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Protective effects of germinated and fermented soybean extract against *tert*-butyl hydroperoxide-induced hepatotoxicity in HepG2 cells and in rats

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Abstract

The aim of the current study is to investigate the antioxidant and hepatoprotective effects of germinated and fermented soybean extract (GFSE) on *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative stress in HepG2 cells and in the rat liver. High performance liquid chromatography (HPLC) analysis showed that genistin (3.40 ± 0.14 μg mg^{-1}) was the most abundant isoflavone in the GFSE. Coumestrol (1.00 ± 0.04 μg mg^{-1}), daidzin (0.78 ± 0.14 g μ mg^{-1}), genistein (0.68 ± 0.05 μg mg^{-1}), glycitin (0.54 ± 0.02 μg mg^{-1}), glycitein (0.41 ±0.02 μg mg^{-1}), and daidzein (0.02 ± 0.0 g mg^{-1}) are also contained in decreasing order of content. GFSE significantly inhibited *t*-BHP-induced reactive oxygen species (ROS) production in HepG2 cells. This GFSE-induced ROS reduction was associated with the down-regulation of nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4), a pro-oxidant enzyme, and up-regulation of the mRNA levels of antioxidant enzymes, including catalase, superoxide dismutase (SOD), glutathione reductase (GR), and glutathione peroxidase (Gpx) in the rat liver. In addition, increased levels of antioxidant enzyme mRNAs correlated with the enhanced enzymatic activities of SOD, catalase, and glutathione-S-transferase (GST). The antioxidant effect of GFSE was supported by the reduction in levels of malondialdehyde (MDA), a hydroperoxide, and the serum levels of lactate dehydrogenase (LDH), a biomarker of cell damage, was also lowered by GFSE. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) which are clinical biomarkers of liver function were shown to be improved with GFSE administration. The effects of GFSE were attributable to an improvement in liver tissue morphology. Taken together, GFSE protected liver from *t*-BHP-induced oxidative stress by regulating ROS-related enzymes. Our results suggest that GFSE might be hepatoprotective sources against oxidative stress.

Keywords: germinated and fermented soybean, *t*-BHP, rat, HepG2, antioxidant activity, hepatoprotective effect.

1. Introduction

Reactive oxygen species play a role as signaling molecules in complex cellular processes, including gene expression and regulation of cell proliferation [1, 2]. ROS production is a naturally occurring process in cells but in excess it leads to oxidative stress, which has been known to mediate the development of chronic diseases as well as the loss of cell function [3, 4]. Oxidative stress also occurs via exposure to various environmental factors such as pollutants, alcohol, smoking, stress, and unhealthy diets [5]. The radical oxidizing chemical *tert*-butyl hydroperoxide (*t*-BHP) has been reported to directly induce oxidative stress [6]. This chemical has been widely adopted for the study of cellular physiology and the mechanisms of cell damage induced via oxidative stress [7], because it can be metabolized to free radical intermediates [8]. Oxidative stress is considered to be associated with the development of pathological conditions such as inflammation, aging, and cancer [9]. The body has protective mechanisms for preventing excessive ROS production or for detoxifying ROS. These mechanisms involve protective enzymes and molecules referred to as antioxidants [2].

Food-derived phytochemicals are known to be potent antioxidants that can scavenge and intercept free radicals and prevent cellular damage [10]. The ROS scavenging effect of phytochemicals include mechanistic actions such as deactivation of detoxifying enzymes, gene expression of anti or pro-oxidant enzymes, modulation of cell signaling, and other cellular effects [10].

Soybean is considered as a plant source of complete proteins and a good substitute for animal proteins. Soybean crops have been processed into soybean and vegetable oils in various ways [11], and soybean has become an important part of the diet in many countries, including China, Japan, and Korea. It has also received attention as a healthy food, which contains beneficial phytochemicals such as isoflavones [12]. Isoflavone, a flavonoid compound in soybeans, has been extensively studied for its pharmacological properties such as antioxidative, anti-inflammatory, neuroprotective, and anticarcinogenic properties, as well as protective effects against bone loss and cardiovascular diseases [13-18]. Different processes that have been used to enrich or obtain soybean-derived active compounds, including germination, fermentation, heat treatment, and chemical and enzymatic hydrolysis, have

been studied [19]. In particular, the germination process has been shown to modify the composition of soybean phytochemicals, and a recent study reported that germination of soybean substantially increases the active compounds, including lecithin, phytoesterols, and saponins [20]. Microbial fermentation has been demonstrated to cause bioconversions of soybean-derived compounds such as isoflavones during the process [21]. Simon et al. (2011) [22] showed that the fermentation of soybeans by *Rhizopus microsporus* during the germination process markedly induced the formation of phytoalexins, altered isoflavones composition, and enhanced the estrogenic potential of soybeans. A recent study reported that germination and fermentation process enhanced the level of soybean phytochemicals which has a potent antioxidant effect [23]. However, the study on biological effect of germinated and fermented soybean using *in vivo* system was yet to be investigated.

In this study, germinated and fermented soybean (GFS) was generated using the food-grade fungus *Aspergillus oryzae*. We investigated the antioxidant properties and protective effect of GFS ethanol extracts (GFSE) against *t*-BHP-induced hepatotoxicity in rats, as well as the underlying molecular events involved. The results from this study can be used to facilitate the utilization of soybean and its processed products.

2. Materials and methods

2.1. Preparation of GFSE

The soybean *Glycine max* (L) Merr. specimens provided by the Jeju Agricultural Research and Extension Services (Jeju, Korea) were harvested in 2013 and used for the present study conducted in 2014. The seed germinations and fungal inoculations were carried out according to a previously described method [24]. Briefly, soybeans were surface-sterilized with 70% ethanol for 3 min and then rinsed with sterile water before they were soaked in sterile water for 24 h. The fungal spore suspension was prepared by suspending 0.2 g of *A. oryzae* culture powder (1×10^9 conidia g^{-1}; Mediogen; Seoul, Korea) in 20 mL of sterilized water for 24 h at 25 °C in an incubator. The soaked soybeans were placed in sterile plastic containers (30 cm × 15 cm). The soybeans were then inoculated with the sporangiospore suspension (0.5 mL g^{-1}) by gently distributing the suspension

manually in the soaked soybeans. The containers were placed on a clean bench at room temperature in the dark for 5 days and sprayed with sterile water 5 times a day. The *A. oryzae*-challenged soybeans were dehydrated in a drying oven at 60 °C for 24 h. A total of 10 g of the soybean sample was homogenized in 80% ethanol (7 mL g^{-1}) and then heated at 50 °C for 3 h in a water bath with sonication. Three replicates were performed for each sample. After the samples had cooled down, the mixtures were centrifuged at 10,000×*g* for 10 min. The supernatants were collected and filtered with a polytetrafluoroethylene membrane (0.45 μM) and evaporated. Finally, the extract solution was lyophilized to obtain the powdered soybean extract sample with a yield of 12.3%.

2.2. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay

The DPPH assay was performed as described previously [25]. In brief, reaction mixtures containing an ethanolic solution of 200 μM DPPH (100 μL) and 2-fold serial dilutions of the sample (dissolved in 100 μL ethanol, ranging from 2 to 1000 μg mL^{-1}), were placed in a 96-well microplate and incubated at 37 °C for 30 min. After incubation, the absorbance was read at 517 nm, and the mean value was obtained from 3 duplicated readings. Ascorbic acid was used as the positive control. The scavenging activity was determined using the following equation: percentage (%) scavenging activity = $[A_{control} - A_{sample}]/A_{control} \times 100$. The half-maximal stimulation concentration (SC_{50}) value was extrapolated from the linear regression analysis and defined as the concentration of the sample required to scavenge 50% of the DPPH radicals.

2.3. The 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity

The ABTS radical scavenging activity was measured according to the method described by Re et al. (1999) [26] with some modifications. The ABTS radical cations were generated by adding 7 mM ABTS to a 2.45 mM potassium persulfate solution and then allowing the mixture to stand overnight in the dark at room temperature. This solution was then diluted with distilled water to obtain an absorbance of 1.4–1.5 at 414 nm (molar extinction coefficient, $\varepsilon = 3.6 \times 104$ mol^{-1} cm^{-1}). Next, 1 mL of the

diluted ABTS radical cation solution was added to 50 μL of the sample. After 90 min, the absorbance was measured at 414 nm.

2.4. Ferric reducing antioxidant power (FRAP) activity

The FRAP of the GFSE was determined based on the method of Benzie and Strain [27]. The assay system contained 2.5 mL of 300 mM acetate buffer (pH 3.6), 0.25 mL of 10 mM 2,4,6-tripyridyl-s-triazine solution in 40 mM HCl, 0.25 mL of 20 mM $FeCl_3$, and GFSE in 0.1 mL methanol. The mixture was incubated at room temperature for 30 min and the absorbance was measured at 593 nm. The standard curves were prepared using known concentrations of ferrous salt ($FeSO_4$) in methanol to replace $FeCl_3$. Ascorbic acid was used as the reference sample.

2.5. Cell viability assay

Effect of GFSE on the HepG2 cell viability was determined using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay [28]. In brief, HepG2 cells were plated on 24-well plates at a density of 1×10^4 cells per well. Cells were treated with GFSE samples for 24h and subsequently with 200 μM t-BHP for 6 h. MTT (Life Technologies) solution was added to each well and incubated for 4 h at 37 ℃, after which 100 g L^{-1} sodium dodecyl sulfate (SDS) (Sigma) in 0.01 mol L^{-1} HCl was added to each well. The plates were incubated overnight to dissolve the formazan crystals. The absorbance of the samples at 570nm was then measured. Relative cell viability was expressed as % of non-treated control cells

2.6. Determination of ROS in HepG2 cells

Cellular ROS level was examined by the DCFH assay [29]. In brief, HepG2 cells were plated to a black 96-well plate at a density of 2×10^5 per well for 24 h. Different concentrations of GFSE samples were treated, after which cells were washed with phosphate-buffered saline (PBS) and incubated with 1 mM of *t*-BHP for 2 h to generate ROS. DCFH of 25 μM was added, and fluorescent formation of DCF was measured at 485 and 530 nm excitation and emission, respectively, over 30 min in a

Molecular devices fluorescence (Gemini EM) at 37 ℃.

2.7. Protein determination

The protein concentration was determined using a standard commercial kit (Pierce; Rockford, IL, USA) for the bicinchoninic acid method with bovine serum albumin as the standard.

2.8. Isoflavone analysis by high-performance liquid chromatography (HPLC)

The HPLC system used was an Agilent 1260 Infinity HPLC system (Santa Clara, CA, USA) equipped with a reversed phase column (Luna C18, 250 × 4.6 mm, 5 μm diameter, Phenomenex; Torrance, CA, USA). The flow rate and injection volume were 1 mL/min and 10 μL, respectively. The chromatograms were detected at 260 nm and collected at 40 °C. The mobile phase used for the separation consisted of solvent A (water:methyl alcohol:acetate, 88:10:2) and solvent B (methyl alcohol:acetate, 98:2). The gradient elution procedure used was as follows: 0 min, 90% A; 0–25 min, 60% A; 25–32 min, 60% A; 32–35 min, 40% A; and 40–55 min, 90% A. The peak identifications were based on the retention times and comparisons with the injected standard samples. All solutions were filtered through 0.45-μm membrane syringe filters (Millipore; Bedford, MA, USA) prior to analysis. To determine the calibration curves, the isoflavone standards glycitin, genistin, daidzin, glycitein, daidzein, genistein, and coumestrol (Sigma Chemical Co.; St. Louis, MO, USA) were dissolved individually in HPLC-grade methanol and adjusted to the appropriate concentrations and quantities. The level of total isoflavone was determined by adding the levels of the 7 isoflavones.

2.9. Animal maintenance and treatments

Male Sprague-Dawley rats (5 weeks old) were obtained from Daehan Biolink (Chungbuk, Korea). The animals were allowed access to Purina rodent chow and tap water ad libitum. They were maintained in a controlled environment at 21 ± 2 °C and 50 ± 5% relative humidity with a 12-h dark/light cycle, and acclimatized for at least 1 week prior to use. All experiments were approved by Ethical Committee and performed according to the guideline and regulations of the Animal Ethics Committee, Korea University. The rats were divided into 5 groups of 6 animals each. To evaluate the

effect of the GFSE, the samples were dissolved in saline and administered intragastrically at 0.5–1 g kg^{-1} once daily for 7 consecutive days. Three hours after the final administration of GFSE, the animals were treated intraperitoneally with 0.5 mmol kg^{-1} *t*-BHP (dissolved in saline), and after 18 h they were anesthetized with CO_2. Blood samples were collected by cardiac puncture and the serum alanine transaminase (ALT), aspartate transaminase (AST), total cholesterol (TCHO), and lactate dehydrogenase (LDH) activities were determined. Following blood collection, the animals were euthanized by cervical dislocation and the livers were weighed, sliced, snap frozen on dry ice, and stored at −70 °C until the lipid peroxidation levels and enzymatic activities were determined.

2.10. Serum biochemical analysis

The serum biomarkers for the liver function analysis, including ALT, AST, TCHO, and LDH levels, were assessed using standard assay kits (Roche Diagnostics GmbH, USA) and analyzed using Fuji Dri-Chem 3500 (Fuji Photo Film Co.; Osaka, Japan).

2.11. Lipid peroxidation assay

The rats were euthanized by decapitation, and the liver tissues were promptly removed and placed immediately on ice. The tissue homogenates were prepared in a 50 mM phosphate buffer containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and centrifuged at 10,000 ×*g* at 4 °C for 20 min. The supernatants were assayed for malondialdehyde (MDA) using an improved thiobarbituric acid fluorometric method at 553 nm with excitation at 515 nm, and 1,1,3,3-tetramethoxypropane was the standard. The results are expressed as mmol MDA formation per mg of liver tissue.

2.12. Determination of catalase and superoxide dismutase (SOD) activities

The rats were euthanized by decapitation, and the liver tissues were removed promptly and placed immediately on ice. Tissue homogenates were prepared in a 50 mM phosphate buffer containing 0.1 mM EDTA and centrifuged at 10,000 ×*g* at 4 °C for 20 min. The supernatants were analyzed for catalase, SOD, and GST activities. The catalase activity was measured according to the method of Aebi [30]. The reaction was started by adding 0.3 mL of 30 mM hydrogen peroxide (H_2O_2) to 0.65 mL

of 50 mM potassium phosphate buffer and 50 mL (10 mg protein) of the sample. The H_2O_2 decomposition was monitored at 240 nm and 37 °C for 3 min. The catalase activity was expressed as the unit of H_2O_2/mg of protein, using the molar absorption coefficient of 36 $M^{-1}cm^{-1}$. The SOD activity was determined using the xanthine–xanthine oxidase 3-(4-iodophenyl)-2-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) system, as previously described [31]. The reaction mixture containing 0.05 mM xanthine, 0.025 mM INT, 1 mM EDTA, and 0.1 mL of the liver supernatants was mixed with 0.1 mL xanthine oxidase (80 units mL^{-1}). The change in absorbance in 20 min was monitored at 450 nm.

2.13. Determination of glutathione (GSH) and glutathione S-transferase (GST) activity

The GSH content was assayed according to the method of Ellman [32]. An aliquot of 1.0 mL of hepatic post mitochondria supernatant was precipitated with 1.0 mL of 10% metaphosphoric acid. The assay mixture contained 0.1 mL of the aliquot, 2.7 mL of phosphate buffer (0.1 M, pH 7.4), and 0.2 mL of 5,5'-dithiobis-(2-nitrobenzoic acid) (1 mg/mL in phosphate buffer, 0.1 M, pH 7.4). The yellow color that developed was read at 412 nm. The activity of the cytosolic GST was measured using the method of Habig et al. (1974) [33]. The reactions were carried out in the presence of 1 to 10 mg of the cytosolic protein, 1 mM 1-chloro-2, 4-dintrobenzene (CDNB, GST substrate), 1 mM reduced GSH, and 100 mM potassium phosphate buffer (pH 7.5) at 37 °C in a final volume of 2.5 mL. A complete assay mixture without the enzyme served as a control. The conjugation of CDNB with the GSH was monitored at 340 nm, using the molar absorption coefficient of 9600 M^{-1} cm^{-1} and expressed as mmol of GSH-CDNB conjugate/units/mg of sample protein.

2.14. RNA isolation and real-time polymerase chain reaction (qPCR)

Total RNA was isolated from the rat liver samples using the Trizol® reagent (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's protocol. The concentration of total RNA was determined photometrically using a NanoQuant plate (Tecan Infinite 2000; Männedorf, Switzerland). The complementary cDNA was synthesized by the reverse transcription of 1.0 µg of total RNA (Invitrogen) using superscript polymerase. The RQ1 RNase-free DNase I (Promega; Madison, WI,

USA) was treated according to the manufacturer's instruction. After cDNA synthesis, the qPCR was performed using a power Taqman PCR master mix kit (Applied Biosystems, Foster City, CA). The sequence-specific PCR primer sets and the TaqMan MGB probe (FAM™ dye-labeled) were purchased from Applied Biosystems. For qPCR, the cycling conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The data were analyzed using the 2-ΔΔCT method [34]. The data are presented as the fold change in the gene expression normalized to an endogenous reference gene (β-actin), and relative to the normal control sample. The primers and probes included SOD: NM _017050.1, CAT: NM _012520.1, GPX: NM_001039849.1, GR: NM_001106609.2, NOX4: NADPH oxidase 4: NM_001106609.2, and β-actin: NM_031144.2 as an internal standard.

2.15. Histopathological evaluation

The livers from the rats in the different groups were fixed in 10% neutral formalin solution, dehydrated in graded alcohol, and embedded in paraffin. The fine sections obtained were mounted on glass slides and counter-stained with hematoxylin and eosin (H&E) dye for light microscopic observation. The histological indices of hepatic inflammation and necrosis were analyzed based on the method of Knodell *et al*. [35].

2.16. Statistical analysis

The quantitative data are expressed as the mean ± standard deviation (SD) and all statistical comparisons were made using a one-way analysis of variance (ANOVA) test followed by the Tukey's test. The differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. *In vitro* ROS scavenging effects of GFSE

We examined the radical scavenging effects of GFSE using DPPH, ABTS, and FRAP assays. The IC_{50} values of GFSE in the DPPH and ABTS assays were 2.43 ± 0.04 mg mL^{-1} and 4.29 ± 0.03 mg mL^{-1}, respectively (Table 1). The *in vitro* radical scavenging activity of GFSE was lower than that of

ascorbic acid (positive control) with IC_{50} values of 21.70 ± 0.14 μg mL^{-1} and 63.72 ± 0.40 μg mL^{-1}, respectively. In addition, the FRAP-based antioxidant activities of GFSE and ascorbic acid were 0.26 ± 0.01 and 10.53 ± 0.1 mmol g^{-1}, respectively (Table 1).

3.2. Isoflavone composition of GFSE

We determined the isoflavones contained in GFSE by comparing them with 7 standard isoflavones using HPLC analysis. Genistin was the most abundant isoflavone in the GFSE (3.40 ± 0.14 μg mg^{-1}, Table 2). The GFSE also contained the following isoflavones in this decreasing order of content; coumestrol (1.00 ± 0.04 μg mg-1), daidzin (0.78 ± 0.14 μg mg^{-1}), genistein (0.68 ± 0.05 μg mg^{-1}), glycitin (0.54 ± 0.02 μg mg^{-1}), glycitein (0.41 ±0.02 μg mg^{-1}), and daidzein (0.02 ± 0.0 g mg^{-1}). The glycosides genistin and daidzin were determined to be major constituents of the isoflavones in GFSE, and the aglycones genistein and daidzein were present at relatively lower levels. The level of the coumestan isoflavone coumesterol was relatively higher than the level of the other isoflavones (Table 2). The high content of coumestrol in GFSE is attributable to the germination and fermentation procedures used to process the soybean [23]. This result indicates that the compositions of the GFSE components were modified by germination and fermentation, which produced the genistin as a major compound.

3.3. Effects of GFSE on viability of HepG2 cells

The HepG2 cells exposed to different doses of GFSE did not show any cytotoxic effects at the tested concentrations (~0.5 mg mL^{-1}, Fig. 1A). The *t*-BHP decreased the viability of the hepatocytes by about 40%. However, the addition of GFSE ameliorated the *t*-BHP-induced cytotoxicity (Fig. 1B). The cell viability was recovered by about 20% with GFSE treatment (0.125–0.5 mg mL^{-1}, Fig. 1B). This result showed that the GFSE protected hepatocytes against *t*-BHP-induced cytotoxicity.

3.4. Effects of GFSE on generation of intracellular ROS in HepG2 cells

We examined the effects of GFSE on *t*-BHP-induced ROS production. Treatment of cells with *t*-BHP increased ROS generation over 2-fold more than the untreated normal group. However, the *t*-BHP-

induced increase in ROS level was decreased following GFSE treatment dose-dependently. The GFSE treatment (0.5 mg mL^{-1}) decreased ROS production 50% more than the *t*-BHP treatment (Fig. 2). This result suggests that the GFSE has ROS scavenging activities.

3.5. Effects of GFSE on serum biomarkers and MDA in the rat

We examined the effects of GFSE administration on liver damage induced by *t*-BHP in rats. The serum levels of ALT and AST, which indicate liver function, were increased more in the *t*-BHP-treated group than control group. The increase in the biomarkers was significantly reduced in the rats treated with GFSE (Table 3). Treatment with *t*-BHP increased the AST level in the control group from 75.33 ± 6.35 U L^{-1} to 96.00 ± 7.94 U L^{-1}, while treatment with GFSE (1 g + *t*-BHP) effectively inhibited this increase to a level of 79.00 ± 0.71 U L^{-1}. The ALT level was also significantly decreased following treatment with 1 g kg^{-1} GFSE (32.50 ± 4.40 U L^{-1}), while the *t*-BHP-treated group showed a level of 42.25 ± 4.24 U L-1. This result indicated that GFSE suppressed the damage of liver function induced by *t*-BHP treatment. In addition, the level of a biomarker of cellular cytotoxicity, LDH, was markedly decreased by GFSE administration. The GFSE treatment (1 g kg^{-1}) reduced the *t*-BHP-induced elevation of LDH by over 50% (from 227.0 ± 46.66 to 102.2 ± 16.83) (Table 3). Silymarin also decreased the level of LDH in *t*-BHP group by around 37% (141.33 ± 24.34 U L^{-1}). This result showed that the GFSE played a similar effect to the positive control silymarin and normal groups, which showed levels at 141.33 ± 24 U L^{-1} and 132.33 ± 21.83 U L^{-1}, respectively (Table 3). Furthermore, the TCHO was effectively decreased from 85.25 ± 3.77 to 64.63 ± 7.8 U L^{-1} following treatment with 1 g kg^{-1} of GFSE, However, *t*-BHP group failed to show a significant increase in TCHO level compared to normal control group (Table 3). This GFSE-induced lowering of TCHO was greater than that observed with silymarin treatment (Table 3). In addition, the *t*-BHP-induced increase in MDA (a by-product of lipid peroxidation) was also reduced to normal levels in the silymarin-treated and GFSE-treated groups (Fig. 3). These data suggest that GFSE treatment has beneficial effects on the function and metabolism of the liver by protecting liver from *t*-BHP-induced damage.

3.6. Effect of GFSE on expression of antioxidant and prooxidant enzymes in the rat liver

The cytotoxicity induced by *t*-BHP has been shown to be caused by oxidative damage associated with an increase in ROS in cells [7]. Therefore, we examined the expression levels of enzymes involved in ROS production and scavenging in rats treated with *t*-BHP and GFSE. Treatment with *t*-BHP significantly decreased the mRNA level of catalase by around 30% compared to the control group, while GFSE treatment raised the expression levels, reverting them back to normal (Fig. 4A). The mRNA levels of SOD, another antioxidant enzyme, showed a similar trend to that of catalase (Fig. 4B). The mRNA expression of Gpx, an enzyme that catalyzes the removal of free H_2O_2 and lipid hydroperoxides in cells, was reduced by 40% in the *t*-BHP group (Fig. 4C). However, the GFSE reversed the reduction in Gpx mRNA to the normal level (Fig. 4C). In addition, GFSE treatment greatly increased the mRNA level of GR, which catalyzes the synthesis of the antioxidant molecule GSH, compared with *t*-BHP treatment (TC), more than silymarin-treated group (Fig. 4D). These results indicated that GFSE upregulates the antioxidant enzymes, thereby protecting the liver from oxidative damage. Next, we examined the effects of GFSE on the gene expression of the ROS-producing enzyme NOX4. Interestingly, the mRNA expression of NOX4 was increased by around 30% in the *t*-BHP group while GFSE and silymarin suppressed the *t*-BHP-induced up-regulation of NOX4 (Fig. 4E). This result correlated with the data (Fig. 3) showing the ROS scavenging effect of GFSE and indicated that this effect may be attributable, at least in part, to the suppression of the NOX4 gene expression.

3.7. Effect of GFSE on antioxidant enzyme activities and antioxidant level in rat livers

We sought to determine if the genetic regulation of the antioxidant enzymes by GFSE is associated with cellular activities. Therefore, we examined the activities of the antioxidant enzymes and the level of antioxidant molecules. Treatment with *t*-BHP greatly decreased the activity of catalase, and this decrease was effectively reversed by GFSE (Table 4). In particular, the GFSE group treated with 1 g kg^{-1} + *t*-BHP showed a catalase level (321.44 ± 28.21 U mg $protein^{-1}$), which was 60% more than that of the *t*-BHP group (198.55 ± 6.22 U mg $protein^{-1}$, Table 4). The SOD enzymatic activity, which was reduced in the *t*-BHP-treated group (21.26 ± 1.71 U mg $protein^{-1}$) was also reversed by treatment with

0.5 g kg^{-1} of GFSE (27.35 ± 3.32 U mg $protein^{-1}$). This result agrees with the gene expression data of CAT and SOD (Fig. 4A and B), which showed regulation by GFSE treatment. These data indicated that the GFSE-induced activation of the enzymatic activities of SOD and CAT was, at least in part, caused by the up-regulation of gene expression. In addition, the level of GST, a detoxifying enzyme, was decreased more in the *t*-BHP treated group (24.55 ± 5.01 U mg $protein^{-1}$) than in the normal group, but this reduction was greatly suppressed by GFSE (Table 4). GFSE at 1 g kg^{-1} increased the activity of GST by around two-fold compared to *t*-BHP treated group (Table 4). In addition, the level of GSH, a cellular antioxidant molecule, was significantly increased in the GFSE-treated than the *t*-BHP-treated group; GFSE at 1 g kg^{-1} increased GSH level by 43% compared to *t*-BHP group (Table 4). These data collectively showed that GFSE increased the overall antioxidant effects at the cellular level, and thereby suppresses the *t*-BHP-induced cytotoxicity and liver damage.

3.8. Effect of GFSE on liver histopathology of *t*-BHP-treated rats

We examined the histopathology of liver sections from rats using H&E staining. The liver sections from the test groups did not show any distinct differences in cell distribution and morphology. However, the surrounding blood vessels showed significant changes between the groups. The liver section of the normal rats that were not treated with *t*-BHP exhibited clear blood vessels with well-preserved cell distribution in the tissues (arrow, Fig. 5A). However, the liver sections of the *t*-BHP-treated rats showed that the peripheral regions of the blood vessels were infiltrated by cells (arrow, Fig. 5B), indicating that the cellular and vascular boundaries were damaged by *t*-BHP. The cellular infiltration in the surrounding blood vessels was significantly decreased or disappeared in the silymarin or GFSE-treated groups (Fig, 5 C–D). This result indicated that GFSE suppressed the *t*-BHP-induced damage of the liver in rats.

4. Discussion

This study showed the antioxidant and hepatoprotective effects of the extracts of soybean processed by germination and fermentation, using a rat model. The damage in the hepatocytes and liver was induced with *t*-BHP, a radical enhancer. Oxidative stress is induced by an imbalanced ROS status and

is closely associated with liver damage [36]. When the uncontrolled generation of free radicals overrides the capacity of cellular antioxidative responses to offset them, the resultant excessive production of radicals causes oxidative stress to organs as well as damage to DNA, lipids, and proteins [36]. Our data showed that GFSE effectively suppressed the increase in ROS production induced by *t*-BHP (Fig. 2), thereby ameliorating hepatotoxicity (Fig. 1). This result indicated that the GFSE-mediated protection against *t*-BHP-induced cytotoxicity in hepatocytes was a result of radical scavenging effect. Radical-induced oxidative stress has adverse effects on the biomolecules. DNA is easily damaged by radicals, which leads to its breakage and subsequent mutation. This DNA mutation is a fundamental cause of cancer and other chronic diseases [37]. Proteins can also be oxidized by radicals leading to the production of protein carbonyl or protein hydroperoxides, which can cause physiological disorders via modulation of the amino acid sequence or structure [38]. Oxidative stress can be an important causal factor for lipid peroxidation, which leads to the formation of MDA, a highly reactive aldehyde molecule [39]. In this study, GFSE effectively decreased the formation of MDA induced by *t*-BHP (Fig. 2). However, the effects of GFSE on radical-induced damages to DNA or proteins were not addressed in this study. Analysis of DNA breakages and protein hydroperoxide levels would further support the protective effects of GFSE observed against radical-induced damages to these cellular components in future studies. We evaluated the hepatoprotective effects of GFSE *in vivo* by examining serum biomarkers related to liver function in the absence or presence of GFSE. In addition, we determined the activities and gene expression levels of antioxidant enzymes in the liver to identify the relationship between the liver damage and antioxidant effects. Our results showed that GFSE effectively reversed the *t*-BHP-mediated elevation of ALT, AST and LDH levels to normal (Table 3). These results suggest that GFSE protects the liver against *t*-BHP-induced damage by suppressing the leakage of cellular enzymes. The increase in serum AST and ALT could occur as a result of cellular damage by the *t*-BHP-derived increase in radicals in the liver. In particular, GFSE effectively inhibited the expression of NOX4, which is responsible for the synthesis of ROS (Fig. 4E). The down-regulation of NOX4 by GFSE partially contributed to the decrease in intracellular ROS levels in hepatocytes (Fig. 2). In contrast, GFSE up-regulated the mRNA levels of the antioxidant enzymes, including catalase and

SOD (Fig. 4), with a resultant increase in enzymatic activities (Table 4). This result indicated that GFSE regulates ROS-producing and scavenging enzymes at the genetic and biochemical level to exhibit hepatoprotective effects. However, GFSE did not allow the significant differences on the effects between treated doses as seen in mRNA levels of antioxidant enzymes such as SOD and CAT, and some serum biomarkers such as ALT and AST (Fig. 4 and Table 4). This result is thought to be due to the small dose difference of GFSE groups (0.5 g kg^{-1} and 1.0 g kg^{-1}). Therefore, wider ranges of GFSE doses need to be examined to get a dose-dependent result. Finally, the antioxidant effect of GFSE ameliorated the *t*-BHP-induced liver damage (Fig. 5). However, GFSE itself was not shown to influence on liver function because the levels of serum biomarkers (ALT and AST) in GFSE only-treated groups were not significantly different from normal group (data not shown). In most cases, liver damage is accompanied by inflammation because ROS-induced oxidative stress is associated with an increase in the proinflammatory cytokines [40]. Therefore, GFSE, which showed radical-reducing effects, is also considered to possess anti-inflammatory effects. A recent study reported that soybean-derived glyceollin has an anti-inflammatory effect and inhibits the activation of inflammatory mediators, including NF-kB [41]. ROS generation is also associated with mitogen-activated protein kinase (MAPK) signaling. In this signaling pathway, the ROS-induced activation of p-38, c-Jun-N-terminal kinase (JNK), or extracellular signal-regulated kinase (ERK) promotes nuclear translocation of AP-1, a transcription factor, and thereby stimulates the expression of various inflammatory mediators [42]. Chiang et al. (2007) showed that the isoflavone extract from soybean cake inhibited the phosphorylation of ERK and p-38, thereby providing protection against UV-induced photoaging in human keratinocytes In addition, the nuclear transcription factor E2-related factor-2 (Nrf2), an ROS-sensing transcription factor, is activated in the nucleus by binding to the antioxidant responsive element (ARE) region in the DNA, thereby inducing the expression of antioxidant enzymes during oxidative stress conditions [43]. Accordingly, the GFSE-induced up-regulation of antioxidant enzymes (Fig. 3) suggests that the antioxidant effects of GFSE may be mediated via the Nrf2-ARE signaling pathway. Therefore, we have proposed to investigate the involvement of various ROS-related signaling cascades in the GFSE-mediated effects in future studies.

There are a number of studies on the hepatoprotective effects of plant materials [44-46], and several studies reported the hepatoprotective effect of soybean [44, 46]. However, studies on the hepatoprotective effects of germinated and fermented soybean are limited. Studies on various methods of processing, including germination and fermentation have been performed in different food sources [47, 48]. Mohd Ali et al. showed that the aqueous extract of germinated and fermented mung beans exhibited antioxidant and hepatoprotective effects on ethanol-mediated liver damage. The present study describes the hepatoprotective properties and antioxidant effects of germinated and fermented soybean using a rat model. Our data showed that GFSE effectively protected the rat liver from the *t*-BHP-induced hepatocytotoxicity with antioxidant properties at the genetic and biochemical levels. However, our data did not address the specific active compounds responsible for the hepatoprotective effect. Soybean contains various active compounds like isoflavones, phytic acids, and phytoalexin, etc., and their levels can be modulated via germination and fermentation processes [21, 22, 47]. A recent study showed that a standardized isoflavone mixture attenuates carbon tetrachloride (CCl_4)-induced oxidative stress in Wistar rats [44]. However, the active compound responsible for the hepatoprotective effect has not been identified yet. Our data revealed that genistin, a glycoside of genistein, is the most abundant isoflavone present in the GFSE used in this study (Table 2). This result is in agreement with the other studies [19, 49]. Genistin could be a strong candidate for a hepatoprotective compound, and likely contributes, at least partially, to the hepatoprotective effect of GFSE. In addition, coumestrol was observed at a high level in GFSE (1.0 ± 0.04 μg mg^{-1}, Table 2). Coumestrol is not detected in unprocessed soybean, and germination and fermentation are known to enhance its presence [23]. Jeon et al. (2012) showed that *Aspergillus oryzae*-challenged germination increased the levels of coumestrol and antioxidant activity in soybean. Therefore, the level of coumestrol and the antioxidant activity of GFSE are considered to be attributable to the germination and fermentation processes. Furthermore, the hepatoprotective effects of GFSE may be due to the increase in coumestrol caused by these processes. Further studies on the constituents would reveal the specific GFSE-derived active compounds responsible for the antioxidant and hepatoprotective effects.

In conclusion, GFSE, which was processed by germination and fermentation, showed protective properties against the *t*-BHP-induced hepatotoxicity in rats. Its hepatoprotective effect was evidenced by improvement in the levels of the serum biomarkers, including ALT, AST, and LDH, as well as by the analysis of the H &E staining. The GFSE-mediated hepatoprotective properties are considered to be a result of the antioxidant effects, including decrease in MDA level, up-regulation of antioxidant enzymes, and down-regulation of prooxidant enzymes, in the liver. Therefore, GFSE has a potential to be a hepatoprotective agent developed using soybean and its processed product.

Conflict of interest statement

Authors declare that there is no conflict of interest.

Acknowledgement

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Figure legends

Fig. 1 Effects of germinated and fermented soybean extract (GFSE) on the viability of HepG2 cells. Cells (1×10^4 cells per well) were treated with GFSE for 24 h and then 200 μM tert-butyl hydroperoxide (*t*-BHP) for 6 h. Cell viability was determined by the MTT assay. Absorbance of the samples was measured at 570 nm. Cell viability is expressed as a percentage (%) of the non-treated control. (A) Cells treated with GFSE only (B) Cells incubated with GFSE for 24 h prior to *t*-BHP exposure. ns: not significant, NC: normal control (no treatment), TC: (*t*-BHP treated control). Values are means ± SD, n = 3. Different letters indicate significant differences, $p < 0.05$.

Fig. 2 Effect of germinated and fermented soybean extract (GFSE) on *tert*-butyl hydroperoxide (*t*-BHP)-induced reactive oxygen species production in HepG2 cells. After pretreatment with GFSE (0.0625, 0.125, 0.25, 0.5 mg mL^{-1}) for 12 h, cells (2×10^5 cells per well) were incubated with 25 μM dichlorofluorescin diacetate (DCFH) for 60 min. Cells were washed and exposed to 200 μM *t*-BHP for 30 min, and the ROS level was measured with a fluorescence microplate reader. NC: normal control (no treatment), TC: (*t*-BHP treated control). Bars represent means ± SD of triplicate experiments. Values not sharing the same letter are significantly different, $p < 0.001$.

Fig. 3 Effect of germinated and fermented soybean extract (GFSE) on *tert*-butyl hydroperoxide (*t*-BHP)-induced production of hepatic malondialdehyde (MDA) in the rat liver. The Sprague Dawley (SD) rats were administered GFSE (0.5, 1 g kg^{-1} body weight) or silymarin (50 mg kg^{-1} body weight) for 7 days, and *t*-BHP (0.5 mmol kg^{-1}) was administered for 18 h, followed by euthanization of rats for liver collection. Hepatic malondialdehyde (MDA) was assayed using the thiobarbituric acid fluorometric method (TBARS). NC: normal control (no treatment), TC: (*t*-BHP treated control). Values are expressed as mean ± SD for 6 rats. Different letters indicate significant differences at $p < 0.05$.

Fig. 4 Effect of germinated and fermented soybean extract (GFSE) on the expression of mRNA levels of antioxidant enzymes. (A) CAT, (B), SOD (C) Gpx, (D) GR, and (E) NOX4 mRNA in *t*-BHP-

557 treated rats. The SD rats were administered GFSE (0.5, 1 g kg^{-1} body weight) or silymarin (50 mg kg^{-1}
558 body weight) for 7 days, and *t*-BHP (0.5 mmol kg^{-1}) was administered for 18 h, followed by
559 euthanization of rats for liver collection. Total RNA was isolated from the liver samples using the
560 Trizol® reagent. After cDNA synthesis, qPCR was performed using a power Taqman PCR master mix
561 kit (Applied Biosystems; Foster City, CA, USA). NC: normal control (no treatment), TC: (*t*-BHP
562 treated control). Values are expressed as mean ± SD for 6 rats. Different letters indicate significant
563 differences at $p < 0.05$.

564 Fig. 5 Effects of germinated and fermented soybean extract (GFSE) on liver histology in rats treated
565 with *tert*-butyl hydroperoxide (*t*-BHP). Liver sections were stained with hematoxylin and eosin
566 (H&E) and examined under a microscope. (A) Control (untreated) group, (B) *t*-BHP-treated group,
567 (C) GFSE (0.5 g kg^{-1}) pretreated group, and (D) GFSE (1.0 g kg^{-1}) pretreated group.
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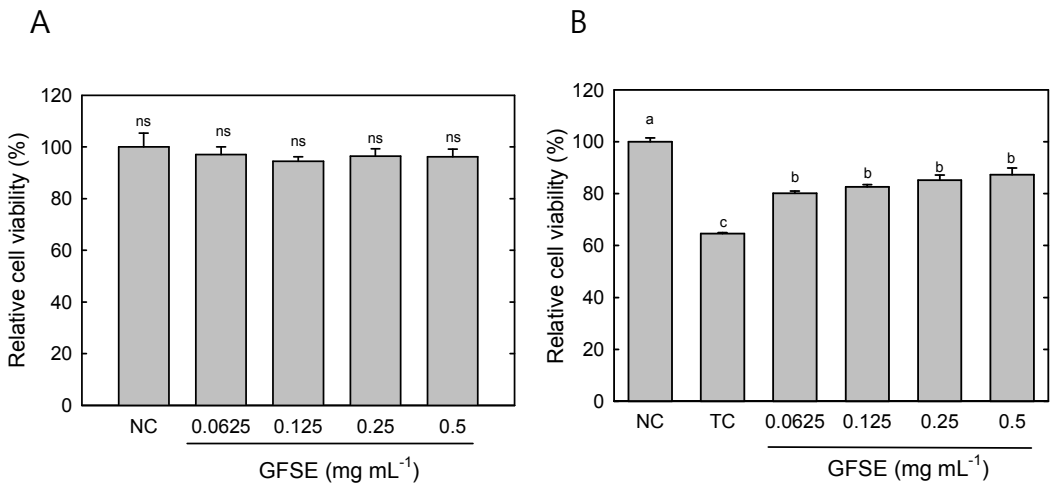

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592 Fig. 1

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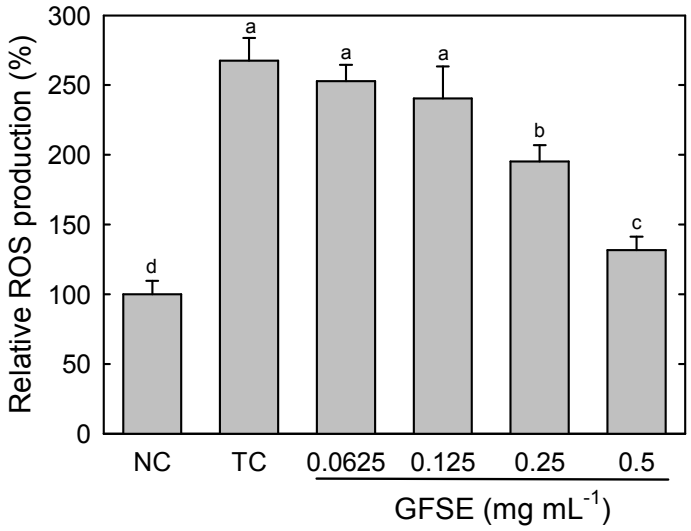
Relative ROS production (%)
300
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200
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a
a
b
c
d
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TC
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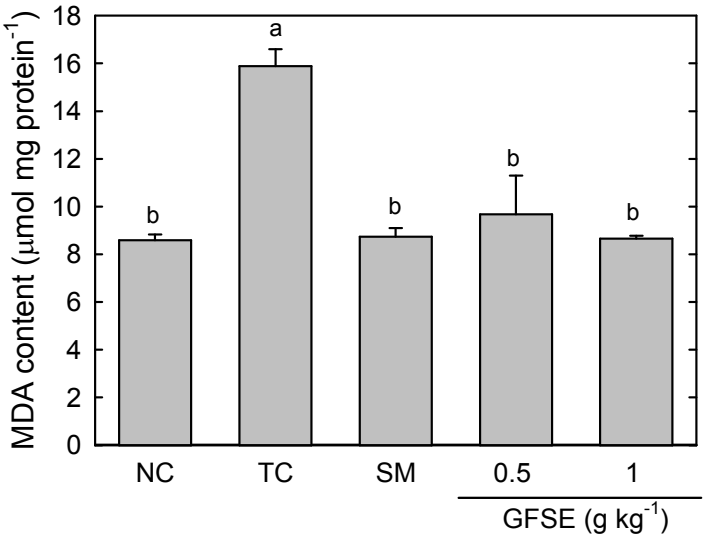
0.25
0.5
GFSE (mg mL-1)

Fig. 2


MDA content (μmol mg protein-1)
18
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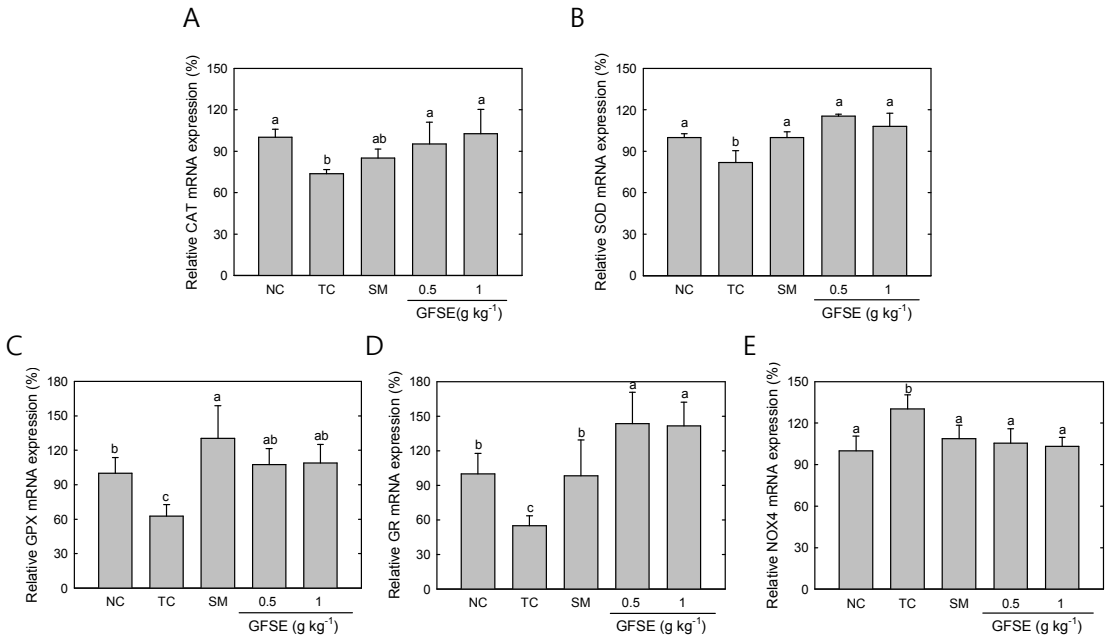
b
b
b
NC
TC
SM
0.5
1
GFSE (g kg-1)

Fig. 3


A
Relative CAT mRNA expression (%)
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a
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ab
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a
NC
TC
SM
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1
GFSE(g kg-1)
B
Relative SOD mRNA expression (%)
a
b
a
a
a
NC
TC
SM
0.5
1
GFSE (g kg-1)
C
Relative GPX mRNA expression (%)
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150
120
90
60
30
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b
c
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ab
ab
NC
TC
SM
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1
GFSE (g kg-1)
D
Relative GR mRNA expression (%)
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c
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NC
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SM
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1
GFSE (g kg-1)
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Relative NOX4 mRNA expression (%)
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NC
TC
SM
0.5
1
GFSE (g kg-1)

Fig. 4

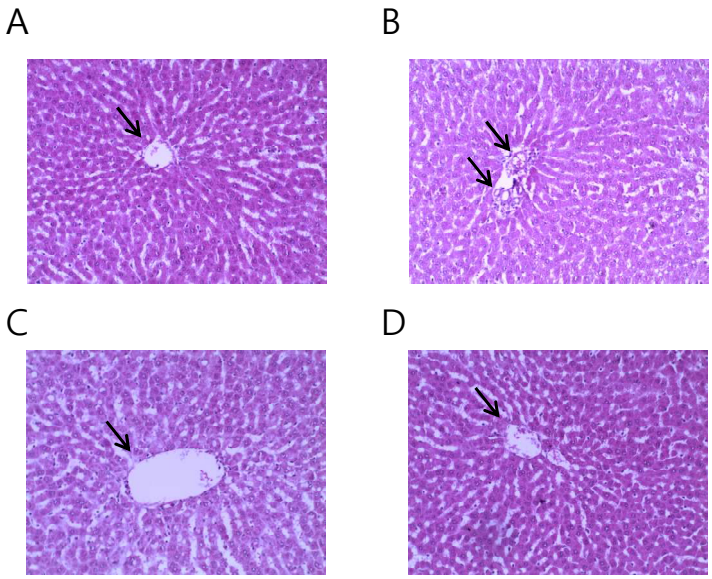


Fig. 5

Table 1 Radical scavenging activities of germinated and fermented soybean extract (GFSE)

	DPPH (IC_{50})	ABTS (IC_{50})	FRAP
GFSE	2.43 ± 0.04 mg mL^{-1}	4.29 ± 0.03 mg mL^{-1}	0.26 ± 0.01 mmol g^{-1}
Ascorbic acid	21.70 ± 0.14 μg mL^{-1}	63.72 ±0.40 μg mL^{-1}	10.53 ± 0.1 mmol g^{-1}

GFSE was analyzed by each assay according to the above methods. DPPH and ABTS are expressed as IC_{50} values, and FRAP is expressed as the ferric reducing ability equivalent to that of mmol g^{-1} ferrous salt.

Table 2 Isoflavone composition of germinated and fermented soybean extract (GFSE)

Composition	Isoflavone contents (μg/mg)
Glycitin	0.54 ± 0.02
Genistin	3.40 ± 0.14
Daidzin	0.78 ± 0.04
Glycitein	0.41 ± 0.02
Daidzein	0.02 ± 0.00
Genistein	0.68 ± 0.05
Coumestrol	1.01 ± 0.04
Total	6.87 ± 0.31

Table 3 Biochemical analysis of serum biomarkers

Treatment	ALT (U L^{-1})	AST (U L^{-1})	TCHO (mg dL^{-1})	LDH (U L^{-1})
Normal control	34.20 ± 2.00^{b}	75.33 ± 6.35^{b}	80.00 ± 7.81^{ab}	132.33 ± 21.83^{b}
t-BHP	42.25 ± 4.24^{a}	96.00 ± 7.94^{a}	85.25 ± 3.77^{a}	227.0 ± 46.66^{a}
Silymarin	37.0 ± 2.83^{ab}	80.33 ± 6.08^{b}	80.8 ± 6.87^{ab}	141.33 ± 24.34^{b}
GFSE 0.5g+*t*-BHP	33.33 ± 0.58^{b}	79.67 ± 1.53^{b}	66.29 ± 9.89^{bc}	127.0 ± 27.38^{b}
GFSE 1g +*t*-BHP	32.50 ± 4.49^{b}	79.00 ± 0.71^{b}	64.63 ± 7.89c	102.20 ± 16.83^{b}

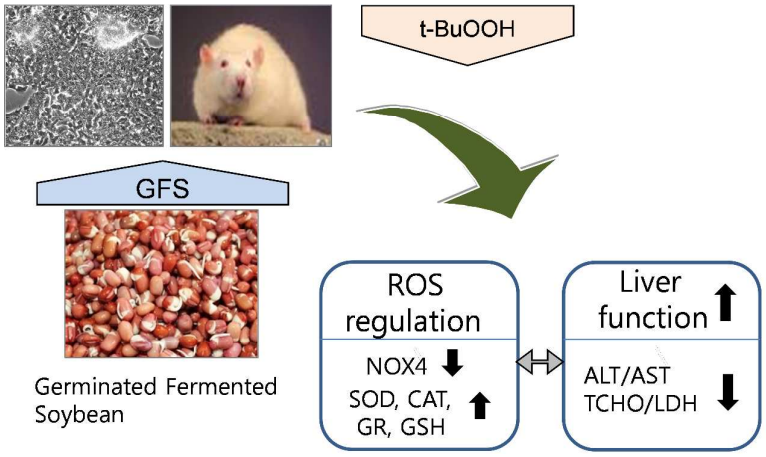
Rats were pretreated with soybean fermented after germination (0.5, 1 g kg^{-1}, intragastrically) once daily for 7 consecutive days. Normal control rats were given saline. Three hours after the final treatment, the rats were treated with *t*-BHP (0.5 mmol/kg, intraperitoneal). Hepatotoxicity was determined 18 h later by quantifying the serum activities of alanine aminotransferase (ALT), and aspartate aminotransferase (AST), total cholesterol (TCHO), and lactate dehydrogenase (LDH). Values are expressed as mean ± SD for 6 rats. Values not sharing the same letter are significantly different, $p < 0.05$.

Table 4 Enzymatic activities and levels of hepatic antioxidant proteins (SOD, CAT, GST, and GSH) by GFSE treatment

Treatment	SOD (unit mg protein^{-1})	CAT (unit mg protein^{-1})	GST (unit mg protein^{-1})	GSH (mmol mg protein^{-1})
Normal control	29.61 ±0.11^{a}	258.08 ± 5.60^{bc}	45.89 ± 8.22^{ab}	28.37 ± 2.43^{a}
t-BHP	21.26 ±1.71^{c}	198.55 ± 6.22^{d}	24.55 ± 5.01^{c}	18.43 ± 2.85^{c}
Silymarin	28.63 ±1.97^{a}	295.22 ± 24.71^{ab}	53.09 ± 4.28^{a}	24.43 ± 0.33^{b}
GFSE 0.5 g + *t*-BHP	27.35 ± 3.32^{a}	239.04 ± 14.74^{c}	52.60 ± 1.19^{a}	28.51 ± 2.11^{a}
GFSE 1.0 g + *t*-BHP	25.67 ± 0.21^{b}	321.44 ± 28.21^{a}	54.38 ± 2.61^{a}	26.42 ± 1.81^{ab}

SOD, superoxide dismutase; CAT, catalase; GFSE, germinated and fermented soybean extract (0.5, 1 g kg^{-1} of body weight). Data are expressed as mean ± SD in each group, n = 6. Values not sharing the same letter are significantly different, $p < 0.05$.

Graphical abstract



Germinated and fermented soybean extract (GFSE) suppresses reactive oxygen species production via genetic regulation of anti/prooxidant enzymes for recovery of liver function in HepG2 cells and in rats.