Integrative Medicine Research 10 (2021) 100466

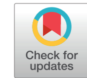
Contents lists available at ScienceDirect

Integrative Medicine Research

journal homepage: www.imr-journal.com

Original Article

Hepatoprotective effects of *Gamisoyo-san* against acetaminophen-induced liver injuries



Seong Eun Jin, Hyeun-Kyoo Shin, Hyekyung Ha*

Herbal Medicine Research Division, Korea Institute of Oriental Medicine, Daejeon, Republic of Korea

ARTICLE INFO

Article history:
Received 11 March 2020
Received in revised form 18 June 2020
Accepted 24 June 2020
Available online 1 July 2020

Keywords:
Acetaminophen
Acute liver injury
Gamisoyo-san
Hepatoprotection

ABSTRACT

Background: Acetaminophen (*N*-acetyl-*p*-aminophenol, APAP) is a safe and effective analgesic at therapeutic dosage. However, APAP overdose is a major cause of acute liver injury. *Gamisoyo-san* (GMSYS; *Jiaweixiaoyao-san* in Chinese, *Kamishoyo-san* in Japanese), a traditional herbal formula, is used to treat phlegm and cough in Korea. The purpose of this study was to investigate the hepatoprotective effect of GMSYS against APAP-induced liver injury *in vitro* and *in vivo*.

Methods: We evaluated the effect of GMSYS on APAP-induced hepatotoxicity by measuring cell viability in murine BNL CL.2 liver cells. Additionally, BALB/c mice were orally administered with GMSYS once daily for 7 days. Eighteen hours after the last administration, mice were intraperitoneally injected with 200 mg/kg APAP. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, hepatic antioxidants, and histological changes were examined.

Results: Pretreatment with GMSYS attenuated the decrease in cell viability induced by APAP in BNL CL.2 cells. In mice, pre-administration with GMSYS alleviated APAP-induced hepatotoxicity by decreasing plasma ALT and AST activities and hepatic malondialdehyde, and by increasing the total glutathione (GSH)/reduced GSH ratio and the activities of several antioxidants such as superoxide dismutase, catalase, GSH peroxidase, GSH reductase, GSH-S-transferase, and heme oxygenase-1.

Conclusion: GMSYS has a protective effect against APAP-induced acute liver injury by decreasing plasma transaminases and increasing antioxidants. GMSYS may be an effective candidate for the prevention of acute liver injury.

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1. Introduction

The liver plays a central role in the metabolic, vascular, immunological, secretory, and excretory functions of the body.[1] Drug-induced liver injury causes clinically severe effects and seriously endangers human health.[2,3] The most clinically relevant drug in this class is acetaminophen (*N*-acetyl-*p*-aminophenol, APAP).[2] APAP is widely used globally as an effective analgesic and antipyretic agent. At therapeutic doses, APAP is mainly excreted in the urine via conjugation with glucuronide or sulfate, and small concentrations are metabolized to *N*-acetyl-*p*-benzoquinone imine (NAPQI), a reactive metabolite, through the cytochrome P450 (CYP450)-dependent oxidative enzyme pathway.[4] Although NAPQI is detoxified by glutathione (L-γ-glutamyl-L-cysteinyl glycine, GSH), an overdose of APAP leads to the depletion of GSH through the accumulation of NAPQI and saturates the glucuronidation and sulfation pathways.[5–7] Consequently, excess NAPQI binds with free thiols on intracellular proteins to generate reactive oxygen species (ROS).[8,9] Previous reports have shown that oxidative stress is closely related to APAP hepatotoxicity. Additionally, lipid peroxidation resulting from oxidative stress is a pivotal marker for cell death due to APAP overdose.[10] Recently, much research has demonstrated that various natural products have the potential to prevent liver injury by stimulating the defense system against oxidative damage by APAP.[11]

Gamisoyo-san (GMSYS; *Jiaweixiaoyao-san* in Chinese, *Kamishoyo-san* in Japanese) is traditional herbal formula comprising twelve different medicinal herbs (Paeoniae Radix, Atractylodis Rhizoma Alba, Anemarrhenae Rhizoma, Lycii Radicis Cortex, Angelicae Gigantis Radix, Poria Sclerotium, Liriope Tuber, Rehmanniae Radix Crudus, Gardeniae Fructus, Phellodendri Cortex, Platycodi Radix, and Glycyrrhizae Radix et Rhizoma). According to "Donguibogam",[12] a traditional Korean medicinal book, GMSYS has been used to alleviate blood deficiency, irritability heat, night sweats, flushing, phlegm and cough. In Korea, GMSYS is one of the most popular used formula for the treatment of menopausal

* Corresponding author at: Herbal Medicine Research Division, Korea Institute of Oriental Medicine, 1672 Yuseong-daero, Yuseong-gu, Daejeon 34054, Republic of Korea.

E-mail address: hkha@kiom.re.kr (H. Ha).

https://doi.org/10.1016/j.imr.2020.100466

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Table 1
Composition of GMSYS.

Latin name	Scientific name	Amount (g)	Origin
Paeoniae Radix	*Paeonia lactiflora* Pallas	4.500	Uiseong, Korea
Atractylodis Rhizoma Alba	*Atractylodes macrocephala* Koidzumi	4.500	China
Anemarrhenae Rhizoma	*Anemarrhena asphodeloides* Bunge	3.750	Kangjin, Korea
Lycii Radicis Cortex	*Lycium chinense* Miller	3.750	China
Angelicae Gigantis Radix	*Angelica gigas* Nakai	3.750	Bonghwa, Korea
Poria Sclerotium	*Poria cocos* Wolf	3.000	Pyeongchang, Korea
Liriope Tuber	*Liriope platyphylla* Wang et Tang	3.000	Miryang, Korea
Rehmanniae Radix Crudus	*Rehmannia glutinosa* Liboschitz var. *purpurea* Makino	3.000	Gunwi, Korea
Gardeniae Fructus	*Gardenia jasminoides* Ellis	1.875	Gurye, Korea
Phellodendri Cortex	*Phellodendron amurense* Ruprecht	1.875	China
Platycodi Radix	*Platycodon grandiflorum* A. De Candolle	1.125	Muju, Korea
Glycyrrhizae Radix et Rhizoma	*Glycyrrhiza uralensis* Fischer	1.125	China
Total		35.25	

symptoms and their associated patterns.[13] Our previous studies showed that GMSYS possesses anti-inflammatory effects by reducing the expression of inducible nitric oxide synthase and cyclooxygenase-2, as well as the release of nitric oxide, prostaglandin E_2, tumor necrosis factor alpha, and interleukin-6 in RAW 264.7 macrophages.[14] In addition, Go et al.[15] reported that GMSYS inhibited lipid accumulation induced by oleic acid in HepG2 cells.[15] However, the hepatoprotective effect of GMSYS has not yet been investigated. In the present study, we investigate the protective efficacy and mechanisms of GMSYS on APAP-induced hepatotoxicity using BNL CL.2 cells and a mouse model.

2. Methods

2.1. Preparation of GMSYS

GMSYS was prepared as previously reported[14] and the ratio of 12 medicinal herbs composing GMSYS was presented in Table 1. Voucher specimens (2012-KE45-1~KE45-11) were deposited at the Herbal Medicine Research Division, Korea Institute of Oriental Medicine, Republic of Korea. The high-performance liquid chromatography (HPLC) profile of GMSYS has been previously reported,[14] and the contents of gallic acid, neomangiferin, chlorogenic acid, mangiferin, geniposide, paeoniflorin, berberine, liquiritin, nodakenin, glycyrrhizin, and atractylenolide III–the eleven marker compounds in GMSYS–were 0.58 ± 0.01, 0.45 ± 0.01, 0.90 ± 0.01, 1.99 ± 0.01, 8.37 ± 0.05, 8.91 ± 0.09, 2.01 ± 0.001, 0.94 ± 0.002, 1.43 ± 0.01, 0.81 ± 0.01, and 0.04 ± 0.001 mg/g, respectively. The correlation coefficients (r^2) of these compounds exhibited good linearity (≥ 0.9996).

2.2. Chemicals and materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo (Kumamoto, Japan). APAP, silymarin, and RIPA buffer were purchased from Sigma Aldrich (St. Louis, MO, USA). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity assay kits were obtained from BioVision, Inc. (Milpitas, CA, USA), and assay kits for thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST) were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Microplate assay kits for total GSH (GSH_t)/oxidized GSH (glutathione disulfide; GSSG) and Heme Oxygenase-1 (HO-1) Mouse SimpleStep ELISA kits were purchased from Oxford Biomedical Research (Rochester Hills, MI, USA) and Abcam (Cambridge, UK & Cambridge, MA, USA), respectively. Protease inhibitor cocktail and Bio-Rad Protein Assay Reagent were obtained from Roche Applied Science (Indianapolis, IN, USA) and Bio-Rad Laboratories (Hercules, CA, USA), respectively.

2.3. Cell culture

The murine liver cell line, BNL CL.2, was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). BNL CL.2 cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) in a 5% CO_2 incubator at 37°C.

2.4. Cell viability assay

Cell viability was determined using the CCK-8 kit (Dojindo). BNL CL.2 cells (2.5×10^3 cells/well) were plated into 96-well plates and left to adhere overnight at 37°C under a humidified atmosphere with 5% CO_2. Cells were pretreated with GMSYS at various concentrations ranging from 100–400 μg/mL for 2 h and treated with/without APAP (20 mM) for an additional 22 h. At the end of the incubation period, CCK-8 reagent was added to each well and incubated for 4 h. The absorbance (Abs) at 450 nm was measured using a Benchmark plus microplate reader (Bio-Rad Laboratories). Cell viability was calculated using the following equation:

$$\text{cell viability (\%)} = \frac{\text{mean Abs in test sample wells}}{\text{mean Abs in control wells}} \times 100$$

Data are expressed as the mean ± standard error of the mean (SEM; n = 4).

2.5. Animals

Male BALB/c mice (5 weeks of age) were obtained from DBL Co., Ltd. (Chungcheongbuk-do, Republic of Korea) and used for experiments after a week of acclimatization. Animals were kept in a temperature-controlled room at 23 ± 3°C, relative humidity of ~40–60%, and a 12-h light/dark cycle. Water and commercial rodent chow were provided *ad libitum*. This study was conducted according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals,[16] and all experiments were approved by the Institutional Animal Care and Use Committee of the Korea Institute of Oriental Medicine. The approval number for this study is #17-013.

2.6. Animal treatments

According to the literature, one pack of GMSYS is equivalent to 35.25 g.[12] The yield of GMSYS extract used in this study was about 19.41%. Therefore, a human of 60 kg would need to consume about 114.03 mg/kg of our GMSYS extract per pack. In the present study, the highest dose for the mice was calculated according to the

conversion principle of mice dose to human equivalent dose (HED) based on body surface area.[17] Therefore, 1400 mg/kg was selected as the high dose, and the low dose was 700 mg/kg. The mice were divided into seven groups (n = 8 animals/group) as follows:

1) *Control group:* distilled water (oral administration; p.o.) once daily for 7 days followed by warm distilled water (intraperitoneal injection; i.p.)
2) APAP group: distilled water (p.o.) once daily for 7 days followed by APAP (200 mg/kg, i.p.)
3) Silymarin + APAP group: silymarin (200 mg/kg, p.o.) once daily for 7 days followed by APAP (200 mg/kg, i.p.)
4) Silymarin group: silymarin (200 mg/kg, p.o.) once daily for 7 days followed by warm distilled water (i.p.)
5) GMSYS 700 + APAP group: GMSYS (700 mg/kg, p.o.) once daily for 7 days followed by APAP (200 mg/kg, i.p.)
6) GMSYS 1400 + APAP group: GMSYS (1400 mg/kg, p.o.) once daily for 7 days followed by APAP (200 mg/kg, i.p.)
7) GMSYS 1400 group: GMSYS (1400 mg/kg, p.o.) once daily for 7 days followed by warm distilled water (i.p.).

Silymarin is a standardized extract of the milk thistle (*Silybum marianum*), and chiefly contains flavonoids including silybin, silybinin, silydianin, and silychristin.[18] In our previous study, we confirmed the protective effect of silymarin against APAP-induced liver injury.[19] Silymarin was therefore used as a positive control. Silymarin and GMSYS were prepared in distilled water, and mice in the control and APAP groups were orally pre-administered with distilled water. Mice in the other groups were orally pre-administered with silymarin or different doses of GMSYS once daily for 7 consecutive days. At 6 h after the final administration, acute liver injury was induced by i.p. with 200 mg/kg APAP prepared in warm distilled water. Mice were fasted for 18 h after APAP injection and then sacrificed with Entobar (pentobarbital sodium; Hanlim Pharm. Co., Ltd., Seoul, Republic of Korea). Blood was collected in BD Microtainer blood collection tubes containing EDTA-2 K (Becton Dickinson Co., Franklin Lakes, NJ, USA) from the abdominal veins; plasma was separated (3,000 rpm for 15 min at 4°C) and then stored at −80°C for subsequent assays. The liver and kidneys were immediately excised and weighed. The right lobe of the liver and the kidneys were immediately fixed in 4% paraformaldehyde for histopathological examinations. The left lobe of the liver was stored at −80°C for other analyses.

2.7. Plasma ALT and AST activity assays

ALT and AST activity was measured in plasma using a commercial kit according to the manufacturer. The Abs at 450 nm and fluorescence intensity at excitation 535 nm and emission 587 nm were measured using the SpectraMax i3 Multi-Mode Platform (Molecular Devices, Sunnyvale, CA, USA).

2.8. Preparation of liver tissue homogenates

Liver tissue (50 mg) was homogenized in 500 μL RIPA buffer containing protease inhibitor cocktail using a rotor-stator homogenizer (T 10 basic ULTRA-TURRAX; IKA works, Staufen, Germany). The homogenates were centrifuged at 10,000 × g for 15 min at 4°C, and the supernatant was collected. The protein concentration was measured using Bio-Rad Protein Assay Reagent. To quantify GSSG activity exclusive of GSH, liver tissue (50 mg) was quickly added to 5 μL 2-vinylpyridine (1 M) and 500 μL RIPA buffer containing the above protease inhibitor cocktail and homogenized using a rotor-stator homogenizer. All subsequent assays were performed as described in this section.

2.9. Hepatic lipid peroxidation assay

Lipid peroxidation in the liver was measured based on the formation of malondialdehyde (MDA). MDA content was measured using a commercial TBARS assay kit. The Abs at 535 nm was measured using a microplate reader.

2.10. Hepatic antioxidative activity assay

To investigate the antioxidative effects of GMSYS, the concentrations of GSH_t, GSSG, and HO-1, as well as the activities of antioxidants including catalase, SOD, GPx, GR, and GST in the liver were measured using a commercial kit. The Abs was measured using a microplate reader.

2.11. Histopathological examination

Liver and kidney tissues were fixed in 4% paraformaldehyde, and then embedded in paraffin, sectioned into 4-μm slides and stained with hematoxylin and eosin (H&E; Sigma Aldrich). Histopathological changes were assessed by microscopy according to the previously described criteria.[20]

2.12. Statistical analysis

Data were expressed as the mean ± SEM. All statistical analyses were performed by one-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test using SYSTAT (Version 10.0; Systat Software Inc., San Jose, CA, USA). A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. In vitro *effect of GMSYS against APAP-induced hepatotoxicity*

As shown in Fig. 1, no significant cytotoxicity was showed in GMSYS at doses of up to 400 μg/mL. APAP significantly decreased cell viability to 43.68 ± 1.04% of the negative control ($P < 0.01$). In contrast, pretreatment with GMSYS at concentrations of 100 μg/mL (48.09 ± 1.07 % of the negative control, $P < 0.05$ when compared with the APAP group), 200 μg/mL (52.47 ± 0.99% of the negative control, $P < 0.01$ when compared with the APAP group), or 400 μg/mL (51.24 ± 1.88% of the negative control, $P < 0.01$ when compared with the APAP group) attenuated the rates of cell death caused by APAP.

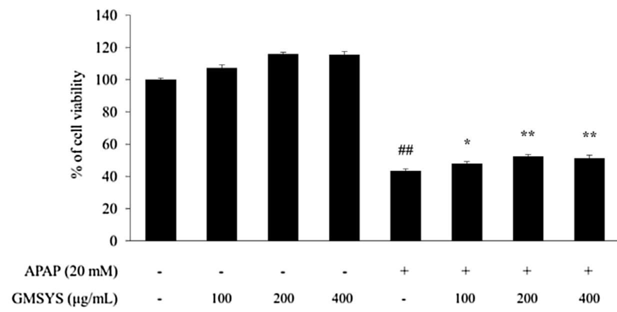
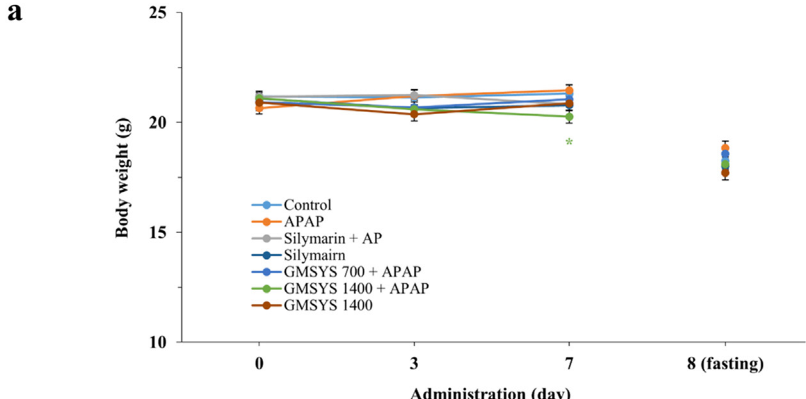
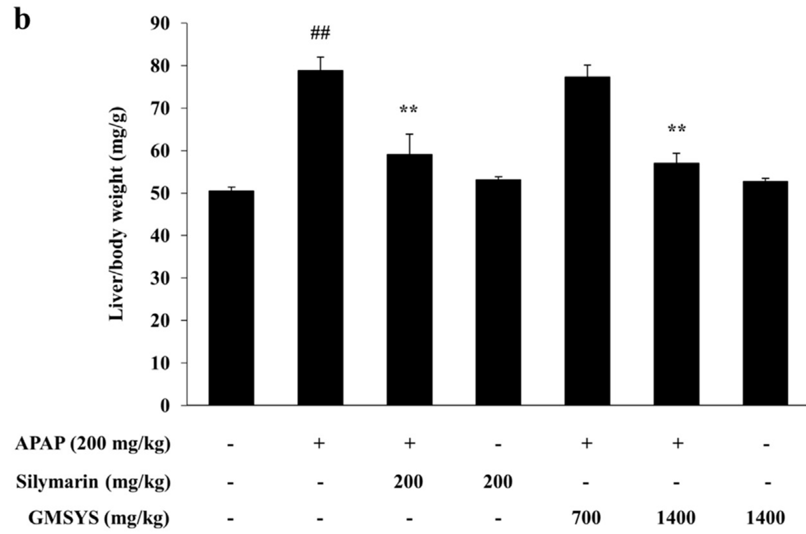


Fig. 1. Effect of GMSYS on APAP-induced hepatotoxicity in BNL CL.2 cells.
Cells were pretreated with the various concentrations of GMSYS for 2 h and treated with APAP (20 mM) for 22 h. Cell viability was measured using CCK-8 kit. Data are presented as mean ± SEM (n = 4). ## indicates a statistical significant difference at $P < 0.01$, when compared with negative control cells; * and ** indicates a statistical significant difference at $P < 0.05$ and $P < 0.01$, respectively, when compared with APAP-alone treated cells.





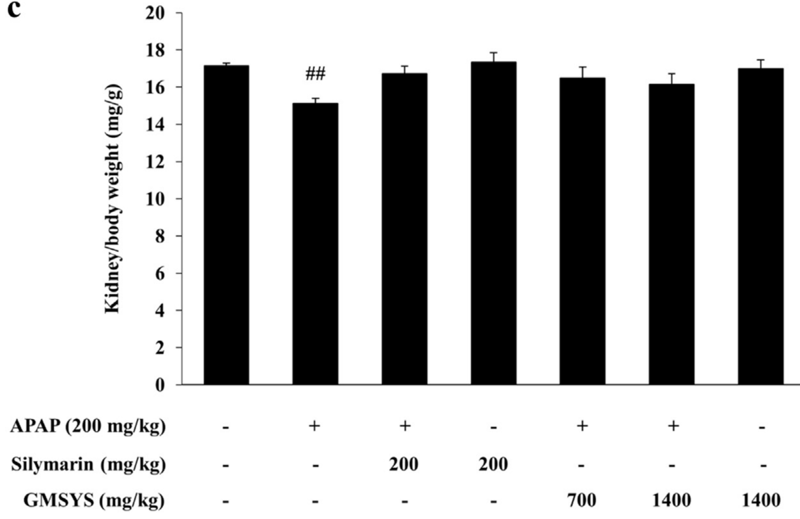


Fig. 2. Effect of GMSYS on the weight of body (a), liver (b) and kidneys (c) in APAP-treated mice.
Data are presented as mean ± SEM (n = 8). ## indicates a statistical significant difference at P < 0.01, when compared with the control group; * and ** indicates a statistical significant difference at $P < 0.05$ and $P < 0.01$, respectively, when compared with the APAP group.

3.2. Changes in body weight and relative organ weight

As shown in Fig. 2a, a significant decrease in body weight was observed on day 7 in the GMSYS 1400 + APAP group when compared to the APAP group. However, no significant changes in body weight were observed in the GMSYS 1400 mg/kg/day alone-treated group compared to the control group.

Relative liver weight was significantly increased by APAP injection compared to that in the control group ($P < 0.01$); however, both the silymarin and GMSYS 1400 mg/kg/day treatments significantly

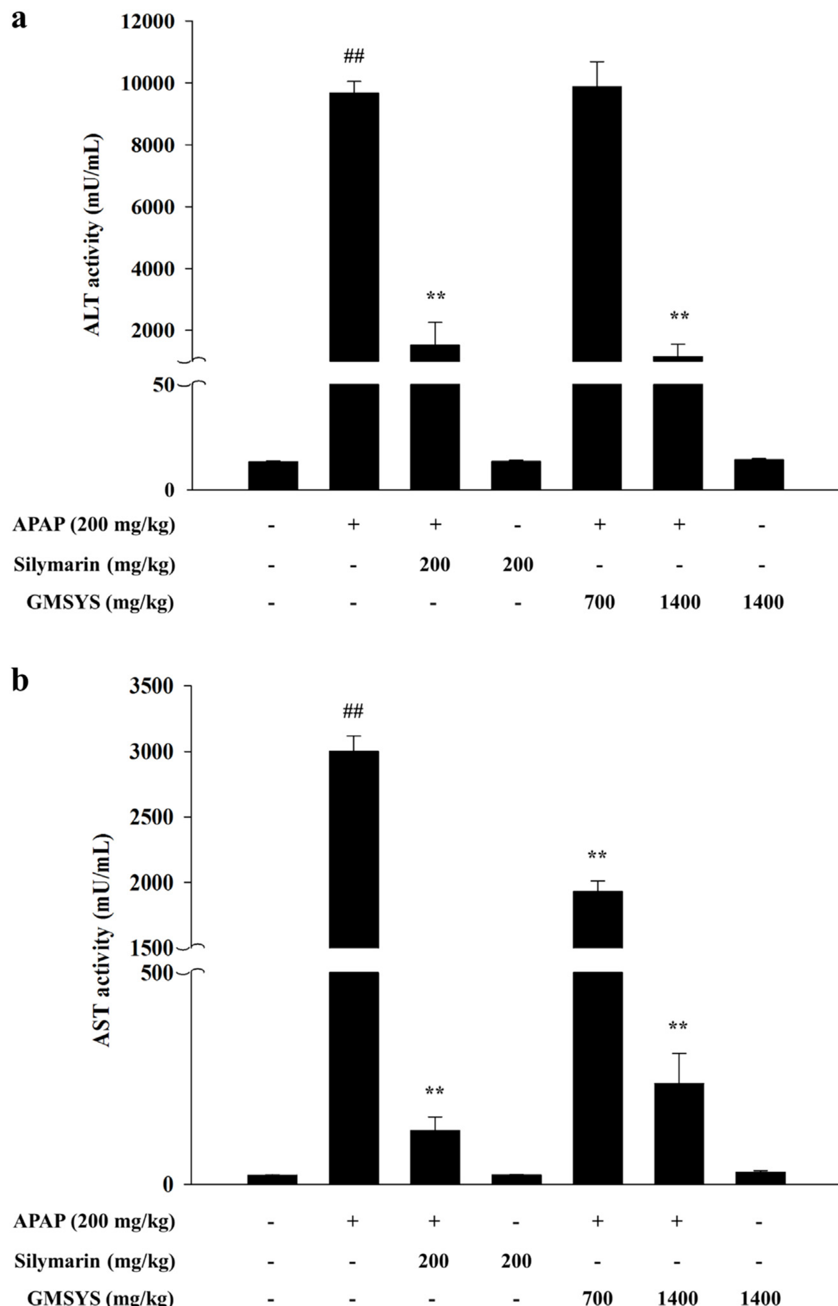


Fig. 3. Effect of GMSYS on the activities of plasma ALT (a) and AST (b) in APAP-treated mice.
Data are presented as mean ± SEM (n = 8). ## indicates a statistical significant difference at $P < 0.01$, when compared with the control group; ** indicates a statistical significant difference at $P < 0.01$, when compared with the APAP group.

lowered the relative liver weight compared to that in the APAP group (Fig. 2b, $P < 0.01$). Relative kidney weight was significantly decreased in the APAP group compared to the control group ($P < 0.01$); however, this sign was alleviated in the silymarin or GMSYS treatment groups (Fig. 2c).

3.3. *Effect of GMSYS on plasma ALT and AST activities*

As shown in Fig. 3 and Supplementary data 1, the activities of ALT (9674.60 ± 369.65 mU/mL, Fig. 3a) and AST (3002.73 ± 114.69 mU/mL, Fig. 3b) in plasma were significantly increased after APAP injection ($P < 0.01$). In contrast, administration with GMSYS at a dose of 1400 mg/kg/day significantly inhibited the activities of ALT (1147.24 ± 401.82 mU/mL) and AST (238.00 ± 70.98 mU/mL) in plasma in APAP-treated mice ($P < 0.01$). The plasma level of ALT in the GMSYS 700 + APAP group (9880.03 ± 807.05 mU/mL) was not different compared to that in the APAP group (Fig. 3a). Treatment with GMSYS at a dose of 700 mg/kg/day (1930.27 ± 79.74 mU/mL) caused a significant inhibition of AST activity in plasma compared to that in the APAP-treated mice (Fig. 3b, $P < 0.01$), but still remained at a high level of AST. Administration with silymarin at a dose of 200 mg/kg/day suppressed the activities of plasma ALT (1514.98 ± 738.74 mU/mL) and AST (126.98 ± 32.16 mU/mL) in APAP-treated mice ($P < 0.01$). Administration of silymarin alone or 1400 mg/kg/day GMSYS alone did not significantly affect the activities of plasma ALT and AST compared to those in the control group.

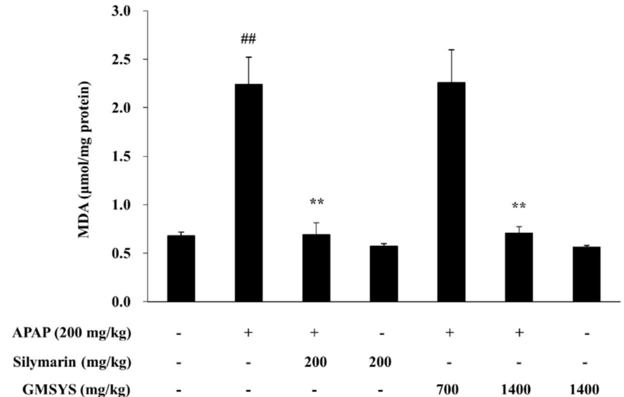


Fig. 4. Effect of GMSYS on lipid peroxidation in the liver of APAP-treated mice. Data are presented as mean ± SEM (n = 8). ## indicates a statistical significant difference at $P < 0.01$, when compared with the control group; ** indicates a statistical significant difference at $P < 0.01$, when compared with the APAP group.

3.4. Effect of GMSYS on hepatic lipid peroxidation

As presented in Fig. 4 and Supplementary data 1, MDA content was markedly increased in the livers of mice in the APAP only-treated group (2.24 ± 0.28 μmol/mg) when compared to those in the control group ($P < 0.01$). In the silymarin- (0.69 ± 0.12 μmol/mg) or 1400 mg/kg/day GMSYS-treated (0.71 ± 0.07 μmol/mg) groups, a significant decrease in the MDA content increased by APAP injection was observed ($P < 0.01$). The administration of silymarin alone or 1400 mg/kg/day GMSYS alone caused no significant changes in MDA content compared to that of the control group.

3.5. Effect of GMSYS on hepatic antioxidative activity

The activities of SOD (3.30 ± 0.35 U/mg), catalase (833.98 ± 154.66 U/mg), GPx (250.88 ± 18.11 nmol/min/mg), GR (12.59 ± 1.86 nmol/min/mg), and GST (10.58 ± 1.62 nmol/min/mg) in the APAP group were decreased compared to those in the control group (Fig. 5 and Supplementary data 1, $P < 0.05$). Conversely, treatment with 1400 mg/kg/day GMSYS significantly increased the activities of SOD (4.55 ± 0.16 U/mg, Fig. 5a), catalase (3172.76 ± 359.08 U/mg, Fig. 5b), GPx (481.82 ± 25.37 nmol/min/mg, Fig. 5c), GR (21.18 ± 1.01 nmol/min/mg, Fig. 5d), and GST (22.61 ± 1.90 nmol/min/mg, Fig. 5e) compared to that in the APAP group ($P < 0.01$). Meanwhile, the catalase activity tended to increase in 700 mg/kg/day GMSYS-treated group (2078.50 ± 358.95 U/mg) compared to the APAP group, but there was no significance. Additionally, there was no difference in the activities of SOD (3.02 ± 0.19 U/mg), GPx (282.30 ± 23.11 nmol/min/mg), GR (13.78 ± 1.48 nmol/min/mg), and GST (10.32 ± 1.08 nmol/min/mg) between the APAP group and 700 mg/kg/day GMSYS-treated group. Silymarin increased the activities of SOD (4.01 ± 0.70 U/mg), catalase (1279.58 ± 95.83 U/mg), and GST (16.65 ± 1.54 nmol/min/mg) compared to that in the APAP group, but not significantly. The activities of GPx (407.08 ± 30.89 nmol/min/mg, $P < 0.01$) and GR (18.71 ± 1.36 nmol/min/mg, $P < 0.05$) significantly increased by administration with silymarin compared to that in the APAP group.

APAP treatment caused a marked decrease in the GSH_t/GSSG ratio (13.24 ± 2.37, Fig. 5f and Supplementary data 1, $P < 0.01$). In APAP-treated mice, administration with silymarin (74.92 ± 13.60) or 1400 mg/kg/day GMSYS (71.67 ± 8.48) significantly increased the GSH_t/GSSG ratio compared to that in the APAP group ($P < 0.05$). Treatment with 700 mg/kg/day GMSYS increased the GSH_t/GSSG ratio (39.87 ± 12.87) but not significantly, compared to that in the APAP group. Treatment with silymarin alone or 1400 mg/kg/day

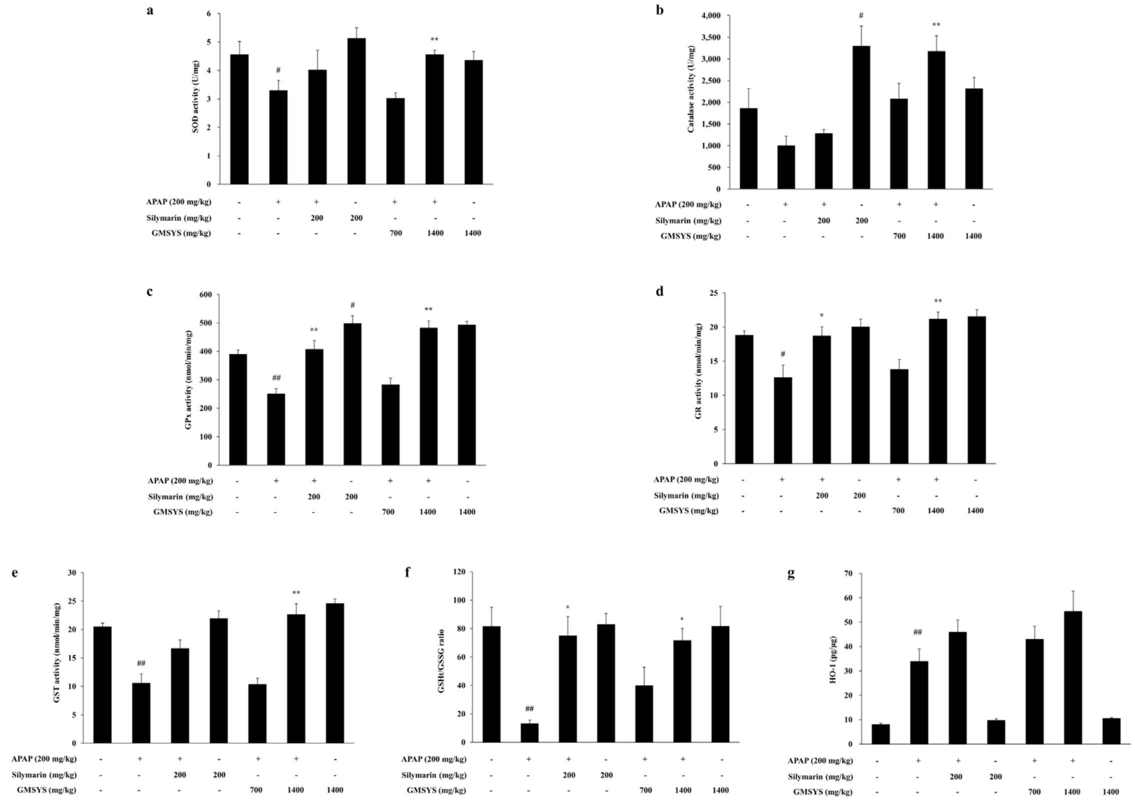


Fig. 5. Effect of GMSYS on the activities of SOD (a), catalase (b), GPx (c), GR (d), and GST (e), GSH_t/GSSG ratio (f), and HO-1 level (g) in the liver of APAP-treated mice. Data are presented as mean ± SEM (n = 8). # and ## indicate a statistical significant difference at $P < 0.05$ and $P < 0.01$, respectively, when compared with the control group; * and ** indicate a statistical significant difference at $P < 0.05$ and $P < 0.01$, respectively, when compared with the APAP group.

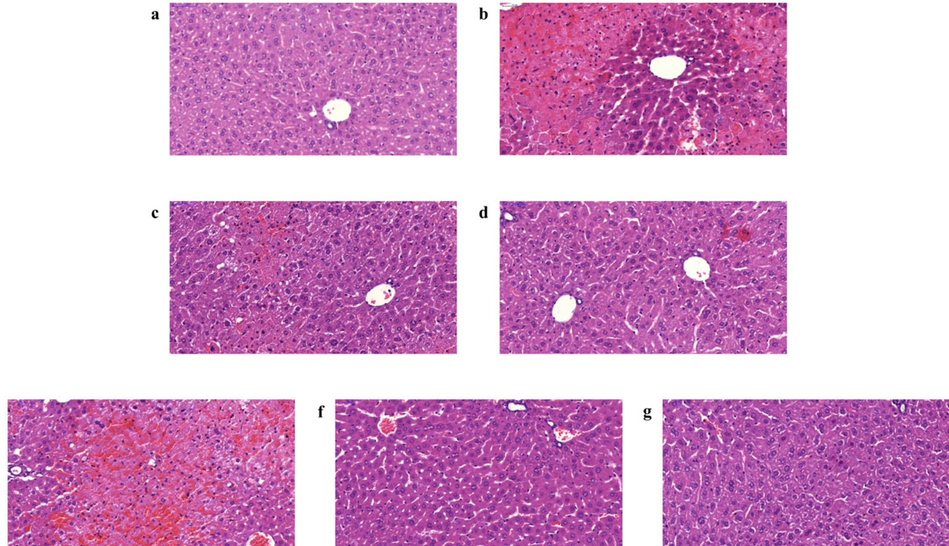


Fig. 6. Histopathological micrograph of the liver in APAP-treated mice.
(a) Control; (b) APAP; (c) silymarin 200 mg/kg/day + APAP; (d) silymarin 200 mg/kg/day; (e) GMSYS 700 mg/kg/day + APAP; (f) GMSYS 1400 mg/kg/day + APAP; (g) GMSYS 1400 mg/kg/day. The liver sections were stained with H&E ($\times$ 40).

GMSYS alone showed no significant changes in the GSH_t/GSSG ratio compared to that in the control group.

APAP treatment resulted in a significant elevation of HO-1 level compared to that in the control group (33.87 $\pm$ 5.15 pg/μg, Fig. 5g and Supplementary data 1, $P < 0.01$). HO-1 level was increasedin the silymarin + APAP (45.85 $\pm$ 5.10 pg/μg), GMSYS 700 + APAP (42.98 $\pm$ 5.39 pg/μg), and GMSYS 1400 + APAP (54.37 $\pm$ 8.35 pg/μg) groups compared to that in the APAP group, but not significantly.

3.6. Histopathological examination

As shown by H&E staining (Fig. 6), the livers of mice from the control group showed no pathological abnormalities (Fig. 6a). In contrast, APAP treatment resulted in several histopathological changes in the liver, such as major cell necrosis, loss of hepatocyte architecture around the blood vessels, and the infiltration of lymphocytes (Fig. 6b). However, APAP-induced hepatic necrosis was ameliorated by pre-administration with silymarin or 1400 mg/kg/day GMSYS (Fig. 6c and 6f). Meanwhile, no histopathological changes associated with renal toxicity in all groups (Supplementary data 2).

4. Discussion

Pharmaceutical drugs used in the treatment of liver diseases not only provide limited benefits, but are also not effective in all patients; some patients cannot tolerate certain therapies. Therefore, it is important to develop effective and non-toxic pharmaceuticals for the treatment of hepatic diseases. The use of herbal medicine to treat hepatic diseases has been previously described in the literature, and a considerable number of medicinal plants and formulas have been known to show hepatoprotective effects.[21,22] In the present study, we found that GMSYS showed protective effects against APAP-induced liver injury both *in vitro* and *in vivo*.

In BNL CL.2 cells, APAP-induced cell death was significantly reduced when cells were pretreated with GMSYS. These results imply that GMSYS pretreatment had a protective effect against APAP-induced liver damage. Therefore, we used APAP-induced liver injury mice model to verify the hepatoprotective effect of GMSYS *in vivo*.

Organ weights are one of the important toxicological parameter for evaluating drug toxicity.[23] In this study, although there were no significant differences in body weights in the control and APAP group, higher liver/body weight ratio had been observed in the APAP-treated mice compared to those in the control group. The enlargement of liver in the APAP-treated mice indicate that hepatic lesions and liver injury related to the accumulation of collagen and extra cellular matrix protein in liver.[24] Pre-administration with 1400 mg/kg/day GMSYS significantly decreased the liver/body weight ratio. Histopathologically, acute renal toxicity is characterized by injury of the proximal tubule cell and decreases of glomerular filtration rate.[25] Although the kidney weight was reduced by APAP in the present study, there was no histopathological changes associated with renal toxicity in all groups (Supplementary data 2). Therefore, it is thought that the kidney weight decreased by APAP in this study was considered to be not associated with toxicity.

ALT and AST are primarily found in the liver, and their activity is majorly affected when cells are damaged by APAP.[26] Consequently, the levels of ALT and AST are commonly measured to verify liver damage.[27] The results of this study revealed that pre-administration with 1400 mg/kg/day GMSYS inhibited plasma ALT and AST activity after its elevation by APAP.

APAP overdose triggers oxidative stress, mitochondrial dysfunction, and liver cell death (apoptosis/necrosis) through a decreased GSH_t/GSSG ratio and increased MDA content, which is a sign of lipid peroxidation.[28,29] In contrast, it has been reported that antioxidants can prevent hepatic damage by eliminating free radicals and preventing lipid peroxidation.[30,31] The hepatoprotective effects of natural products have been frequently attributed to their antioxidant activity and their ability to mobilize the endogenous antioxidant defense system.[32] Our results revealed that the antioxidative effect of GMSYS reduced liver injury; we also provided evidence for some mechanisms underlying the protective effect of GMSYS against APAP-induced liver injury.

Lipid peroxidation is one of the most frequently activated mechanisms in ROS-induced cell death and liver injury.[33] In this study, we demonstrated that pre-administration with GMSYS at a dose of 1400 mg/kg/day reduced the content of hepatic MDA in APAP-treated mice.

Aerobic organisms have an efficient biochemical defense system that includes antioxidative enzymes such as SOD, catalase, and GPx to prevent oxidative damage and redox imbalance.[34] SOD can convert O_2^- into hydrogen peroxide (H_2O_2) as a metal-containing protein that catalyzes the removal of superoxide. H_2O_2 is then converted to H_2O and O_2 through catalase, a heme-containing enzyme, and GPx catalyzes the reduction of H_2O_2 by utilizing two molecules of GSH.[35,36] The depletion of GSH results in the inability to detoxify many reactive metabolites and may increase the risk of hepatotoxicity. Therefore, the SOD and glutathione redox system are major components of the cellular antioxidant system to neutralize free radicals.[37] GSSG, which is produced during the reduction of H_2O_2 by GPx, is reduced to GSH by GR, and it is the reduced form that exists mainly in biological systems.[36] GSSG is accumulated inside the cells and the ratio of GSH_t/GSSG is a good factor to measure the extent of oxidative stress in an organism.[38] GMSYS at a dose of 1400 mg/kg/day successfully normalized the ratio of GSH_t/GSSG and restored the activities of SOD, catalase, GPx, GR, and GST, indicating that GMSYS showed strong antioxidative activity.

The HO-1 enzyme is known to play a major role in maintaining antioxidant homeostasis under various stress conditions.[39] It has been proven that HO-1 upregulation reduces oxidative stress-mediated cellular injury.[40] Some flavonoids and polyphenols have been reported to show hepatoprotective effects through the upregulation of HO-1 expression.[39] In our study, pre-administration of GMSYS at a dose of 1400 mg/kg/day increased HO-1 protein expression, indicating that HO-1 upregulation by GMSYS protected the liver against injury from APAP-induced oxidative stress. Additionally, our histopathology results indicated that inflammatory cell infiltration and severe necrosis were markedly decreased in the livers of GMSYS 1400 mg/kg/day-treated mice.

In conclusion, the present study demonstrated for the first time GMSYS was effective in preventing APAP-induced liver injury *in vitro* and *in vivo*. Our data indicated that GMSYS prevented APAP-induced liver cell death in BNL CL.2 cells. In addition, GMSYS effectively attenuated APAP-induced liver injury by inhibiting plasma ALT and AST activities and hepatic lipid peroxidation; by reducing the depletion of hepatic antioxidant enzymes such as SOD, catalase, GPx, GR, and GST; and by normalizing the GSH_t/GSSG ratio. These results suggested that GMSYS possessed antioxidative activity that likely contributed to its protective effect against APAP-induced liver injury. Taken together, this study suggests that GMSYS has considerable potential for development as a natural hepatoprotective agent.

Author contributions

Seong Eun Jin: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Hyeun-Kyoo Shin:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Hyekyung Ha:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - review & editing.

Conflicts of interest

The authors declare no conflict of interest.

Funding

This research was funded by the grants 'Construction of safety and efficacy for traditional herbal prescriptions of medicinal institution (KSN2013310)' and 'Construction of scientific evidences for herbal medicine formulas (K17251)' from the Korea Institute of Oriental Medicine (KIOM).

Ethics statement

This research has been approved by the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (National Research Council Committee for the Update of the Guide for the & Use of Laboratory, 2011), and all experiments were approved by the Institutional Animal Care and Use Committee of the Korea Institute of Oriental Medicine. The approval number for this study is #17-013.

Data availability

The data will be made available upon reasonable request.

Appendix A. Supplementary material

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imr.2020.100466.

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