long}}$ (p-Bim$_{\text{L}}$)/Bim$_{\text{L}}$ protein expression increased approximately 3.9- and 15.3-fold again when compared with the control, at 3 h after GalN/LPS injection, respectively (Figure 5). Increases in phospho p-54, phospho p-46, Bid, p-Bim$_{\text{EL}}$, and p-Bim$_{\text{L}}$ were attenuated by treatment with **1**.

In conclusion, luteolin (**1**) exhibited a protective effect on lethality and liver injury induced by GalN/LPS in mice, and its potential mechanism might be associated with the inhibition of intrinsic and extrinsic apoptotic pathways as summarized in Figure S1, Supporting Information. This study provides evidence that **1** may be useful as a potential pharmacological alternative for the prevention of hepatic failure.

EXPERIMENTAL SECTION

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were determined using a JEOL JNM ECP-400 spectrometer (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) in dimethylsulfoxide (DMSO)-d_6. Chemical shifts were referenced to residual solvent peaks (2.49 ppm for ^{1}H NMR and 39.50 ppm for ^{13}C NMR). The EIMS was recorded on a JEOL JMS-700 spectrometer (Tokyo, Japan). Reversed-phase HPLC was performed to check the compound purity on a JASCO HPLC system (Tokyo, Japan), consisting of a PU-1580 Intelligent HPLC pump, a LG-1580-04 quaternary gradient unit, a UV-1575 Intelligent UV/vis detector, a PG-1580-54 4-line degasser, and a CO-1560 Intelligent column thermostat. A Borwin chromatographic system (Le Fontanil, France) was used for HPLC data analysis. Column chromatography was performed using silica gel 60 (70−230 mesh, Merck, Germany), and TLC was conducted on precoated Merck

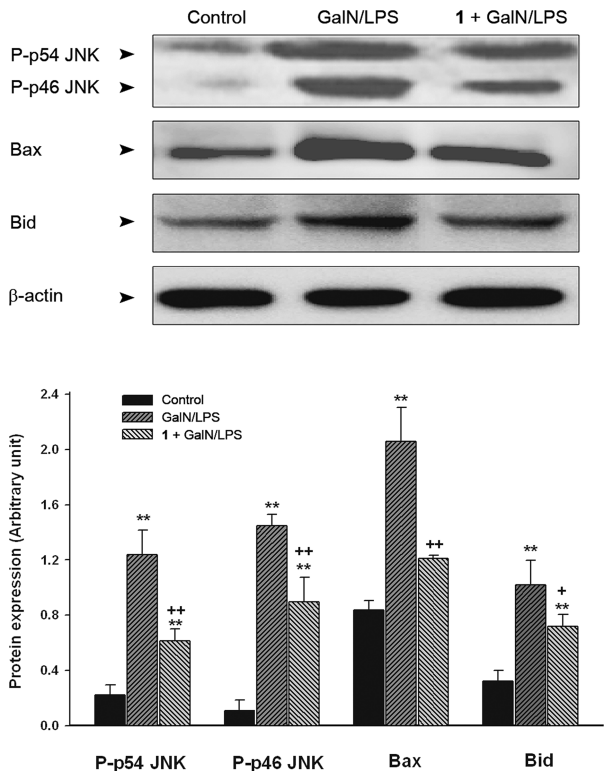

Figure 4. Effect of luteolin (**1**) (200 mg·kg^{-1}) on phospho-JNK, Bax, and Bid protein expression. Cytosolic proteins were extracted from mice at 3 h after GalN/LPS injection. Values are presented as the means ± SEM of 8−10 mice per group. $^{**}p < 0.01$: denotes significant differences from the control group; $^{+}p < 0.05$, $^{+2}p < 0.01$: denotes significant differences from the vehicle-treated GalN/LPS group.

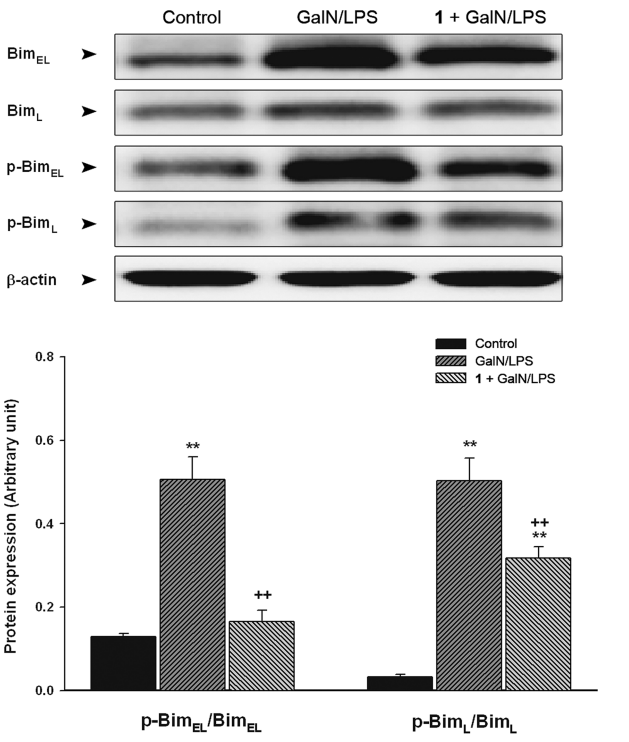

Figure 5. Effect of luteolin (**1**) (200 mg·kg^{-1}) on Bim and phospho-Bim protein expression. Cytosolic proteins were extracted from mice at 3 h after GalN/LPS injection. Values are presented as the means ± SEM of 8−10 mice per group. $^{**}p < 0.01$: denotes significant differences from the control group; $^{+2}p < 0.01$: denotes significant differences from the vehicle-treated GalN/LPS group.

Kieselgel 60 F_{254} plates (20 × 20 cm, 0.25 mm), using 50% H_2SO_4 as a spray reagent. Silymarin, as a positive control, was purchased from Sigma Chemical Co. (St. Louis, MO).

Plant Material. Whole plants of *C. japonicum* were collected in Kyeongju, Republic of Korea, in August 2007. This plant was authenticated by Dr. Je-Hyun Lee of the College of Oriental Medicine, Dongguk University, Kyeongju, Korea. A voucher specimen (20080915) has been deposited in the laboratory of one of the authors (J.S.C.) for future reference.

Isolation and Extraction. Dried whole plants (1.0 kg) were extracted with hot MeOH (2.5 L × 3 times). After filtration, the MeOH extract (220.0 g) was obtained via concentration in vacuo at 40 °C. The MeOH extract (220 g) was then dissolved in a mixture of CH_2Cl_2−MeOH−H_2O (10:1:9), after which the CH_2Cl_2-soluble fraction was concentrated to yield a CH_2Cl_2 fraction (42.0 g). The aqueous fraction was partitioned successively to yield the EtOAc- (58.6 g), *n*-BuOH- (17.2 g), and H_2O-soluble fractions (95.4 g). Repeated column chromatography of the EtOAc fraction (58.6 g) with CH_2Cl_2 and MeOH mixtures (20:1 to 1:1, gradient elution) afforded luteolin (**1**, 6.3 g). Luteolin was identified from its ^{1}H and ^{13}C NMR spectra and EIMS and by comparison with published values,[20] as well as by HPLC and TLC analysis using an authentic sample. The percent purity of the isolated **1** was found to be 99%, as determined by HPLC.

Animals and Treatment Regimens. Male ICR mice weighing 25−30 g (Daehan Biolink Co., Eumseong, Korea) were fasted overnight but given tap water ad libitum. All animal procedures were approved by the Sungkyunkwan University Animal Care Committee and were performed in accordance with the guidelines of the National Institutes of Health. Mice (except for the controls) received an intraperitoneal injection with GalN (700 mg·kg^{-1}, Sigma Chemical Co.) and LPS (10 μg·kg^{-1} *Escherichia coli* 026:B6, Sigma Chemical Co.) dissolved in phosphate-buffered saline. Luteolin (**1**, 99% purity) was suspended in 10% Tween 80−saline (vehicle) and administered intraperitoneally (ip) at a dose of 25, 50, 100, or 200 mg·kg^{-1} 1 h before the GalN/LPS treatment. Animals were assigned randomly to eight groups: Groups I (vehicle) and II (**1**) were treated with saline (10 mL·kg^{-1}, ip). As there were no differences in any of the parameters between group I and group II, they were pooled and are referred to as controls. In groups III to VIII, mice were treated with GalN/LPS. Mice in groups IV to VII were pretreated with **1** (25, 50, 100, and 200 mg·kg^{-1}, ip), and mice in group VIII were pretreated with silymarin (positive control, 200 mg·kg^{-1}, ip). Silymarin (10 mL·kg^{-1}) was suspended in 10% Tween 80 (10 mL·kg^{-1}) and administered 1 h before GalN/LPS. Mice were sacrificed by decapitation at 1, 3, and 8 h after GalN/LPS injection. The dose and timing of **1** treatment were selected based on a previous report[21] and as a result of a preliminary study.

Mortality and Serum Aminotransferase Activity. The survival rate of mice was monitored for 24 h after GalN/LPS injection. Plasma samples were taken from the mice at 8 h after GalN/LPS injection. A Hitachi 747 automatic analyzer (Hitachi, Tokyo, Japan) was used for determination of serum ALT activity.

Histological Analysis. Apoptotic cells were detected by the TUNEL method using an in situ apoptosis detection kit (Takara Co., Shiga, Japan). Under the microscope, the number of TUNEL-positive cells in ×200 histological fields was counted.

Serum TNF-α Level. The circulating level of TNF-α was quantified at 1 h after GalN/LPS injection using commercial mouse ELISA kits (eBioscience, San Diego, CA), conducted according to the manufacturer's instructions.

Preparation of Protein Extracts. Freshly isolated liver tissue was homogenized in lysis buffer for preparation of whole protein extracts. NE-PER (Pierce Biotechnology, Rockford, IL) was used for extraction of nuclear proteins and cytosolic proteins, according to the manufacturer's instructions. Protein concentrations were determined using the BCA Protein Assay kit (Pierce Biotechnology).

Cytosolic Cytochrome *c* Content. For quantification of the cytochrome *c* released from the mitochondria, equal amounts (50 μg) of cytosolic proteins obtained from each experimental mouse were separated on 12% SDS/PAGE and then transferred onto a nitrocellulose membrane. The membrane was immersed in PBS containing 5% nonfat dried milk for 1 h at room temperature to block nonspecific binding and washed three times (5 min each). Subsequently, the membrane was incubated with a monoclonal antibody of cytochrome *c* (Transduction Laboratories, San Jose, CA) or with a monoclonal antibody of β-actin (Sigma Chemical Co.), in PBS containing 5% nonfat dried milk for 1 h at room temperature. After washing three times (5 min each), the membrane was incubated with a secondary antibody conjugated with horseradish peroxidase-coupled anti-IgG (Intron Biotechnology Co., Korea), for 1 h at room temperature. Binding of these antibodies was detected using an ECL detection system (Intron Biotechnology Co.), following the manufacturer's instructions. Intensity of the immunoreactive bands was determined using a densitometric analysis program (Image Gauge V3.12, Fuji film, Kyoto, Japan). Image density of specific bands for cytochrome *c* was normalized with the density of the β-actin band.

Caspase-3 and -8 Activities. Caspase activities were measured using an in vitro colorimetric peptide substrate, namely, Ac-Asp-Glu-Val-Asp *p*-nitroanilide for a caspase-3 substrate peptide and Ac-Ile-Glu-Thr-Asp *p*-nitroaniline for a caspase-8 substrate peptide (DEVD-AFC; BioMol, Plymouth Meeting, PA), according to a previous report.[22] Then 8 h after GalN/LPS treatment, a sample of liver tissue (1 g) was homogenized in 6 mL of buffer containing 25 mM Tris, 5 mM $MgCl_2$, 1 mM EGTA, and 50 μL of protease inhibitor cocktail (Sigma Chemical Co.). The homogenate was centrifuged for 15 min at 40000*g*, and the resulting supernatant was collected for determination of caspase activities. Dithiothreitol (10 mM) was added to the samples immediately before freezing. A 30 μg sample of cytosolic protein was incubated at room temperature for 15 min in buffer containing 30 mM HEPES, 0.3 mM EDTA, 100 mM NaCl, 0.15% Triton X-100, and 10 mM dithiothreitol. The reaction was initiated by addition of 200 μM DEVD-AFC, and the samples were then incubated at 37 °C. Change in fluorescence (extraction at 400 nm and emission at 490 nm) was monitored after 120 min.

Western Blot Immunoassay. Freshly isolated liver tissue was homogenized in a lysis buffer. For determination of the levels of TRADD, Bid, Bim, p-Bim, Bax, and p-JNK protein expression, 10 μg of protein samples from liver homogenates was loaded per lane on 10% polyacrylamide gels. The protein samples were then separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes using a semidry transfer process. After transfer, the membranes were washed with Tris-buffered saline (TBS) and blocked for 1 h at room temperature with 5% (w/v) skim milk powder in TBS. Blots were then incubated overnight at 4 °C with polyclonal antibodies against mouse TRADD (Cell Signaling Technology, Irvine, CA), bid (Santa Cruz Biotechnology, Santa Cruz, CA), bim (Santa Cruz Biotechnology), p-Bim (Cell Signaling Technology), Bax (Cell Signaling Technology), and p-JNK (Santa Cruz Biotechnology), with monoclonal antibodies against mouse β-actin (Sigma Chemical Co.). The next day, the primary antibody was removed and the blots were washed with TBST (0.05% Tween 20 in TBS). Binding of all of the antibodies was detected using an ECL detection system (Intron Biotechnology Co.), according to the manufacturer's instructions. ImageQuant TL software version 2005 (Amersham Biosciences, Piscataway, NJ) was used for densitometric evaluation of visualized immunoreactive bands.

Statistical Analysis. Survival data were analyzed by Kaplan−Meier curves and the log-rank test. All other data were analyzed by one-way ANOVA. Differences between compared groups were considered significant at $p < 0.05$, and the appropriate Bonferroni correction was made for multiple comparisons. All results are presented as the mean ± SEM.

■ ASSOCIATED CONTENT

Supporting Information. Summary of postulated protective effect of luteolin (**1**) associated with inhibition of intrinsic and extrinsic apoptotic pathways. This material is available free of charge via the Internet at http://pubs.acs.org.

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