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Original Research Article

Bergenin Improves Antioxidative System in *Tert*-butyl Hydroperoxide-Induced Oxidative Stress in Mice

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ABSTRACT

Tert-butyl hydroperoxide (TBHP) is a pro-oxidant implicating oxidative stress due to excessive formation of free radicals from hepatic metabolism via CYP2E1 and glutathione peroxidase-glutathione reductase system. Bergenin is a C-glucoside derivative of gallic acid which has been claimed to exert antioxidant, anti-inflammatory, and hepatoprotective activities. The present study aimed to investigate the antioxidative effect of bergenin on TBHP-induced hepatic oxidative stress in mice. Male ICR mice received TBHP (18 mg/kg/day, intraperitoneal) with bergenin (10, 50, or 250 mg/kg/day, per oral) or gallic acid (100 mg/kg/day, per oral) for 7 consecutive days. Mouse plasma was investigated for the levels of liver injury and oxidative stress markers. Mice livers were examined for histopathology, antioxidant enzyme activity, glutathione profile, and mRNA expression of antioxidant enzymes and *Cyp2e1* expression. Bergenin and gallic acid attenuated TBHP-induced hepatic oxidative stress through reduction of prominent histopathological markers, including nuclear pyknosis and necrotic areas, decreases in plasma aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and reactive oxygen species levels, and inhibition of lipid peroxidation in the plasma and livers. Moreover, both bergenin and gallic acid restored the hepatic activities of antioxidant enzymes, namely superoxide dismutase, catalase, and glutathione peroxidase, improved glutathione homeostasis, and returned mRNA expression of the hepatic antioxidant enzymes and *Cyp2e1* to normal. Bergenin exhibited hepatoprotective/antioxidant effects comparable to gallic acid in the TBHP-induced oxidative stress in mice. Therefore, bergenin is a beneficial antioxidant that can improve the hepatic antioxidative system.

Keywords: Antioxidant enzymes, Bergenin, Glutathione homeostasis, Lipid peroxidation, *Tert*-butyl hydroperoxide.

Introduction

Xenobiotics are the major causes of liver toxicity and damage due to capability to generate free radical by-products, especially reactive oxygen species (ROS), from hepatic metabolism.[1,2] The high level of ROS can directly damage cells, tissues, and organs, particularly liver, via impairment of their structures and functions.[3,4] As a toxic compound, *tert*-butyl hydroperoxide (TBHP) is mostly associated with oxidative stress to damage cellular biomolecules, e.g. lipids, proteins, and nucleic acids, and liver tissue.[5] TBHP is a potent oxidant typically encountered via exposure to industrial air pollution[6] and is often applied as a model to induce hepatotoxicity.[7] TBHP is metabolized in the liver to generate ROS via cytochrome P450 2E1 (CYP2E1) and the glutathione peroxidase-glutathione reductase system.[7] This phenomenon causes ROS over-production and oxidant and antioxidant imbalance, leading to oxidative stress, which is crucially associated with pathogenesis, especially liver diseases. Since the liver plays a key role in metabolism and detoxification, it is a major target of ROS production and damage.[8] A decrease in hepatic oxidative stress might be a strategy for attenuation of ROS-induced liver damage, by which consumption of dietary/natural antioxidants may help to maintain an optimal oxidant-antioxidant balance in the body.[9-11]

In recent years, dietary supplements have generated considerable interest as sources of exogenous antioxidants like gallic acid, which is abundant in vegetables and fruits, e.g. cabbages, grapes, and pomegranates.[12] Gallic acid (Figure 1A) is a phenolic antioxidant, hence its natural derivatives likely possess similar properties.[12] Bergenin (Figure 1B) is a C-glucoside derivative of polyphenol 4-*O*-methylgallic acid.[13] Many families of medicinal plants, namely Euphorbiaceae, e.g. *Mallotus japonicas*, *M. philippensis*, and *M. repandus*, Fabaceae, e.g. *Caesalpinia digyna* and *C. mimosoides*, Myrsinaceae, e.g. *Ardisia colorata* and *A. crenata*, Saxifragaceae, e.g. *Bergenia cordifolia*, *B. scopulosa*, and *B. stracheyi*, are bergenin-rich.[13] Bergenin has been documented to possess antioxidant, hepatoprotective, antidiabetic, anti-inflammatory, and gastroprotective activities.[13,14] Due to its range of pharmacological activities, bergenin could be a potential natural treatment for many ailments. In particular, the antioxidant property of bergenin might inhibit the effects of free radicals, especially ROS, which are associated with chronic diseases such as diabetes, cardiovascular disorders, liver diseases, and cancer.[15] The mechanism of bergenin's antioxidative activity remains unclear and its impact on TBHP-induced oxidative damage has never been established. Therefore, the present study examined the ability of bergenin to improve hepatopathology in TBHP-induced oxidative stress in mouse livers by monitoring oxidative stress markers, antioxidative status, and expression of antioxidant enzymes.

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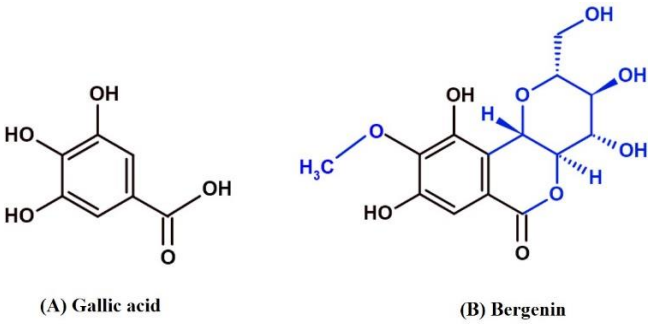

Figure 1: The chemical structures of **(A)** gallic acid and **(B)** bergenin.

Materials and Methods

Chemicals

Aqueous solutions of 1% Eosin Y and Mayer's hematoxylin were the products of Bio Optica (Milan, Italy). Bergenin (Cat. No. BP0258, purity > 98%) was a product of Biopurify Phytochemicals (Chengdu, China). Catalase (CAT), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), 2,4-dinitrophenylhydrazine (DNPH), and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich Chemical (St. Louis, Missouri, USA). Forward and reverse primers of *CuZn-Sod*, *Mn-Sod*, *Cat*, *Gpx*, *Cyp2e1*, and *Gapdh* genes were synthesized by Bio Basic, Inc. (Markham, Ontario, Canada). Gallic acid was obtained from Merck (Darmstadt, Germany). Glutathione reductase (GR) was purchased from Sigma-Aldrich Chemical. α-Ketoglutarate, *L*-alanine, *L*-aspartate, *L*-glutathione oxidized (GSSG), *L*-glutathione reduced (GSH), malondialdehyde (MDA), β-nicotinamide adenine dinucleotide 2′-phosphate (NADPH), and *p*-nitrophenyl phosphate were the products of Sigma-Aldrich Chemical. Random primers were obtained from Vivantis Technologies Sdn. Bhd. (Selangor Darul Ehsan, Malaysia). ReverTraAce® was a product of Toyobo Co., Ltd. (Osaka, Japan). RNase inhibitor was purchased from Vivantis Technologies Sdn. Bhd. Superoxide dismutase (SOD) was a product of Sigma-Aldrich Chemical. SYBR Green I was purchased from Cambrex Bio Science Rockland, Inc. (Rockland, ME, USA). *Taq* DNA polymerase was obtained from Vivantis Technologies Sdn. Bhd. *Tert*-butyl hydroperoxide (TBHP, 70% w/v), thiobarbituric acid (TBA), and 4-vinyl pyridine (4-VP) were purchased from Sigma-Aldrich Chemical.

Animals and experimental design

Male ICR mice (7-week-old) were obtained from Nomura Siam International Co., Ltd., Bangkok, Thailand and housed on corn cob bedding in stainless steel cages with water and commercial mouse diet supplied *ad libitum*. The animals were maintained under controlled temperature of 23 ± 2°C and humidity of 45 ± 2% with a 12 h-dark/light cycle. The protocol was approved by the Institutional Animal Ethics Committee for Use and Care of Animals, Khon Kaen University (IACUC-KKU-86/61).

Mice were divided into normal and TBHP-treated groups. The normal group was randomly divided into 5 groups (n = 5 each); each group was administered gallic acid (100 mg/kg/day, p.o.) or bergenin (10, 50, and 250 mg/kg/day, p.o.) for 7 consecutive days and the control was simply administered distilled water (0.1 mL/mouse/day, p.o.) for the same period. The TBHP-treated group was randomly allocated into 5 groups (n = 5 each); all the mice were administered TBHP (18 mg/kg/day, i.p.) in combination with the administration of gallic acid (100 mg/kg/day, p.o.) or bergenin (10, 50, and 250 mg/kg/day, p.o.) for 7 consecutive days.

After 24 hours of the last treatment, all the mice were sacrificed. Blood was immediately collected from the portal vein and centrifuged at 825 ×*g* for 10 min at 4°C to obtain plasma. Livers were excised and divided for histological examination and further analysis.

Preparation of liver homogenate and determination of protein content in mouse livers

Liver (1 g) was homogenized in 0.01 M phosphate buffered saline (3 mL) with a hand homogenizer in an ice-bath. Protein content was determined using the Bradford method as previously described.[16] Briefly, A 40 µL-aliquot of diluted liver homogenate was mixed with 160 µL of the Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA, USA). Absorbance of the protein-dye complex was measured at a wavelength of 595 nm by comparison with a standard curve of bovine serum albumin (0.0125-0.15 mg/mL).

Determination of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) levels in the mouse plasma

Plasma was incubated with AST substrate (10 mmol/L *L*-aspartate and 1.7 mmol/L α-ketoglutarate) or ALT substrate (300 mmol/L *L*-alanine and 0.7 mmol/L α-ketoglutarate) at 37°C for 30 or 20 min, respectively. Then DNPH was added to a final concentration of 0.5 mmol/L and left for 20 min, followed by the addition of NaOH to a final concentration of 1 mol/L. Absorbance was measured at a wavelength of 505 nm. Activity of AST or ALT was determined as an international unit per liter (IU/L) by comparison with a standard curve of sodium pyruvate (100-500 µmol/L).[17]

For ALP activity, plasma was mixed with buffer substrate solution pH 9.8 containing 0.1 mol/L diethanolamine, 1.25 mmol/L *p*-nitrophenyl phosphate, and 0.5 mmol/L $MgCl_2$. Absorbance was measured at a wavelength of 410 nm at a 2 min-interval for 20 min. ALP activity was determined as IU/L by comparison with a standard curve of *p*-nitrophenol (1-20 µmol/mL).[18]

Determination of reactive oxygen species (ROS) level in the mouse plasma

The amount of ROS was determined by the DCFH-DA method based on the reaction of ROS with DCFH-DA to create highly fluorescent dichlorofluorescein (DCF).[18] Plasma was incubated with 0.06 µmol/L DCFH-DA in the dark for 40 min at room temperature. The DCF was measured by a spectrofluorometer with an excitation wavelength of 484 nm and an emission wavelength of 530 nm. ROS level was calculated by comparison with a standard curve of hydrogen peroxide (2.5-20 µmol/L).

Assessment of lipid peroxidation by thiobarbituric acid reactive substances (TBARS) assay in the mouse plasma and livers

TBARS assay was performed as described previously.[17] In brief, plasma or liver homogenate was mixed with 10% trichloroacetic acid (1:1) followed by centrifugation at 2,300 ×*g* at 4°C for 10 min. The supernatant was transferred to a new tube. An equal volume of 0.8% TBA was added before boiling at 100°C for 15 min, followed by immediate cooling in an ice-bath. Finally, MDA was measured by a spectrofluorometer at an excitation wavelength of 520 nm and an emission wavelength of 590 nm by comparison with a standard curve of MDA (0.25-5 µmol/L for plasma; 5-40 µmol/L for liver homogenate).

Examination of hepatic histology by hematoxylin and eosin (H&E) staining

Hepatic histology was examined as previously described.[17] A small piece of excised liver was washed in phosphate buffered saline and fixed by overnight immersion in 10% neutral-buffered formalin before being dehydrated in ethanol and embedded in paraffin. A 5 µm-paraffin section was cut by a Microm HM315R microtome (Thermo Scientific, Walldorf, Germany), placed on a microscope slide, and stained with H&E. The tissue section was analyzed at 400-fold magnification using an inverted microscope (AE2000; Motic Incoporation, Ltd., Causeway Bay, Hong Kong) and images were recorded with the Moticam 5.0MP digital camera coupled with the Motic images plus 3.0 software (Motic Incoporation, Ltd.).

Assessment of superoxide dismutase (SOD) activity in the mouse livers

To the liver homogenate was added chloroform and ethanol (1:1.67) before centrifugation at 14,000 ×*g* at 4°C for 30 min. The supernatant was incubated with a reaction mixture containing 1.1 mmol/L xanthine, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.6 mmol/L nitroblue tetrazolium, 56 mmol/L Na_2CO_3, and 70 µg/mL bovine serum albumin, followed by 0.00002 unit/µL xanthine oxidase at 25°C for 20 min. After the incubation, $CuCl_2$ was added to a final concentration of 0.1 mmol/L. Absorbance was measured at a wavelength of 550 nm and calculated as the SOD activity by comparison with a SOD standard.[16]

Assessment of catalase (CAT) activity in the mouse livers

Liver homogenate was incubated with 150 µmol/L H_2O_2 at 37°C for 1 min. The reaction was terminated by adding a final concentration of 26 mmol/L ammonium molybdate, followed by absorbance measurement at a wavelength of 405 nm. CAT activity was calculated by comparison with a CAT standard.[16]

Assessment of total glutathione (total GSH), reduced glutathione (GSH), oxidized glutathione (GSSG) contents, and glutathione peroxidase (GPx) activity in the mouse livers

Liver homogenate was extracted in 5% sulfosalicylic acid (SSA) (1:5) and subjected to centrifugation at 10,000 ×*g* at 4°C for 10 min. The supernatant was mixed with a fresh reaction mixture of 7 mmol/L potassium phosphate buffer (pH 7.0), 0.04 units/mL GR, and 0.03 mg/mL DTNB, followed by addition of 0.04 mg/mL NADPH. Absorbance was measured at a wavelength of 405 nm at a 30 sec-interval for 10 min. Total GSH content was calculated by comparison with the slope of a GSH standard curve (6.25-50 µmol/L).[17]

GSSG content assay was performed by incubation of the supernatant with 4-VP for 60 min at room temperature prior to the assessment of total GSH content. GSSG content was calculated by comparison with the slope of a GSSG standard curve (5-30 µmol/L) while GSH content was calculated by subtracting the GSSG content from the total GSH content.[17]

The activity of GPx was performed as previously described with some modifications.[16] Liver homogenate was incubated with a reaction mixture containing 2.5 mmol/L sodium phosphate buffer pH 7.4, 0.01 mmol/L EDTA, and 8.3 µmol/L sodium azide at 30°C for 10 min, followed by the addition of 0.7 mmol/L GSH and 1.2 mmol/L H_2O_2 to start reaction. The reaction was terminated by adding 3.3% SSA before centrifugation at 330 ×*g* for 15 min. The supernatant was performed for the assessment of GPx activity as described for GSSG content. The GPx activity was defined as the amount of GPx that produces 1 µmol of GSSG per 1 min.

Quantitative determination of Cat, CuZn-Sod, Mn-Sod, Gpx, and Cyp2e1 mRNA expression in the mouse livers by real-time polymerase chain reaction (qPCR)

Total RNA was reverse transcribed using ReverTraAce® under the condition recommended by the supplier (Toyobo Co., Ltd., Osaka, Japan) at 25°C for 10 min, 42°C for 60 min, and 95°C for 5 min on a GeneAmp PCR system 2720 ThermalCycler (Applied Biosystem, Singapore). The expression of *Cat*, *CuZn-Sod*, *Mn-Sod*, *Gpx*, and *Cyp2e1* mRNAs was quantified by qPCR using SYBR Green I with specific primers (Table 1).[19,20] The CFX96 real-time PCR detection system (Bio-Rad®, California, USA) was used to monitor the SYBR Green I signal at the end of each extension period of 45 cycles; each cycle consisted of denaturation at 95°C for 20 sec, annealing for 20 sec with the specific annealing temperature of each gene (Table 1),[19,20] and extension at 72°C for 20 sec. The mRNA level of each gene was normalized to a reference glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*).

Statistical analysis

The results are expressed as mean ± standard deviation (SD). All data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey*'s post hoc* test using SPSS, IBM version 22.0 (Armonk, USA). $p < 0.05$ is considered to be statistically significant.

Results and Discussion

Effect of bergenin on markers of liver injury and oxidative stress in TBHP-induced oxidative stress in mice

Increases in AST, ALT, and ALP indicate liver injury and dysfunction[21] while elevation of ROS and MDA, a toxic by-product of lipid peroxidation, reflect hepatic oxidative stress.[8] In this study, bergenin (10, 50, and 250 mg/kg/day) and gallic acid (100 mg/kg/day) alone did not affect plasma levels of AST, ALT, ALP, ROS, and MDA (Figure 2). Treatment with TBHP significantly elevated the plasma levels of AST, ALT, ALP, ROS, and MDA compared with the controls ($p < 0.05$). These observations correspond with previous studies that demonstrated increases in serum AST, ALT, and ALP following a single intraperitoneal (i.p.) TBHP dose (20-50 mg/kg) in male ICR mice,[22,23] and increases in serum AST and ALT in male Sprague-Dawley rats administered a single i.p. TBHP dose (18 mg/kg).[24] The proposed mechanism of TBHP-stimulated liver injury is that TBHP enhances ROS and MDA formation, as demonstrated in both HepG2 cells and primary rat hepatocytes.[22,25] ROS, principally peroxyl and alkoxyl radicals, generated from hepatic metabolism of TBHP damage biological molecules, tissues, and organs, especially the liver itself. These free radicals attack polyunsaturated fatty acids of the cell membrane leading to cell membrane fragility and loss of selective membrane permeability,[8] which results in hepatic structural damage and the leakage of AST, ALT, and ALP from hepatocytes to circulation.[21]

In the TBHP-treated mice, both bergenin, in a dose-dependent manner, and gallic acid significantly reduced the AST, ALT, ALP, ROS, and MDA plasma levels compared with the TBHP group ($p < 0.05$) and the highest dose of bergenin (250 mg/kg/day) restored AST, ALP, and MDA levels to the same as the control (Figure 2). These findings revealed the ability of bergenin to preserve liver homeostasis. The decrease in cell membrane rupture and the release of liver damage biomarker enzymes and oxidative stress markers observed for bergenin were comparable to the potent antioxidant gallic acid.

Effect of bergenin on hepatic histology in TBHP-induced oxidative stress in mice

Normal polygonal shaped-hepatocytes were observed in the control mice (Figure 3A). Bergenin and gallic acid alone did not modify hepatic histological structure compared to the control (Figure 3B-3E). Obviously, the TBHP-induced hepatotoxic effect was demonstrated by multiple hepatic histopathological features consisting of swollen hepatocytes (black arrows), cell membrane disruption (squares), nuclear pyknosis (white arrows), and necrotic areas (circles) (Figure 3F). The hepatic histopathological findings supported the rigorous liver damage due to the excessive generation of ROS by TBHP compared to the control. According to previous studies, TBHP doses ranging from 18 to 50 mg/kg caused liver lesions, including hepatocyte swelling, leukocyte infiltration, pyknosis, and necrosis in mice and rats.[7,22-24] Both bergenin and gallic acid (Figure 3G-3J) extensively attenuated the TBHP-induced liver damage according to the antioxidant property.[12,13]

Effect of bergenin on hepatic antioxidative system in TBHP-induced oxidative stress in mice

The antioxidative system plays a crucial role in both normal and pathological conditions for maintenance of the oxidant-antioxidant balance. This system involves antioxidant enzymes, i.e., superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) and the glutathione system.[8,15] Cytosolic (CuZn-SOD) and mitochondrial (Mn-SOD) SOD enzymes are the first defence against free radicals, mainly superoxide anion ($O_2^{\bullet-}$), which is transformed into hydrogen peroxide (H_2O_2) by SOD and subsequently water and oxygen by CAT and GPx.[10,15] For the glutathione system, reduced glutathione (GSH) is the active form that scavenges ROS to become oxidized glutathione (GSSG) by the action of GPx, GSSG is then returned to GSH by glutathione reductase (GR).[8] Hence, maintaining the GSH-GSSG balance is vital to maintain homeostasis.[15] In this study, bergenin and gallic acid alone did not alter SOD, CAT, and GPx activities or MDA level in mouse livers (Figure 4). Moreover, the

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hepatic glutathione profile (total glutathione (total GSH), GSH, GSSG contents, and the GSH/GSSG ratio) was not disturbed by either bergenin or gallic acid (Table 2).

TBHP is a short-chain organic hydroperoxide pro-oxidant widely used as a bleaching/oxidizing agent or an initiator of polymerization in industrial processes.[6] It is metabolized in the liver by two distinctive pathways involving ROS generation. The first mechanism relates to CYP2E1-mediated ROS production, in which the major ROS generated are peroxyl and alkoxyl. The second mechanism is mediated via the GPx-GR system to convert TBHP into a toxic metabolite, *tert*-butyl alcohol, which subsequently disturbs glutathione homeostasis.[7] In the present study, TBHP (18 mg/kg/day) significantly decreased SOD, CAT, and GPx activities (Figure 4A-4C), depleted total GSH and GSH contents, and elevated GSSG content, resulting in a significant decrease in the hepatic GSH/GSSG ratio (Table 2). In addition, TBHP greatly increased MDA level in the mouse livers (Figure 4D), reflecting lipid peroxidation and liver tissue damage. These findings are consistent with previous studies that showed treatment of rodents with TBHP (18-20 mg/kg) for 18-24 h resulted in hepatotoxicity via an increase in MDA level with a decrease in GSH content, followed by reduction of GSH/GSSG ratio in the livers.[22,26]

The antioxidative system of TBHP-induced mice was improved by treatment with bergenin (10, 50, and 250 mg/kg/day) and gallic acid (100 mg/kg/day). Both compounds significantly increased SOD, CAT, and GPx activities (Figure 4A-4C) and decreased MDA level (Figure 4D). In addition, both bergenin and gallic acid significantly augmented total GSH and GSH contents with an associated decrease in GSSG content in the mouse livers, which resulted in reduced GSH/GSSG ratio (Table 2). Particularly, bergenin at the highest dose (250 mg/kg/day) and gallic acid (100 mg/kg/day) completely restored glutathione stores (including total GSH, GSH, and GSSG contents), resulting in GSH/GSSG ratio comparable to the control. A previous study demonstrated the hepatoprotective effect of gallic acid in paracetamol-treated male Swiss albino mice through decreased MDA level and improved SOD, CAT, and GPx activities.[27] Similarly, gallic acid was shown to sustain hepatic antioxidative status, i.e. SOD, CAT, GPx, GR activities, and GSH level in TBHP-induced hepatotoxicity in male C57BL/6N mice.[28] The present observations that bergenin reduced TBHP-induced hepatocyte damage support the hepatoprotective effect observed for bergenin in carbon tetrachloride-induced liver damage in Sprague-Dawley rats.[14] Bergenin reduced hepatic MDA level, increased glutathione *S*-transferase and GR activities, and restored GSH content.[14] In addition, bergenin exhibited hepatoprotective activity in high fat diet-induced oxidative stress in male C57BL/6J mice by restoring GSH content and elevating SOD, CAT, and GPx activities, and decreasing MDA level in rat livers.[29]

Bergenin and gallic acid alone did not modify mRNA expression of antioxidant enzymes; *CuZn-Sod*, *Mn-Sod*, *Cat*, and *Gpx* (Figure 5A-5D) and *Cyp2e1* (Figure 5E) in the normal mouse livers while in the TBHP-induced oxidative stress mice, the expression of *CuZn-Sod*, *Mn-Sod*, *Cat*, and *Gpx* mRNAs was significantly lessened (Figure 5A-5D) with elevation of *Cyp2e1* mRNA expression (Figure 5E). CYP2E1 is the CYP450 mainly responsible for TBHP metabolism and induction of CYP2E1 contributes to an increase in ROS production.[7] These findings correlated well with the study of Choi et al. (2015),[28] who demonstrated that expression of antioxidant-related genes including *CuZn-Sod*, *Mn-Sod*, *Cat*, and *Gpx* was downregulated in male C57BL/6N mice intraperitoneally administered a single TBHP dose (2.5 mmol/L/kg).[28] Both bergenin (250 mg/kg/day) and gallic acid significantly up-regulated the expression of *CuZn-Sod*, *Mn-Sod*, *Cat*, and *Gpx* mRNAs (Figure 5A-5D), followed by a significant down-regulation of *Cyp2e1* expression (Figure 5E) compared with the TBHP-induced oxidative stress in mice.

The active metabolite of gallic acid, 4-*O*-methylgallic acid is a major part of bergenin structure.[30] The hydroxyl rich-structures of bergenin and gallic acid directly bind with free radicals by an electron donating-mechanism, resulting in a decrease in oxidative stress.[9,11] Our results demonstrate that bergenin possesses similar antioxidant potential to gallic acid. Bergenin exerted its antioxidant effect against TBHP-induced hepatic oxidative stress in mice by multiple pathways. Bergenin decreased AST, ALT, and ALP leakage, reduced ROS and MDA formation, enhanced *Sod*, *Cat*, and *Gpx* expression, restored glutathione stores via a simultaneous increase in the total GSH/GSH contents and decrease in the GSSG content, and down-regulated *Cyp2e1* expression.

Table 1: The specific forward and reverse primers

Genes	Accession number	Primers (5' → 3')	Annealing temperature (°C)
Cat	NM_009804.2	Forward: GCA GAT ACC TGT GAA CTG TC Reverse: GTA GAA TGT CCG CAC CTG AG	55.0
CuZn-Sod	NM_011434.2	Forward: AAG GCC GTG TGC GTG CTG AA Reverse: CAG GTC TCC AAC ATG CCT CT	56.0
Mn-Sod	NM_013671.3	Forward: GCA CAT TAA CGC GCA GAT CA Reverse: AGC CTC CAG CAA CTC TCC TT	55.0
Gpx	NM_008160.6	Forward: CCT CAA GTA CGT CCG ACC TG Reverse: CAA TGT CGT TGC GGC ACA CC	56.0
Cyp2e1	NM_021282.3	Forward: TCC CTA AGT ATC CTC CGT GA Reverse: GTA ATC GAA GCG TTT GTT GA	56.0
Gapdh	NM_008084.3	Forward: CCT CGT CCC GTA GAC AAA ATG Reverse: TGA AGG GGT CGT TGA TGG C	57.4

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Table 2: Effect of bergenin on the hepatic glutathione profile

Treatments		Glutathione contents (nmol/mg protein)			Ratio of GSH/GSSG
		Total GSH[1]	GSH[2]	GSSG[3]	
Normal	Control	67.79 ± 3.03	56.37 ± 3.92	11.60 ± 1.81	4.86 ± 1.19
	Gallic acid				
	100 mg/kg/day	68.76 ± 5.38	58.96 ± 3.63	9.80 ± 1.85	6.01 ± 1.15
	Bergenin				
	10 mg/kg/day	65.03 ± 4.92	54.76 ± 4.38	10.27 ± 0.94	5.33 ± 0.77
	50 mg/kg/day	63.09 ± 3.17	52.64 ± 3.51	10.45 ± 1.60	5.04 ± 1.02
	250 mg/kg/day	67.93 ± 2.25	57.66 ± 2.20	10.27 ± 0.75	5.61 ± 0.60
TBHP induced-oxidative stress	TBHP	34.60 ± 3.02*	9.74 ± 1.95*	24.86 ± 1.87*	0.39 ± 0.09*
(18 mg/kg/day)	Gallic acid				
	100 mg/kg/day	62.77 ± 5.20#	50.34 ± 6.21#	12.43 ± 2.16#	4.05 ± 1.13#
	Bergenin				
	10 mg/kg/day	53.97 ± 2.85*#	41.36 ± 1.90*#	12.61 ± 0.85#	3.28± 0.14*#
	50 mg/kg/day	55.12 ± 2.39*#	42.85 ± 2.04*#	12.27 ± 0.29#	3.49 ± 0.18*#
	250 mg/kg/day	62.86 ± 1.90#	50.82 ± 1.57*#	12.04 ± 0.32#	4.22 ± 0.14#

Note. The results are expressed as mean ± SD (n = 5). [1]Total GSH, total glutathione; [2]GSH, reduced glutathione; [3]GSSG, oxidized glutathione. A significant difference was determined by one-way analysis of variance followed by Tukey's *post hoc* test. *$p < 0.05$ vs Control; #$p < 0.05$ vs TBHP induction.

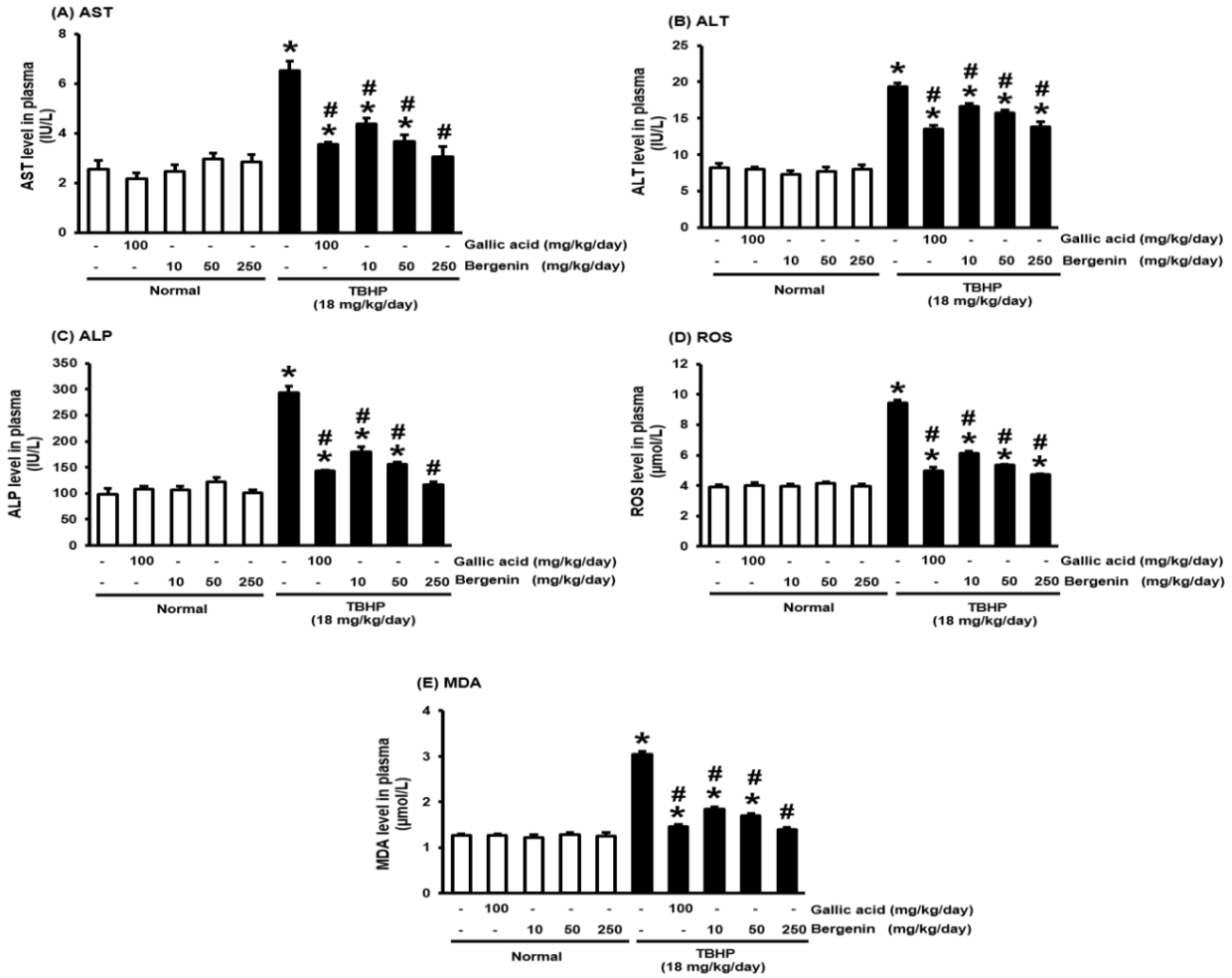

Figure 2: Effect of bergenin on the plasma **(A)** AST, **(B)** ALT, **(C)** ALP, **(D)** ROS, and **(E)** MDA levels in the TBHP-induced oxidative stress in mice. A significant difference was determined by one-way analysis of variance followed by Tukey's *post hoc* test. *$p < 0.05$ vs control; #$p < 0.05$ vs TBHP induction

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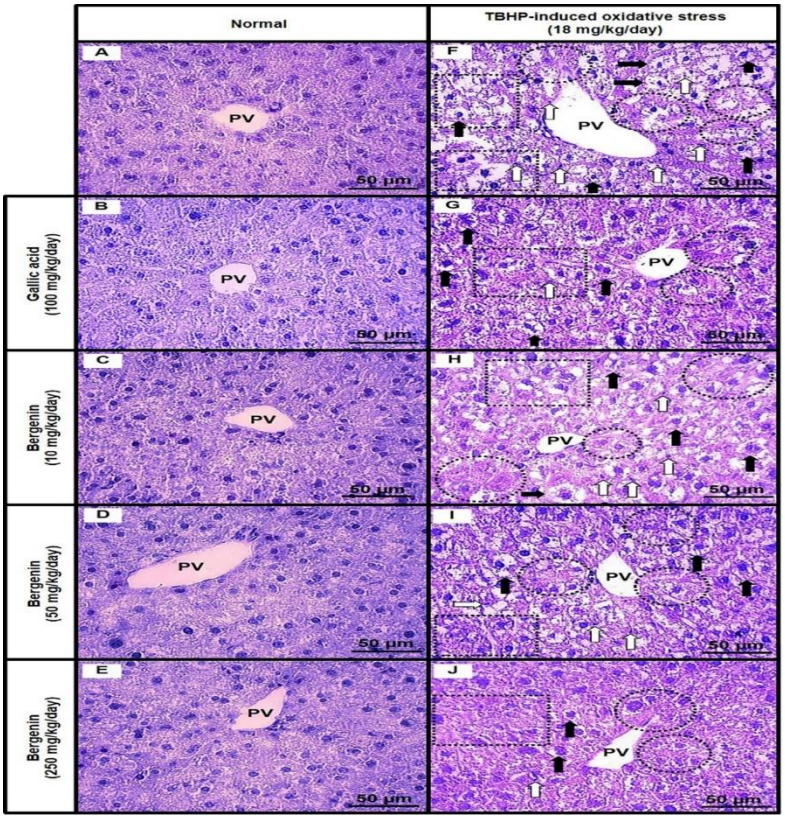

Figure 3: Effect of bergenin on hepatic histology by H&E staining in the TBHP-induced oxidative stress in mice. **(A-E)** are normal mice: **(A)** control and **(B-E)** gallic acid (100 mg/kg/day, p.o.) or bergenin (10, 50, and 250 mg/kg/day, p.o.) for 7 consecutive days. **(F-J)** are TBHP-induced oxidative stress mice: **(F)** TBHP (18 mg/kg/day, i.p.) and **(G-J)** TBHP with gallic acid (100 mg/kg/day, p.o.) or bergenin (10, 50 and 250 mg/kg/day, p.o.) for 7 consecutive days. Images are expressed at 400-fold magnification. PV, portal vein; black arrow, swollen hepatocyte; white arrow, nuclear pyknosis; circle, necrotic area; square, cell membrane disruption.

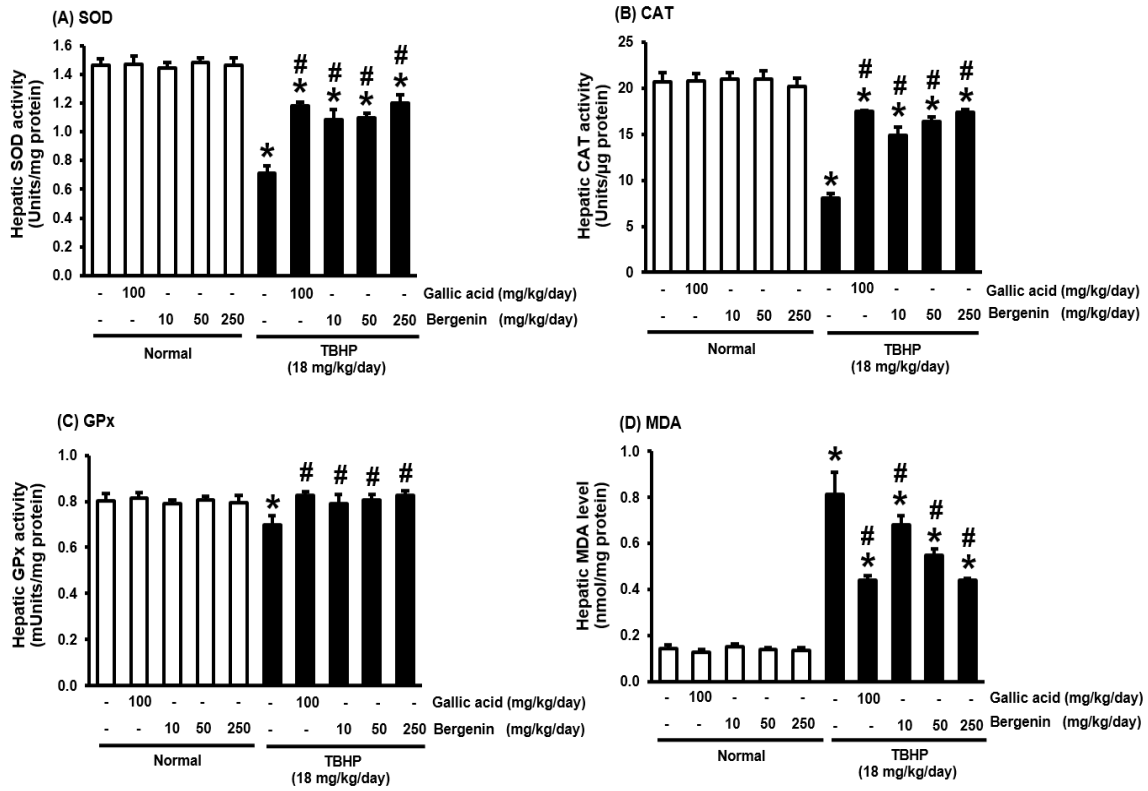

Figure 4: Effect of bergenin on the hepatic **(A)** SOD, **(B)** CAT, and **(C)** GPx activities and **(D)** the MDA level in the TBHP-induced oxidative stress in mice. A significant difference was determined by one-way analysis of variance followed by Tukey's *post hoc* test. $^{*}p < 0.05$ vs control; $^{\#}p < 0.05$ vs TBHP induction

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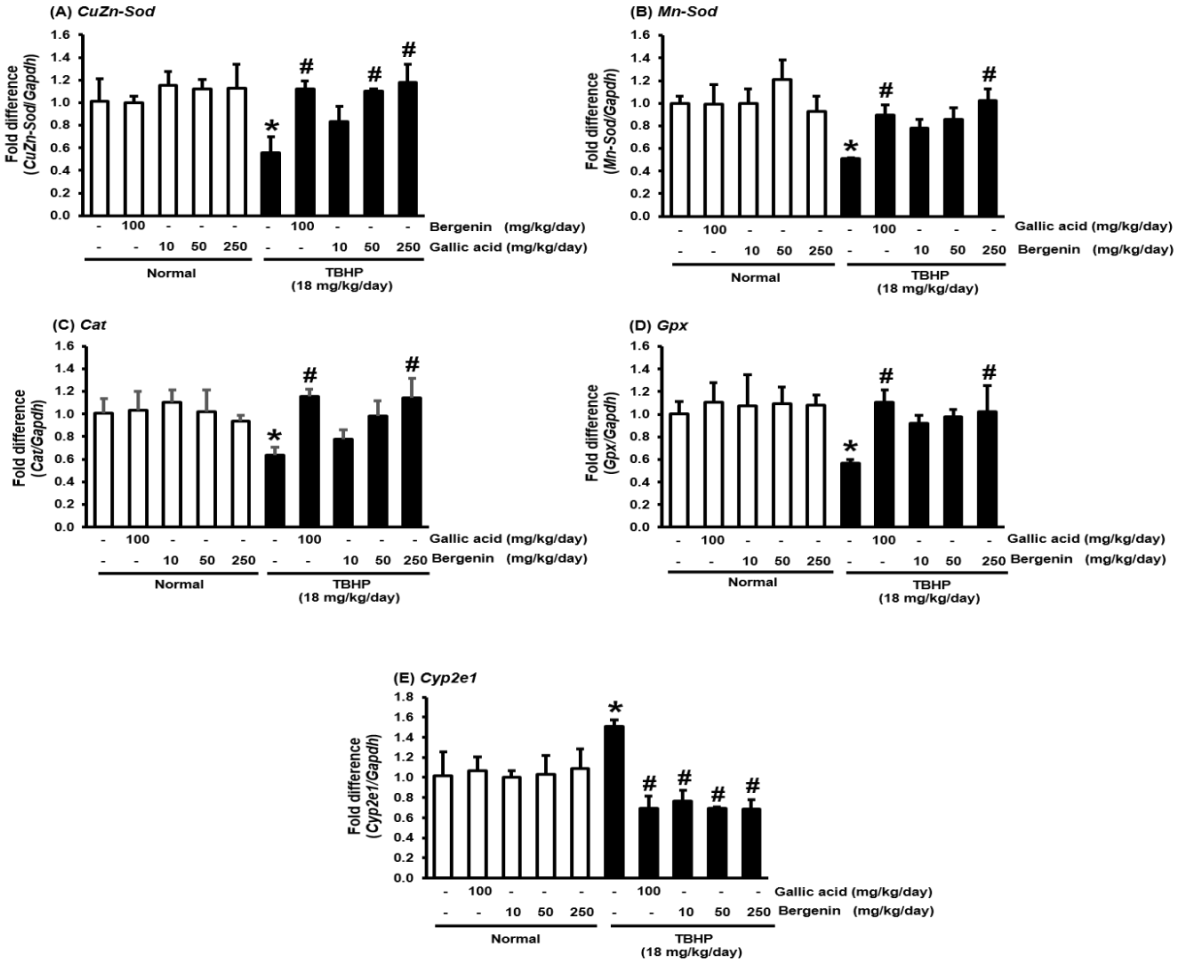

Figure 5: Effect of bergenin on the expression of hepatic **(A)** *CuZn-Sod*, **(B)** *Mn-Sod*, **(C)** *Cat*, *Gp***(D)** *x*, and **(E)** *Cyp2e1* mRNAs in the TBHP-induced oxidative stress in mice. A significant difference was determined by one-way analysis of variance followed by Tukey's *post hoc* test. *$p < 0.05$ vs control; $^{\#}p < 0.05$ vs TBHP induction.

Conclusion

Bergenin, particularly 250 mg/kg/day, maintained the hepatic oxidant-antioxidant balance in TBHP-induced oxidative stress in mice in a manner comparable to the potent antioxidant gallic acid. As an active metabolite of gallic acid, bergenin is a novel antioxidant with the potential for use to prevent hepatic oxidative injury/damage. Therefore, bergenin is a promising candidate to further develop as a natural supplement for antioxidation and hepatoprotection.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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