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Research article

Hepatoprotective Activity of Bergenin against Xenobiotics-Induced Oxidative Stress in Human Hepatoma (HepG2) Cells

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Abstract Bergenin, a natural derivative of gallic acid, has been shown to exert anti-oxidant and anti-inflammatory activities. This study aimed to determine hepatoprotective activity of bergenin against ethanol and *tert*-butyl hydroperoxide (TBHP) induced oxidative stress in human hepatoma (HepG2) cells. HepG2 cells (5×10^5 cells/well) were co-treated with ethanol (100 mM) or TBHP (100 μ M) and either bergenin (75, 150, and 300 μ M) or gallic acid (60 μ M, positive control) for 24 h. Cell viability, hematoxylin and eosin staining of cell morphology, cellular injury biomarkers: lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and malondialdehyde (MDA), and antioxidant biomarkers: superoxide dismutase (SOD), catalase (CAT), and total glutathione (GSH) content, were determined. Ethanol and TBHP decreased cell viability (86.67% and 84.49% of control, respectively), increased LDH toxicity (12.53% and 15.91% of control, respectively), and increased AST, ALT, and MDA levels, compared with the control. Based on cell morphology, both ethanol and TBHP injured HepG2 cells causing the loss of cell nuclei. Treatment of HepG2 cells with either ethanol or TBHP reduced SOD and CAT activities and depleted total GSH content, compared with the control. Bergenin and gallic acid improved the cell morphology, elevated cell viability (95.94-99.20% and 97.72-99.62% of control, respectively), lowered LDH toxicity (8.14-9.10% and 7.82-8.92% of control, respectively), restored AST, ALT, and MDA levels, promoted SOD and CAT activities, and enhanced the total GSH content of ethanol- and TBHP-treated HepG2 cells. Bergenin exhibited hepatoprotective activity via restoration of the oxidant-antioxidant system and is a potential candidate for hepatoprotective treatment.

Keywords: Antioxidant biomarkers, Cellular injury biomarkers, Ethanol, Glutathione, *Tert*-butyl hydroperoxide

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INTRODUCTION

The liver is a major organ for metabolism, detoxification, and regulation of physiological homeostasis (Polson and Lee, 2005). The liver is easily damaged by xenobiotics because it is the primary organ for their biotransformation, which can subsequently produce excessive free radicals, especially reactive oxygen species (ROS) (Birben et al., 2012). Ethanol is a xenobiotic-related to lifestyle that is mostly found in beverages and diet, but is also present in household and industrial products (Zakhari, 2013). *Tert*-butyl hydroperoxide (TBHP) is a powerful oxidizing agent used as a bleach and an initiator of polymerization in industrial processes and it is typically related to environmental pro-oxidant pollutant (Liu et al., 2002). Ethanol and TBHP present a high potential risk to the liver due to the creation of ROS by-products from hepatic metabolism (Liu et al., 2002; Zakhari, 2013). This phenomenon can result in an imbalance of the oxidant-antioxidant system, leading to oxidative stress-induced liver injury, and subsequent damage to biological structures through liver malfunction (Cichoż-Lach and Michalak, 2014). Hence, oxidative stress is closely associated with the pathogenesis of liver diseases. Augmentation of hepatic antioxidant activity has been proposed as an important strategy to protect the liver against oxidative stress (Li et al., 2015).

Polyphenols are known to provide health-improving benefits due to their strong antioxidant effects (Li et al., 2015). Bergenin (*C-glucoside* of polyphenol 4-O-methylgallic acid, Figure 1A), a natural derivative of gallic acid, is an isocoumarin found in *Mallotus japonicus*, *M. repandus*, *M. philippinensis*, *Caesalpinia digyna*, *Ardisia colorata*, *Bergenia ciliata*, and *B. ligulata* (Bajracharya, 2015). Bergenin has been claimed to possess antioxidant (Nazir et al., 2011), hepatoprotective (Ambika and Saravanan, 2016), nephroprotective (Aggarwal et al., 2016), antidiabetic (Kumar et al., 2012a), anti-inflammatory (Gao et al., 2015), and gastroprotective properties (Zhang et al., 2003). Previously, extracts of *M. philippinensis* and *M. repandus* have been shown to exhibit hepatoprotective activity in ethanol- and D-galactosamine-induced hepatotoxicity in rats (Geetha et al., 2011; Mondal et al., 2020) and both are good sources of bergenin; *M. repandus* contained bergenin 19% dry weight (Sriset et al., 2018) and *M. philippinensis* reported 6% dry weight (Haribabu et al., 2012). However, there are few studies examining the hepatoprotective effects of bergenin, and there is lack of evidence establishing the hepatoprotective mechanism of bergenin in the human hepatic system. In the present study, the human hepatoma (HepG2) cell line was employed as an in vitro model to investigate the mechanism of bergenin activity against xenobiotics-induced hepatotoxicity. HepG2 cells are considered to be similar to the human hepatic system since they possess several specialized functions of normal human hepatocytes such as xenobiotic metabolism and glutathione (GSH) synthesis (Scharf et al., 2003). As gallic acid (Figure 1B) has shown potential against hydrogen peroxide (H₂O₂), TBHP, and carbon tetrachloride-induced oxidative stress in HepG2 cells, mice, and rats (Choi et al., 2015; Jadon et al., 2007; Kong et al., 2016), it was chosen as a positive control.

The present study, therefore, aimed to evaluate the hepatoprotective mechanism of bergenin by examining its effects on cell morphology, cellular injury, oxidative stress biomarkers, and the antioxidative status of HepG2 cells subjected to ethanol- and TBHP-induced oxidative stress.

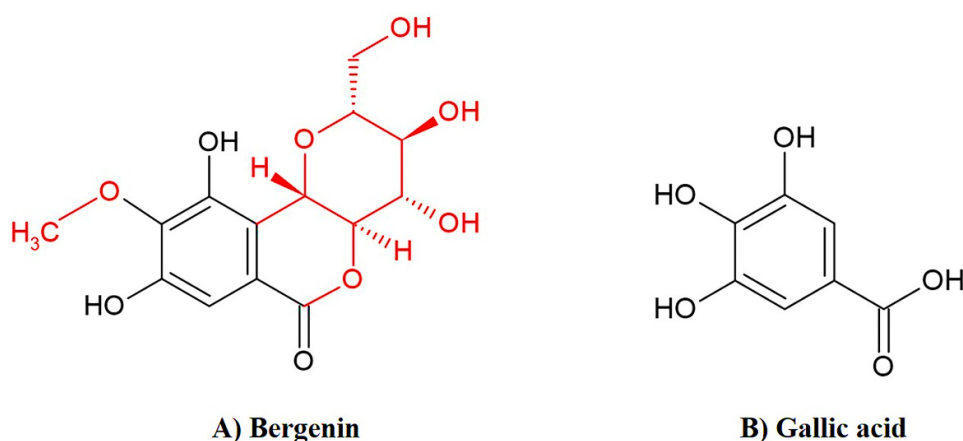


Figure 1. Chemical structure of A) bergenin and B) gallic acid.

MATERIALS AND METHODS

Materials

Bergenin (Cat. No. BP0258, purity > 98%) was a product of Biopurify Phytochemicals (Chengdu, China). Absolute ethanol ($\geq 99.9\%$ v/v) and gallic acid were obtained from Merck (Darmstadt, Germany). Catalase (CAT), 2,4-dinitrophenylhydrazine (DNPH), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), glutathione reductase, L-glutathione reduced, β -nicotinamide adenine dinucleotide 2'-phosphate (NADPH), resazurin sodium salt, superoxide dismutase (SOD), and *tert*-butyl hydroperoxide (TBHP, 70% w/v) were products of Sigma-Aldrich Chemical (St. Louis, Missouri, USA). α -Ketoglutarate, L-alanine, L-aspartate, malondialdehyde (MDA), and thiobarbituric acid (TBA) were purchased from Sigma-Aldrich Chemical. Aqueous solutions of 1% Eosin Y and Mayer's hematoxylin were the products of Bio Optica (Milan, Italy). Dulbecco's modified Eagle medium (DMEM, Cat. No. 11885-084), Dulbecco's modified Eagle medium nutrient mixture F-12 (DMEM/F-12 without phenol red, Cat. No. 21041-025), and fetal bovine serum (FBS) were products of Gibco[®] (New York, USA). All other laboratory chemicals were of the highest purity and obtained from chemical suppliers.

Cell culture and treatment protocol

HepG2 cells (ATCC[®] HB-8065[™], Lot No. 61983117) were purchased from the American Type Culture Collection (Manassas, Virginia, USA). The cells were cultured in DMEM supplemented with 10% v/v FBS, 1 $\times$ Glutamax[®], and 1 $\times$ penicillin-streptomycin-neomycin antibiotic mixture under 95% humidity in an atmosphere of 5% CO₂ at 37°C.

The cells were seeded into 6-well plates (5 $\times 10^5$ cells/well) in DMEM. After 48 h, the medium was replaced with DMEM/F-12 without phenol-red (supplemented with 10% FBS, 1 $\times$ Glutamax[®], and antibiotic mixture) containing ethanol (100 mM) or TBHP (100 μ M) and either bergenin (75, 150, and 300 μ M) or gallic acid (60 μ M; positive control). Stock solutions of bergenin and gallic acid were prepared in 0.2% dimethyl sulfoxide (DMSO). Control was incubated with DMEM/F-12 without phenol-red containing 0.2% DMSO. After 24 h, %cell viability was determined by resazurin assay and the medium was kept to measure %lactate dehydrogenase (LDH) toxicity, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. Cell morphology was examined using hematoxylin and eosin (H & E) staining. The cells were harvested in 1 $\times$ phosphate buffered saline (PBS) by a cell scraper to determine SOD and CAT activities, total glutathione (total GSH), and MDA contents.

Assessment of cell viability

The percentage cell viability was fluorometrically measured by resazurin assay based on the ability of cellular enzymes to reduce resazurin (blue and non-fluorescent

dye) to resorufin, a pink fluorescent compound (Miret et al., 2006). Briefly, 1 mM resazurin solution was added to culture medium (1:10) before incubation in 5%CO₂ at 37°C for 1 h. The percentage cell viability was calculated from the amount of resorufin, which was measured at 530 nm excitation and 580 nm emission (Chatuphonprasert et al., 2020).

Assessment of LDH toxicity

The percentage LDH toxicity was assessed by determining LDH activity in culture medium using an LDH assay kit (Roche®, Mannheim, Germany). This assay measures activity of LDH released into culture medium upon a loss of cell membrane integrity. The percentage of HepG2 cell toxicity was calculated by comparison with a standard curve of Triton X-100 (6.25-50% toxicity) (Sriset et al., 2019).

Determination of AST and ALT levels

Culture medium was incubated with either AST substrate (10 mM *L*-aspartate and 1.7 mM α -ketoglutarate) or ALT substrate (300 mM *L*-alanine and 0.7 mM α -ketoglutarate) at 37°C for 30 or 20 min, respectively. Then DNPH was added to a final concentration of 0.5 mM and left for 20 min, followed by addition of sodium hydroxide to a final concentration of 1 M. Absorbance was measured at 505 nm. The levels of AST and ALT were determined as international units per liter (IU/L) by comparison with a standard curve of sodium pyruvate (100-500 μ M) (Sriset et al., 2019).

Examination of HepG2 cell morphology by H & E staining

Culture medium was removed, and cells were washed with 0.01 M phosphate buffered saline (PBS) 3 times. The cells were immersed in 10% v/v neutral-buffered formalin for 30 min, followed by 60% v/v isopropanol for 1 min. Then the cells were stained with hematoxylin for 3 min and eosin for 30 sec. Morphological features of HepG2 hepatocytes were examined at 200-fold magnification using an inverted microscope (AE2000; Motic Incorporation, Ltd., Causeway Bay, Hong Kong). Images were recorded with the Moticam 5.0MP digital camera coupled with the Motic images plus 3.0 software (Motic Incorporation, Ltd.) (Qin et al., 2010).

Determination of protein content

HepG2 cells were lysed in 0.01 M phosphate buffered saline with a hand homogenizer in an ice-bath. Protein content of HepG2 cell lysate was quantified using the Bradford method with a few modifications (Chatuphonprasert et al., 2013). Briefly, 40 μ L of the diluted HepG2 cell lysate was mixed with 160 μ L of the Bradford reagent (Bio-Rad, Hercules, CA, USA). The measurement of protein-dye complexes at a wavelength of 595 nm was performed and protein content was calculated by comparison with a standard curve of bovine serum albumin (BSA; 12.5-150 μ g/mL).

Assessment of SOD activity

SOD activity was evaluated based on the inhibition of formation of nitroblue tetrazolium (NBT) products (formazan), as described previously (Sriset et al., 2019). In brief, 50 μ L of HepG2 cell lysate was mixed with chloroform and ethanol (1:1.67) and subsequently centrifuged at 14,000 $\times$ g at 4°C for 30 min. A reaction mixture of 1.1 mM xanthine, 0.1 mM ethylenediamine tetraacetic acid, 0.6 mM NBT, 56 mM sodium carbonate, and 70 μ g/mL BSA was mixed with the supernatant before incubation with 20 μ Units/ μ L xanthine oxidase at room temperature for 20 min, followed by addition of 0.1 mM copper (II) chloride to stop the reaction. Formazan product was measured at 550 nm and SOD activity was calculated by comparison with the SOD standard (40 mUnits/ μ L).

Assessment of CAT activity

CAT activity was assessed by the colorimetric method using H₂O₂ as the substrate. HepG2 cell lysate was incubated with 150 μ M H₂O₂ at 37°C for 1 min. After incubation, ammonium molybdate was added to a final concentration of 26 mM, followed by measurement at 405 nm. CAT activity was calculated by comparison with the CAT standard (2.5 Units/ μ L) (Sriset et al., 2019).

Assessment of total glutathione (GSH) content

HepG2 cell lysate was extracted with 5% w/v sulfosalicylic acid (1:5) and centrifuged at 10,000×g at 4°C for 10 min to obtain supernatant. A freshly prepared reaction mixture of 7 mM potassium phosphate buffer (pH 7.0), 0.04 Units/mL glutathione reductase, and 30 µg/mL DTNB was mixed with the supernatant, followed by addition of 40 µg/mL NADPH to start the reaction. Absorbance was measured at 405 nm every 30 sec for 10 min. Total GSH content was calculated by comparison with a slope of GSH standard curve (6.25-50 µM) (Sriset et al., 2019).

Assessment of lipid peroxidation by thiobarbituric acid reactive substances (TBARS) assay

TBARS assay was performed as described previously (Sriset et al., 2019). In brief, HepG2 cell lysate was mixed with 10% w/v trichloroacetic acid (1:1) and subjected to centrifugation at 2,300×g at 4°C for 10 min. Supernatant was transferred to a new tube and mixed with an equal volume of 0.8% w/v TBA, before boiling at 100°C for 15 min, followed by immediate cooling in an ice-bath. TBARS formed by the reaction of TBA and MDA (a by-product of lipid peroxidation) were measured at 520 nm excitation and 590 nm emission and content was calculated by comparison with a standard curve of MDA (0.125-1 µM).

Statistical analysis

Results are expressed as mean ± standard deviation (SD) (n = 4). All data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test using Statistical Package for Social Studies (SPSS) version 22.0 (Armonk, New York). *P* < 0.05 was considered statistically significant.

RESULTS

Effect of bergenin on cell viability and cytotoxicity in ethanol- and TBHP-treated HepG2 cells

HepG2 cells treated with ethanol and TBHP showed significant decreases in %cell viability compared with the control (*P* < 0.05), but cell viability remained more than 80% (Table 1). Substances can be considered cytotoxic at % LDH over 10 (Sriset et al., 2019), and exposure to ethanol and TBHP was certainly toxic to HepG2 cells compared to the control (*P* < 0.05) (Table 1). All concentrations of bergenin (ranging from 75 to 300 µM) and gallic acid (60 µM) improved %cell viability and decreased %LDH toxicity in HepG2 cells treated with ethanol and TBHP to levels comparable to the control (*P* < 0.05).

Table 1. Effect of bergenin on cell viability and LDH toxicity in ethanol- and TBHP-treated HepG2 cells

Treatment		Cell viability (%)	LDH ^a toxicity (%)
Normal	Control	100.00 ± 0.00	6.55 ± 0.34
Ethanol induction (100 mM)	Ethanol	86.67 ± 3.88*	12.53 ± 0.84*
	Gallic acid 60 µM	99.62 ± 1.56 [#]	7.82 ± 1.49 [#]
	Bergenin 75 µM	98.67 ± 1.41 [#]	8.53 ± 1.34 [#]
	150 µM	98.76 ± 0.67 [#]	8.41 ± 0.61 [#]
	300 µM	99.20 ± 0.21 [#]	8.66 ± 1.33 [#]
TBHP induction (100 µM)	TBHP	84.49 ± 1.30*	15.91 ± 0.73*
	Gallic acid 60 µM	97.72 ± 0.94 ^{\$}	8.92 ± 1.46 ^{\$}
	Bergenin 75 µM	95.94 ± 0.98 ^{\$}	9.10 ± 0.07 ^{\$}
	150 µM	96.11 ± 1.07 ^{\$}	8.59 ± 0.25 ^{\$}
	300 µM	96.66 ± 2.89 ^{\$}	8.14 ± 0.11 ^{\$}

Note: The data are presented as mean ± SD (n = 4). a, lactate dehydrogenase. A significant difference was determined by one-way analysis of variance followed by Tukey's *post hoc* test. **P* < 0.05 versus control; [#]*P* < 0.05 versus ethanol induction; ^{\$}*P* < 0.05 versus TBHP induction.

Effect of bergenin on AST and ALT levels in ethanol- and TBHP-treated HepG2 cells

Ethanol and TBHP dramatically increased AST (Figure 2A) and ALT (Figure 2B) levels in HepG2 cells, compared with the control ($P < 0.05$). Bergenin concentration-dependently reduced AST and ALT levels in ethanol- and TBHP-treated HepG2 cells. Correspondingly, 60 μ M gallic acid also significantly lowered levels of both enzymes in ethanol- and TBHP-treated HepG2 cells.

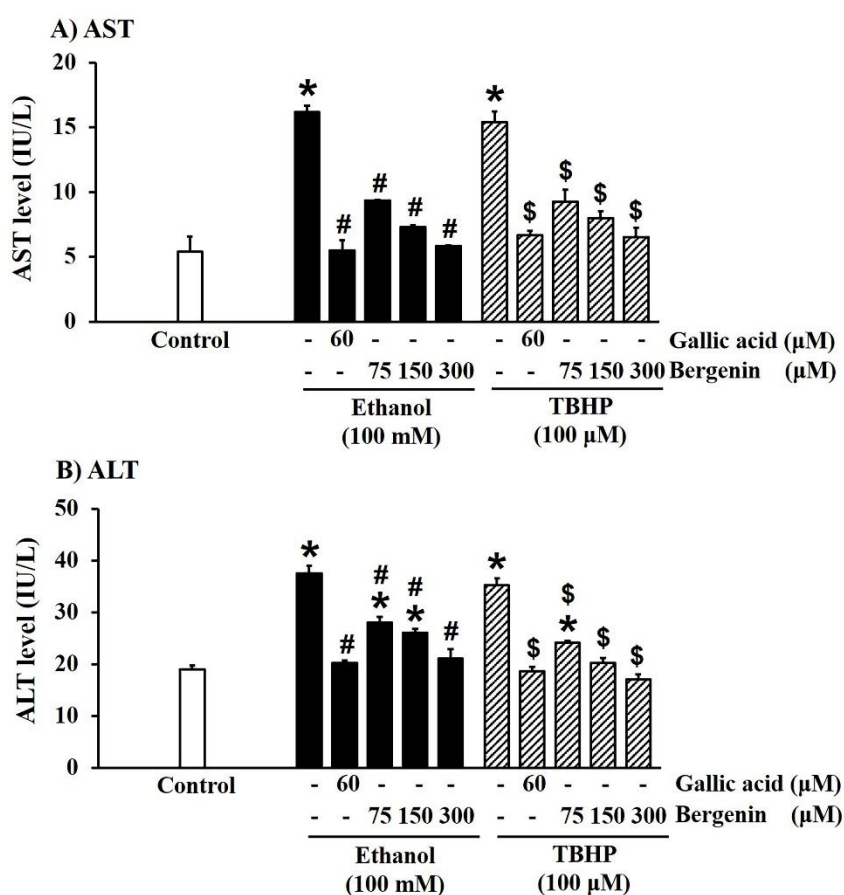


Figure 2. Effect of bergenin on cellular injury biomarkers in ethanol- and TBHP-treated HepG2 cells. A) AST and B) ALT levels. The data are presented as mean $\pm$ SD ($n = 4$). A significant difference was determined by one-way analysis of variance followed by Tukey's post *hoc* test. * $P < 0.05$ versus control; # $P < 0.05$ versus ethanol induction; \$ $P < 0.05$ versus TBHP induction.

Effect of bergenin on cell morphology in ethanol- and TBHP-treated HepG2 cells

Normal HepG2 cell morphology is shown in Figures 3A and 4A (control). Ethanol and TBHP induced HepG2 cell morphological changes including modified round cell shape (blue arrows), and a loss of cell nuclei (orange triangles) (Figures 3B and 4B). Bergenin (Figures 3D-3F and 4D-4F) and gallic acid (Figure 3C and 4C) prevented ethanol- and TBHP-induced HepG2 cell damage.

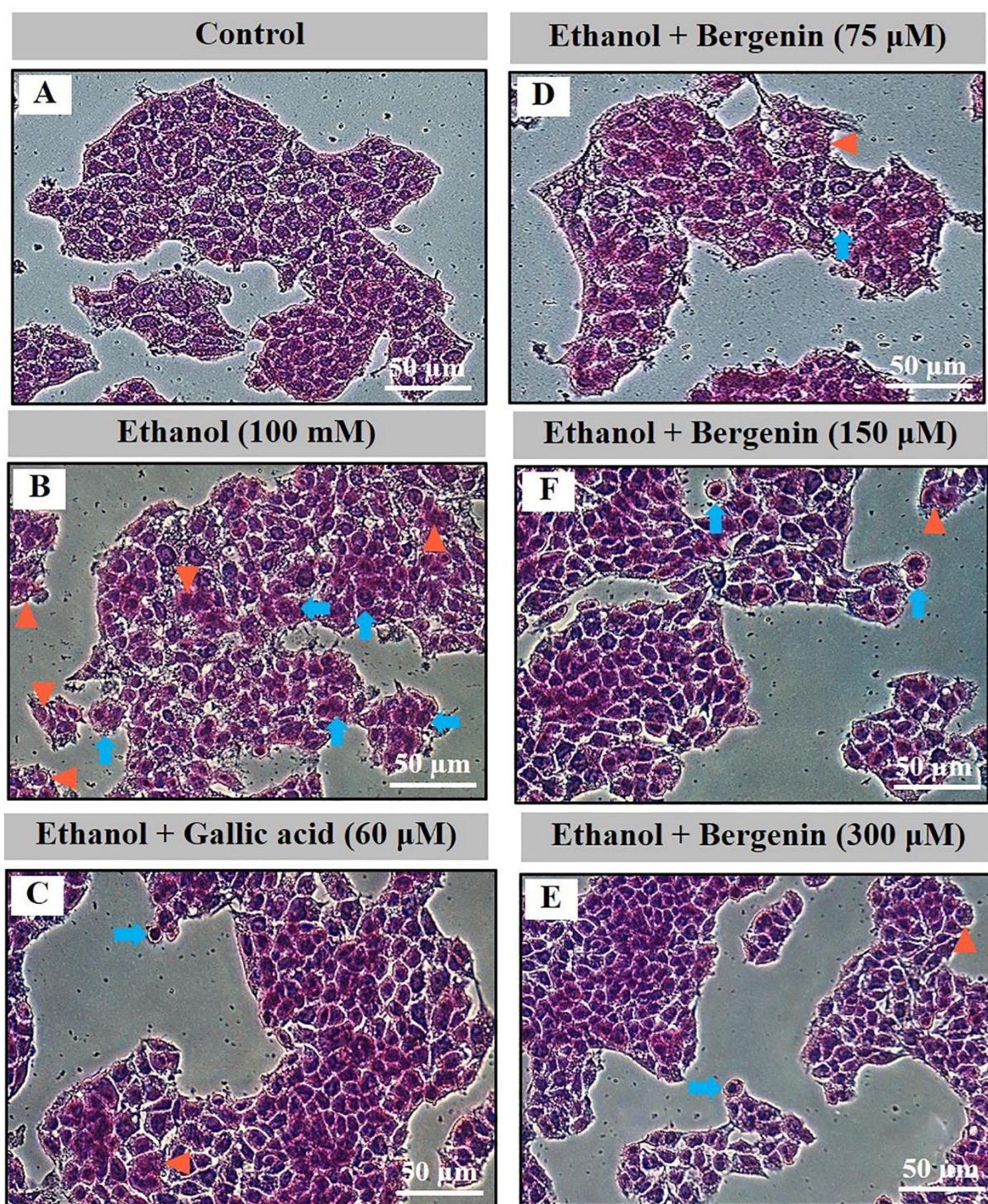


Figure 3. Effect of bergenin on cell morphology by H & E staining in ethanol-treated HepG2 cells. A) control, B) ethanol (100 mM), C) co-treatment of ethanol and gallic acid (60 μ M), and D-F) co-treatment of ethanol and bergenin (75, 150, and 300 μ M) for 24 h. Images are 200-fold magnification. Blue arrows, round-shaped cells; Orange triangles, loss of cell nuclei.

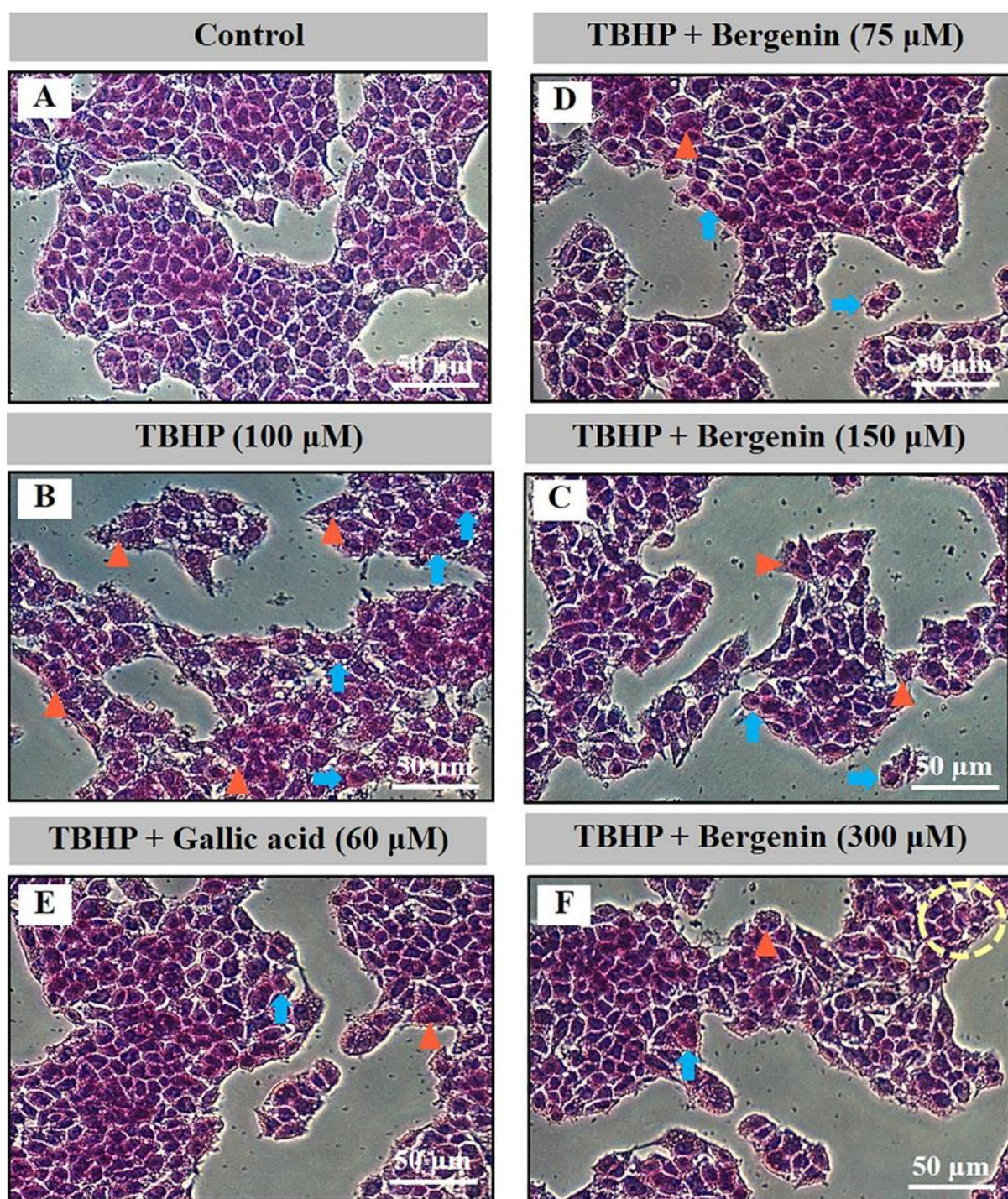


Figure 4. Effect of bergenin on cell morphology by H & E staining in TBHP-treated HepG2 cells. A) control, B) TBHP (100 μ M), C) co-treatment of TBHP and gallic acid (60 μ M), and D-F) co-treatment of TBHP and bergenin (75, 150, and 300 μ M) for 24 h. Images are 200-fold magnification. Blue arrows, round-shaped cells; Orange triangles, loss of cell nuclei.

Effect of bergenin on cellular antioxidant defenses in ethanol- and TBHP-treated HepG2 cells

Cellular antioxidant defenses, including SOD and CAT activities and total GSH content, were examined in ethanol- and TBHP- induced HepG2 cells. Ethanol and TBHP significantly decreased SOD and CAT activities (Figure 5A and 5B) and total GSH content (Figure 6A), compared with the control ($p < 0.05$). Gallic acid (60 μM) and bergenin treatment significantly increased SOD and CAT activities (Figure 5A and 5B) and total GSH content (Figure 6A) in HepG2 cells treated with ethanol and TBHP ($p < 0.05$). The effects of bergenin on the cellular antioxidant defenses were concentration-dependent.

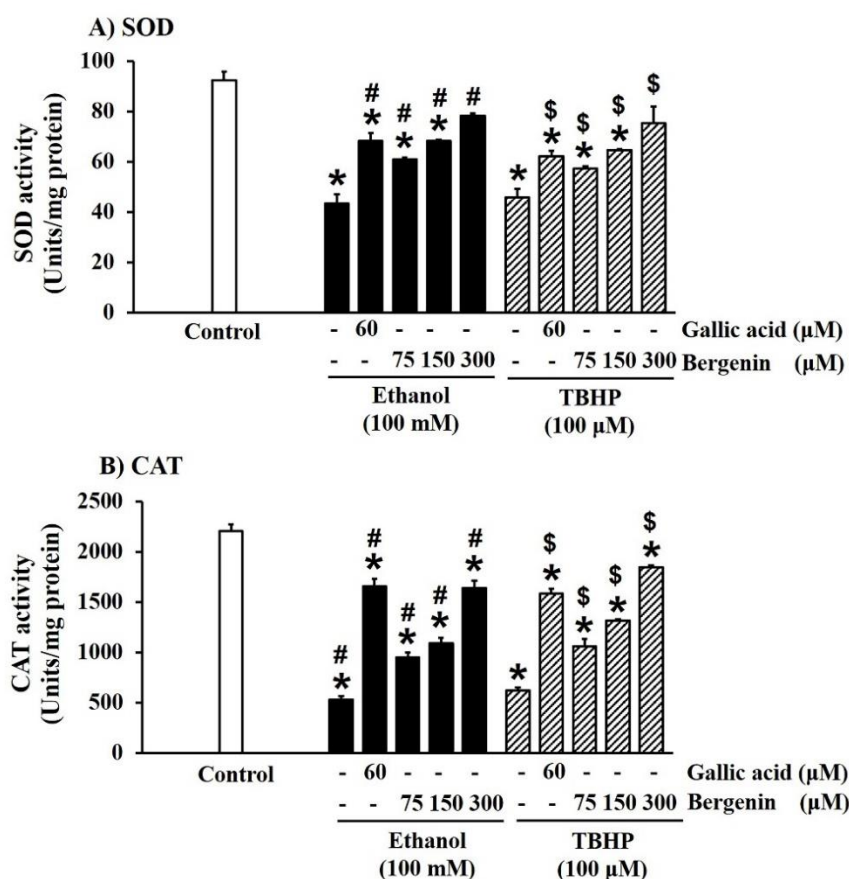


Figure 5. Effect of bergenin on SOD and CAT activities in ethanol- and TBHP-treated HepG2 cells. The data are presented as mean $\pm$ SD ($n = 4$). A significant difference was determined by one-way analysis of variance followed by Tukey's *post hoc* test. * $P < 0.05$ versus control; # $P < 0.05$ versus ethanol induction; \$ $P < 0.05$ versus TBHP induction.

Effect of bergenin on lipid peroxidation in ethanol- and TBHP-treated HepG2 cells

Lipid peroxidation was assessed by quantification of MDA, a final product of this reaction (Sriset et al., 2019). Ethanol and TBHP significantly increased MDA levels in HepG2 cells, compared with the control ($P < 0.05$) (Figure 6B). Gallic acid and bergenin, in a concentration-dependent manner, greatly reduced MDA levels in ethanol- and TBHP-treated HepG2 cells ($P < 0.05$) (Figure 6B).

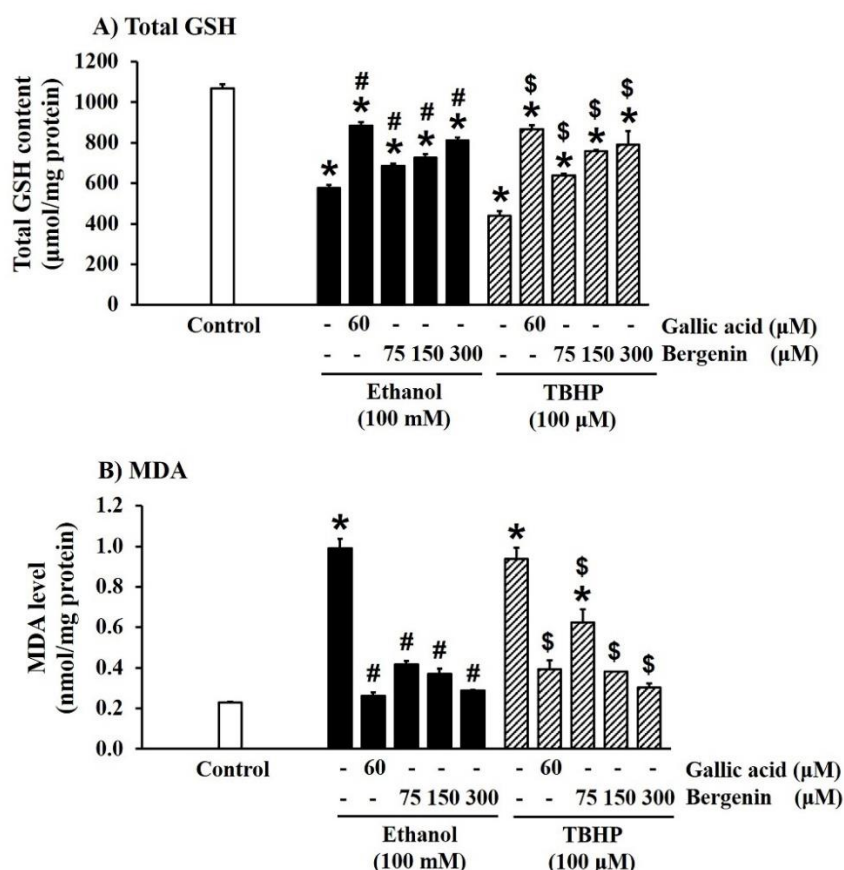


Figure 6. Effect of bergenin on total GSH content and MDA level in ethanol- and TBHP-treated HepG2 cells. The data are presented as mean $\pm$ SD ($n = 4$). A significant difference was determined by one-way analysis of variance followed by Tukey's *post hoc* test. * $P < 0.05$ versus control; # $P < 0.05$ versus ethanol induction; \$ $P < 0.05$ versus TBHP induction.

DISCUSSION

Over-production of ROS contributes to hepatocyte damage (Li et al., 2015). Among the pathophysiological mechanisms of liver injury, xenobiotics-generated ROS are one of the main factors causing oxidant-antioxidant imbalance, leading to oxidative stress which is considered a key step in the progression of liver diseases (Cichoż-Lach and Michalak, 2014). Ethanol and TBHP are well-known xenobiotics encountered in daily life and in industrial pollution, respectively (Liu et al., 2002; Zakhari, 2013). Due to their highly reactive and toxic properties, both ethanol and TBHP are considered hazardous chemicals to humans (Cichoż-Lach and Michalak, 2014). Moreover, hepatic metabolism of ethanol and TBHP can be involved in excessive ROS production, cellular antioxidant depletion, and hepatotoxicity (Liu et al., 2002; Zakhari, 2013). Therefore, ethanol and TBHP can be employed as inducers to evoke hepatocyte damage initiated by oxidative stress and to disturb the cellular antioxidant status in HepG2 cells. Furthermore, the HepG2 cell model is often used to study the cellular antioxidant system, including both the enzymatic and non-enzymatic mechanisms, as the main biological defense in the liver (Alía et al., 2005; Kozics et al., 2013; Pareek et al., 2013).

Ethanol is metabolized in the liver through three pathways consisting of alcohol dehydrogenase (ADH), the microsomal ethanol oxidizing system, and CAT (Zakhari, 2013). The major pathway of ethanol metabolism is ADH, which produces a toxic metabolite acetaldehyde. Furthermore, each pathway generates ROS by-products, especially superoxide anion ($O_2^{\cdot-}$) and hydroxyl radicals (Lee et al., 2008; Zakhari, 2013). Ethanol is known to strongly induce cytochrome P450 2E1 as the major

component of the microsomal ethanol oxidizing system (Liu et al., 2010). This results in the generation of excess ROS that rapidly react with lipid membranes, leading to lipid peroxidation and oxidative stress (Farshori et al., 2015). In the present study, remarkably elevated levels of MDA, the product of lipid peroxidation, were observed in HepG2 cells treated with ethanol. These findings suggest that the microsomal ethanol oxidizing system is a key pathway of ethanol metabolism to induce oxidative stress in HepG2 cells.

TBHP is biotransformed into toxic *tert*-butyl alcohol and ROS, mainly peroxy and alkoxy anions, via activation of cytochrome P450 2E1 and the glutathione peroxidase-glutathione reductase system (Cederbaum et al., 2001; Liu et al., 2002; Park et al., 2009). These ROS and toxic metabolites can induce breakdown of the hepatocyte membrane, releasing liver injury-related enzymes (LDH, AST, and ALT) from the cytoplasm into extracellular fluids (Cichoż-Lach and Michalak, 2014). Furthermore, polyunsaturated fatty acids in the hepatocyte membrane are attacked by ROS, resulting in lipid peroxidation that generates MDA and eventually injures the hepatocyte structure (Niki et al., 2005). Together, these events demonstrate that lipid peroxidation, triggered by ROS generation from xenobiotics such as ethanol, TBHP, and carbon tetrachloride, leads to oxidative damage in HepG2 cells (Gómez-Quiroz et al., 2003; Krithika et al., 2009; Sohn et al., 2005). Therefore, in the present study, the ROS produced by the metabolism of ethanol and TBHP evoked HepG2 cell injury, as seen in the destruction of cell morphology and loss of cell nuclei, the decrease in cell viability, the increase in leakage of LDH, AST, and ALT from the cytoplasm and a surge in MDA levels from increased lipid peroxidation.

SOD is the main antioxidant defense against the O_2^- free radical, which is converted into H_2O_2 and further catalyzed to water and oxygen by CAT (Limón-Pacheco and Gonshebat, 2009). GSH, a non-enzymatic antioxidant, is a reducing agent that directly scavenges and neutralizes free radicals and detoxifies toxic metabolites (Forman et al., 2009). Hence, both enzymatic and non-enzymatic antioxidants are responsible for preventing oxidative stress. Nevertheless, excessive levels of ROS result in an imbalance of this cellular antioxidant system and a deterioration in antioxidant capability (Cichoż-Lach and Michalak, 2014). In this study, ethanol decreased SOD and CAT activities and total GSH content in HepG2 cells. These results correspond with a previous study that showed treatment of human primary hepatocytes with 100 mM ethanol for 24 h increased the amount of AST and LDH and depleted GSH stores, which was followed by an increase in MDA level (Yao et al., 2007). Likewise, HepG2 cells exposed to 100 mM ethanol for 24 h demonstrated increases in AST, ALT, MDA, and ROS levels with a reduction of GSH content (Kumar et al., 2012b). Furthermore, primary rat hepatocytes treated with 100 mM ethanol for 8 h showed increases in LDH and AST activities, followed by increased lipid peroxidation, reduction in SOD and CAT activities, and a decrease in GSH content (Liu et al., 2010). Corresponding to the study of Alía et al., 2005, our study found a decrease in cellular antioxidant status (reduced SOD and CAT activities and total GSH content) due to TBHP in HepG2 cells. Alía et al. found that TBHP at concentrations over 50 μM for 3 h elevated MDA level and reduced SOD and CAT activities in HepG2 cells (Alía et al., 2005). Moreover, TBHP at 200 μM for 3 h decreased GSH content and reduced SOD and CAT activities, followed by increases in MDA, LDH, and ROS in HepG2 cells (Alía et al., 2006; Cuello et al., 2007). TBHP at 250 μM retarded HepG2 cell viability by 47.33%, indicating suppression of cell growth, which was related to increases in ROS and MDA production along with GSH depletion (Sohn et al., 2005). The current study suggests that HepG2 cell damage and toxicity due to exposure to ethanol and TBHP can be mostly attributed to oxidative stress, which accords with our previous study (Sriset et al., 2019). In our previous study, we demonstrated that concentration- and time-dependent oxidative stress could be observed after cells were treated with either 10 to 500 mM ethanol for 24 to 48 h or 50 to 200 μM TBHP for 3 to 24 h, as verified by induction of LDH, AST, and ALT release, increased production of MDA, reduction in SOD and CAT activities, and depletion of total GSH stores (Sriset et al., 2019). Therefore, maintaining the oxidant-antioxidant balance by promoting antioxidant activity is an approach to minimize oxidative stress and liver damage (Li et al., 2015).

Gallic acid at a concentration of 60 μM has previously been shown to be a potent antioxidant and anti-necroptosis agent in human hepatoma HepG2 cells and human hepatocyte LO2 cells (Kong et al., 2016; Zhou et al., 2019). Moreover, our previous study demonstrated that gallic acid at 60 μM restored the antioxidant status (i.e. SOD and CAT activities, and total GSH content) in ethanol-, sodium selenite-, and TBHP-induced oxidative stress in HepG2 cells (Sriset et al., 2019). Therefore, the concentration of 60 μM was selected for gallic acid in the present study. The concentrations of bergenin that were selected for the present study were based on studies examining the inhibitory effects of bergenin against free radicals (Srinivasan et al., 2007). Bergenin showed in vitro antioxidant activity against hydrogen peroxide (Srinivasan et al., 2007) and in vitro anti-inflammatory activity against cyclooxygenase-1 (Nunomura et al., 2009) at concentrations of 75 and 100 μM . In addition, bergenin at 300 μM was reported to have hepatoprotective and antioxidant activities in primary rat hepatocytes (Kim et al., 2000; Lim et al., 2000). Hence, the selected concentrations of bergenin in the present study were 75, 150, and 300 μM .

Gallic acid (30 to 60 μM) reduced ROS and MDA formation and restored SOD and CAT activities in H_2O_2 -induced HepG2 cells (Kong et al., 2016), while gallic acid at 100 μM prevented cumene hydroperoxide-induced ROS generation and lipid peroxidation, and augmented total GSH content in primary rat hepatocytes (Pourahmad et al., 2010). Our previous study demonstrated that 60 μM gallic acid restored the antioxidant status (both SOD and CAT, and total GSH content) in ethanol-, sodium selenite-, and TBHP-induced oxidative stress in HepG2 cells (Sriset et al., 2019). Hence, gallic acid was employed as a positive standard in the present study at a dose of 60 μM , representing the peak antioxidant and hepatoprotective activities. In the present study, LDH, as a stable cytoplasmic enzyme, was employed as a sensitive indicator of cell membrane damage. An increase in extracellular LDH level indicates leakage and can be used as an index of cytotoxicity, which is inverse to cell viability (Nagaraj et al., 2012). Bergenin protected HepG2 cells against ethanol- and TBHP-induced cytotoxicity, elevating cell viability and reducing LDH toxicity. However, these effects were not dose-dependent, with bergenin exerting its maximum effect on the mechanism underlying cell viability and LDH release at 75 μM . Similarly, AST and ALT are specific markers of liver function and can be used to assess liver integrity. The liver can be damaged directly by ROS-induced increases in cell membrane permeability, leading to leakage of AST and ALT (McGill, 2016). Thus, an increase in release of AST and ALT indicates damage to hepatocytes (Giannini et al., 2005). In contrast to LDH, bergenin concentration-dependently lowered ethanol- and TBHP-induced AST and ALT release from HepG2 cells, in our model. Moreover, the hepatoprotective action of bergenin against ethanol- and TBHP-induced liver damage was revealed through the restoration of normal HepG2 cell morphological features following enhancement of SOD and CAT activities, increase in total GSH content, and reduction in MDA level. Correspondingly, the damage to carbon tetrachloride-injured primary rat hepatocytes was attenuated by bergenin at concentrations ranging from 1 to 300 μM via maintenance of glutathione homeostasis (Kim et al., 2000). In addition, the hepatoprotective activity of bergenin (100 μM) could be noted by decreases in the levels of biochemical makers of liver injury including glutamic pyruvic transaminase and sorbitol dehydrogenase in D-galactosamine-intoxicated primary rat hepatocytes (Lim et al., 2000). The hepatoprotective activity of bergenin in a hepatic ischemia reperfusion model in human hepatocyte LO2 cells and male Balb/c mice was recently reported (Xiang et al., 2020). Bergenin improved cell viability by up to 70% by eliminating ROS and up-regulating peroxisome proliferator-activated receptor gamma expression in LO2 cells, which correlates with our findings (Xiang et al., 2020). In the present study, gallic acid attenuated HepG2 cell injury to restore cell viability, LDH, AST, ALT, and MDA to normal levels. The restoration of the antioxidant system (SOD, CAT, and total GSH) by gallic acid was also observed in ethanol- and TBHP-induced oxidative stress in HepG2 cells. The antioxidative potentials of gallic acid and bergenin were not different in the present study. Gallic acid is the active metabolite of 4-O-methylgallic acid and a major part of bergenin's structure (Rastogi and Rawat, 2008; Song et al., 2013). Thus, the hydroxyl rich structures of bergenin and gallic acid (Figure 1) act in the same way to decrease oxidative stress by directly binding with free radicals through an electron donating mechanism (Lü et al., 2010; Chen et al., 2020).

Therefore, it can be concluded that the hepatoprotective potential of bergenin is comparable to gallic acid. Thus, the main finding of the present study is that traditional uses of medicinal plants containing bergenin are related to the hepatoprotective and antioxidant activities of bergenin. For Thai traditional medicine, *M. repandus* has been used to relieve muscle pain and to maintain homeostasis (Rivière et al., 2010; Hasan et al., 2018), while in Bangladesh *M. repandus* has been applied for the treatment of inflammation and liver toxicity (Rivière et al., 2010). In addition, *M. japonicus* and *M. philippinensis* have been traditionally used to treat gastrointestinal diseases in Korea and India, respectively (Rivière et al., 2010; Gangwar et al., 2014).

Bergenin exerted its hepatoprotective effect against ethanol- and TBHP-induced oxidative stress in HepG2 cells by maintenance of the enzymatic and non-enzymatic antioxidant systems. This brought down hepatic oxidative stress and protected the hepatocytes, which inhibited the leakage of LDH, AST and ALT and the formation of MDA. These findings suggest that the hepatoprotective activity of bergenin in HepG2 cells is related to its ability to restore oxidant-antioxidant homeostasis.

CONCLUSION

Ethanol and TBHP induced oxidative stress, suppressed cell viability, accelerated LDH, AST, and ALT leakage, increased production of MDA, decreased SOD and CAT activities and disturbed the glutathione system, resulting in damage to HepG2 cells. Bergenin ameliorated the impact of oxidative stress caused by ethanol and TBHP in HepG2 cells. The highest tested concentration of bergenin at 300 μ M modulated the oxidant-antioxidant imbalance by restoring antioxidant enzyme activities and GSH homeostasis. Therefore, bergenin protected hepatocytes from ethanol- and TBHP-induced damage based on its antioxidant properties. This study provides primary evidence supporting the use of bergenin for hepatoprotection.

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