Hepatoprotective Effect of Gallotannin-enriched Extract Isolated from Gall on Hydrogen Peroxide-induced Cytotoxicity in HepG2 Cells

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ABSTRACT

Background: Gall (Galla Rhois [GR]) is known to have antibacterial, anti-inflammatory, antimitotic, and anti-invasion activities and exert hepatoprotective effects. However, the hepatoprotective effects of gallotannin-enriched GR (GEGR) and their mechanisms have not yet been investigated. Objective: The potential protective effect of GEGR against hepatotoxicity induced by hydrogen peroxide (H2O2) was investigated.

Materials and Methods: Changes in cell viability, apoptosis protein expression, and reactive oxygen species (ROS) generation were determined in HepG2 cells that were pretreated with four different concentrations of GEGR (6.25–50 µg/ml) for 24 h before H2O2 exposure. Results: GEGR consisted of gallotannin (69.2%), gallic acid (26.6%), and methyl gallate (4.2%) and showed remarkable 2,2-diphenyl-1-picrylhydrazyl scavenging activity (inhibitory concentration 50% = 0.212 µg/ml). The lethal dose 50% and effective dose 50% values for the response of HepG2 cells to GEGR were determined to be 178 and 6.85 µg/ml, respectively. Significant reductions in the immunofluorescence intensity indicating apoptosis were also detected in the nuclei of HepG2 cells stained with 4',6-diamidino-2-phenylindole and Annexin V after GEGR treatment. The Bax/Bcl-2 ratio and active caspase-3 level after H2O2 exposure.

Conclusions: The results of this study provide strong evidence that GEGR can prevent cell death induced by H2O2 in HepG2 cells through the induction of antioxidant conditions.

Key words: Antioxidant, Caspase-3, cell death, hepatotoxicity, reactive oxygen species

INTRODUCTION

Gall (Galla Rhois [GR]) is the excrescence formed by parasitic aphids, primarily Schlechtendalia chinensis Bell, on the leaves of the nutgall sumac tree, Rhus javanica L. (Anacardiaceae). GR has long been used as a traditional Korean medicine for the treatment of diarrhea, seminal emissions, excessive sweating, bleeding, and chronic cough although there is little scientific evidence supporting these pharmacological effects. Recent studies have revealed the therapeutic effects of GR against various human diseases and their mechanisms. For example, several compounds and extracts purified from GR exhibited good antibacterial activity against many pathogenic bacteria strains including Salmonella spp., Escherichia coli, Eimeria tenella, Brucella abortus, Staphylococcus aureus, and Clostridium perfringens. Methyl gallate and ethyl gallate isolated from GR were also found to exert significant anti-inflammatory...
activity in lipopolysaccharide-stimulated RAW264.7 macrophages through the induction of heme oxygenase-1 and the suppression of inducible nitric oxide synthase/cyclooxygenase-2 (COX-2). Moreover, galloylglucose (GG6-10) isolated from GR inhibited the invasion of metastatic HT-1080 cells into a reconstituted basement membrane through inhibition of gelationolysis mediated by matrix metalloproteinase (MMP-2) and MMP-9, whereas the ellagic acid extracted from GR showed anticancer activity against nasopharyngeal carcinoma cells through downregulated expression of COX-2 and stathmin. Furthermore, oral administration of GR 85% methanol extract reduced brain infarct volume by 37.5% and lipid peroxidation in middle cerebral artery occlusion, while also improving sensory motor function in a transient focal cerebral ischemia rat model. Moreover, tacrine, nitrofurantoin, and tert-butyl hydroperoxide-induced hepatotoxicity in HepG2 cells were greatly alleviated by two hepatoprotective constituents of GR, an equilibrium mixture of 3-galloyl-gallic acid (3GGA), 4-galloyl-gallic acid isomers (4GGAi), and 1,2,3,4,6-penta-O-galloyl-β-d-glucose (PGG). However, more studies are needed to determine the novel functions and mechanisms of GR extract in hydrogen peroxide (H₂O₂)-induced hepatotoxicity. In this study, we investigated the hepatoprotective effects of galloptannin-enriched GR (GEGR) on H₂O₂-induced hepatotoxicity in HepG2 cells. The results provide novel data indicating that GEGR may associate with protective hepatocytes during apoptosis.

MATERIALS AND METHODS

Preparation of GEGR

Samples of GR were collected from plantations in the Hongcheon area of Korea in October 2013 by Professor Young-Hee Lee, then dried in a hot-air drying machine (JSR, Seoul, Korea) for 24 h at 60°C. Voucher specimens of GR (WPC-14-001) were deposited in the functional materials bank of the PNU-Wellbeing RIS Center at Pusan National University. GEGR was prepared using the modified extract quoting method as previously described. First, dry samples of GR were reduced to powder using an electric blender. Water extract was then obtained by placing the powder in a fixed liquor ratio (solid GR powder/water ratio, 1:10) and heating at 90°C for 9 h using circulating extraction equipment (IKAB Labortechnik, Staufen, Germany). The extracts were subsequently filtered through a 0.4 μm filter, after which they were concentrated by vacuum evaporation and lyophilization using circulating extraction equipment (IKAB Labortechnik, Staufen, Germany). Finally, the powder of GEGR was dissolved in distilled water (dH₂O) to 1 mg/ml, then further diluted with phosphate-buffered saline (PBS) to the required concentration.

Analysis of main components in GEGR

During analysis of the main components of GEGR, gallic acid monohydrate, methyl gallate, and galloptannin were purchased from Sigma-Aldrich Co., St. Louis, MO, USA. The wavelengths of the maximum absorption of pure gallic acid, pure methyl gallate, commercial galloptannin, and nutgall extract were 212/257, 214/268, 213/278, and 212/275 nm, respectively. The ultraviolet-visible (UV-Vis) spectra of pure gallic acid, pure methyl gallate, pure gallotannin, and the nutgall extract showed two bands at 212–214 and 257–278 nm, which were both assigned to the π→π* transitions of the given aromatic units and C=O groups in the UV-Vis region. Finally, the UV-Vis spectra were analyzed using a curve-resolving technique based on linear least squares analysis to fit the combined Lorentzian and Gaussian curves.

Fluorescence-activated cell sorter analysis

Apoptotic cells were detected using a Muse™ Annexin V and Dead Cell Kit (Cat. No. MCH100105, Millipore Co., Billerica, MA, USA) according to the manufacturer’s protocols. Briefly, cells of subset groups were suspended in MEM media (1 × 10⁶ cells/ml), after which 100 μl of the cell suspension (1 × 10⁶ cells/ml) was incubated with Muse™ Annexin V and Dead Cell Reagent (Cat. No. 12-0563, Millipore Co.) for 20 min at room temperature. After the final incubation, the reaction mixture
Analysis of intracellular reactive oxygen species level

Intracellular reactive oxygen species (ROS) levels were measured by staining with 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Cat. No. D6883, Sigma-Aldrich Co.), which is a cell permeable and nonfluorescent agent that can be deacetylated by intracellular esterases to nonfluorescent DCFH. In the presence of ROS, DCFH was converted to highly fluorescent DCF intracellularly. Briefly, HepG2 cells were seeded at a density of 5 × 10^4 cells/2 ml in 6-well plates for 24 h in a 37°C incubator. After washing once with x1 PBS, the cells were incubated with 5 mM H_2O_2 for another 12 h, then fixed in 4% formaldehyde (Cat. No. 69360-0380, Junsei Chemical Co. Ltd., Tokyo, Japan) for 5 min. Next, the DNA-specific fluorochrome DAPI (100 µM, Cat. No. D1306, Invitrogen) was applied to each well, after which samples were incubated for 10 min in the dark at room temperature. Finally, the cells were washed three times with x1 PBS and examined using a fluorescent microscope (Olympus IX71, Tokyo, Japan) at ×400 magnification.

Western blot

Proteins prepared from HepG2 cells were separated by 4%–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 2 h, after which they were transferred to nitrocellulose membranes for 2 h at 40 V. Each membrane was then incubated separately at 4°C with the following primary antibodies overnight: anti-Bax (Cat. No. ab7977, Abcam, Cambridge, UK), anti-Bcl-2 (Cat. No. ab7973, Abcam), anti-Casepase-3 (Cat. No. 9662, Cell Signaling, Danvers, MA, USA), and anti-actin antibody (Cat. No. A5316, Sigma-Aldrich). Next, the membranes were washed with washing buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 0.05% Tween 20) and incubated with 1:1000 diluted horseradish peroxidase-conjugated goat anti-rabbit IgG (Cat. No. G21234, Invitrogen) at room temperature for 1 h. Finally, membrane blots were developed using Amersham ECL Select Western Blotting detection reagent (Cat. No. RPN2235, GE Healthcare, Little Chalfont, UK). The chemiluminescence signals that originated from specific bands were detected using FluorChem™ FC2 (Alpha Innotech Co., San Leandro, CA, USA).

Statistical analysis

One-way ANOVA was used to identify significant differences between No- and H_2O_2-treated groups (SPSS for Windows, Release 10.10, Standard Version, Chicago, IL, USA). Differences in the responses of the H_2O_2+ vehicle and H_2O_2+ GEGR treated groups were evaluated using a post hoc test (SPSS for Windows, Release 10.10, Standard Version). All values are reported as the mean ± standard deviation and P < 0.05 was considered statistically significant.
Free radical scavenging activity of GEGR

To measure the antioxidant activity of GEGR, the free radical scavenging activity of DPPH was analyzed following treatment with various doses of GEGR. The inhibitory activity against DPPH radical was gradually increased by the addition of 0.12–500 µg/ml of GEGR. Based on these data, the IC₅₀ value of GEGR was determined to be 0.212 µg/ml [Figure 1c]. Taken together, these results indicate that GEGR has very strong DPPH radical scavenging activity and therefore has the potential for use as an antioxidant.

Protective effects of GEGR treatment against hydrogen peroxide-induced apoptosis

H₂O₂ treatment induces apoptosis through the production of ROS and oxidative stress in various cells. Therefore, we examined whether GEGR pretreatment can prevent hepatic cell death induced by H₂O₂ exposure. To accomplish this, cell viability was measured in HepG2 cells pretreated with different concentrations of GEGR by MTT assay, fluorescence-activated cell sorter staining, and DAPI staining analysis. Toxicity analysis revealed no significant alterations in cell viability in HepG2 cells pretreated with 6.25 µg/ml to 50 µg/ml of GEGR alone [Figure 2a], while the lethal dose 50% of GEGR was determined to be 178 µg/ml (data not shown). Overall, these findings indicated that GEGR exerted no toxicity at <50 µg/ml.

H₂O₂ + vehicle-treated cells showed low (53%) cell viability relative to the control group. However, their level was significantly higher in HepG2 cells treated with H₂O₂ and four concentrations of GEGR although the maximum level remained consistent in response to pretreatment with 12.5–50 µg/ml of GEGR [Figure 2b]. Based on the above data, the effective dose 50% was determined to be 6.85 µg/ml of GEGR. Similar results were observed upon DAPI staining: Specifically, more irregular nuclei were detected in H₂O₂ + vehicle-treated cells relative to the control group, whereas a lower number of irregular nuclei were observed in H₂O₂ + GEGR-treated cells than H₂O₂ + vehicle-treated cells [Figure 3]. The total number of apoptotic cells showed the reverse pattern. A significant increase in the number (347%–409%) of apoptotic cells was detected in H₂O₂ + vehicle-treated cells relative to the control group. Following pretreatment with GEGR, the number of apoptotic cells gradually decreased although a constant number was detected in HepG2 cells pretreated with 12.5, 25, or 50 µg/ml [Figure 4]. In conclusion, these findings indicate that GEGR pretreatment may prevent the cytotoxicity induced by H₂O₂ treatment in HepG2 cells.

Effects of GEGR on apoptosis-related protein expression

To determine if the increase in apoptotic cells was accompanied by altered expression of apoptosis-related proteins, the expression levels...
of Bax, Bcl-2, and caspase-3 were measured by Western blot analysis in HepG2 cells pretreated with GEGR before \( \text{H}_2\text{O}_2 \) exposure. The Bax/Bcl-2 ratio increased by 19.8% in the \( \text{H}_2\text{O}_2 \) + vehicle-treated group relative to the control group, whereas it decreased significantly in the GEGR pretreated group although cells pretreated with 6.25 \( \mu \text{g/ml} \) of GEGR showed a slight increase in this ratio. Furthermore, a dramatic change in the active form of caspase-3 was observed. Specifically, the intensity of the active form of caspase-3 in the \( \text{H}_2\text{O}_2 \) + vehicle-treated group was about two times higher than that in the control group, whereas it decreased significantly in the GEGR pretreated group relative to the control group [Figure 5]. In conclusion, these results suggest that GEGR pretreatment inhibits ROS production induced by \( \text{H}_2\text{O}_2 \) treatment.

**DISCUSSION**

The liver is a greater target of human toxicants than other organs. Various toxicants induce hepatic damage, necrosis, and apoptosis, and their administration for long periods of time leads to fibrosis, cirrhosis, and hepatic carcinoma. Therefore, novel therapeutic drugs with hepatoprotective activity are of great interest. In this study, we investigated the hepatoprotective effects of GEGR against \( \text{H}_2\text{O}_2 \)-induced cytotoxicity. The results demonstrated that pretreatment with GEGR may prevent apoptosis of HepG2 cells through suppression of caspase-3 activation and ROS production.

Previous studies have shown that several compounds in methanol and ethanol extracts of GR protect against tacrine- and nitrofurantoin-induced cytotoxicity in mammalian cells. Four main compounds, gallic acid methyl ester, gallic acid, an equilibrium mixture of 3GGA + 4GGAI, and PGG were identified in methanol extract of GR. The ethanol extract of GR contained syringic acid (18.5 mg), as well as the phenolics methyl gallate (20.7 mg) and gallic acid (19.5 mg). In addition, a study conducted by Lee et al. revealed that aqueous extract of GR contained gallotannin (69.0%), gallic acid (25.7%), and methyl gallate (5.3%) and showed deodorizing function and antibacterial activity. The concentrations of these components were very similar to those observed in the present study although they did differ slightly, possibly due to the origin of the GR used.

Tannic acid and its derivatives have promising hepatoprotective activity. Specifically, phlorotannins from *Eisenia bicyclis* that showed high contents of total phenolics and strong antioxidant activity
Effects of GEGR on intracellular reactive oxygen species production. Cells in each square of a ×100 magnification image (left column) were further examined under ×400 magnification (right column). Arrows indicate cells stained with 2′,7′-dichlorofluorescein diacetate.

Figure 6: Effects of GEGR on intracellular reactive oxygen species production. Cells in each square of a ×100 magnification image (left column) were further examined under ×400 magnification (right column). Arrows indicate cells stained with 2′,7′-dichlorofluorescein diacetate.

Capsase-3 is considered a key factor responsible for cleavage and inactivation of PPAR, as well as mitochondrial dysfunction (capsase-9 activation) and death receptor pathway activation (capsase-8 activation) in apoptosis of hepatocytes.[16,37] Treatment with PGG-inhibited production of the activated form of caspase-3 in primary rat hepatocytes treated with 100 μM glycodeoxycholic acid.[38] Moreover, the dose-dependent activation of caspase-3 in HepG2 cells by tacrine was inhibited by 2-phloroeckol, one of four types of phlorotannins isolated from the ethyl acetate of E. stolonifera.[39] Similar results were observed in the present study even though the concentration and composition of treatment compounds varied.

Excessive production of ROS can induce oxidative stress, cell dysfunction, and ultimately apoptosis and necrosis.[40] Tannic acid and several related products have been reported to inhibit ROS production. Specifically, ROS production induced by tert-butyl hydroperoxide and tacrine treatment significantly inhibited the procyanidin fractions from defatted grape seeds, as well as 2-phloroeckol from E. stolonifera in HepG2 cells.[38,40] In the present study, the ROS level was decreased in the H2O2 + GEGR-treated group relative to the H2O2 + vehicle-treated group. These findings are similar to those of previous studies although the decrease ratio varied.

CONCLUSION

The results of the present study indicated that GEGR was closely associated with prevention of H2O2-induced HepG2 cell death through regulation of apoptosis-related protein expression. These findings also indicate that GEGR plays a crucial role in the prevention of apoptosis through inhibition of ROS production. Furthermore, GEGR has the potential for use as a food supplement for the prevention of hepatotoxicity.

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Conflicts of interest

There are no conflicts of interest.

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