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Complete List of Authors:	Kim, Yon-Suk; Konkuk University, Department of Biotechnology Kim, Eun-Kyung; Konkuk University, Biotechnology Hwang, Jin-Woo; Konkuk University, Department of Biotechnology Seo, Il-Bok; Semyung University, Department of Anatomy, College of Oriental Medicine Jang, Jae-Hyuk; Korea Research Institute of Bioscience and Biotechnology, Chemical Biology Research Center Son, Sangkeun; Korea Research Institute of Bioscience and Biotechnology, Chemical Biology Research Center Jeong, Jae-Hyun; Korea National University of Transportation, Department of Food Science and Technology Moon, Sang-Ho; Konkuk University, Nokyong Research Center Jeon, Byong-Tae ; Konkuk University, Nokyong Research Center Park, P.-J.; Konkuk University, Biotechnology

Characterization of antioxidant fraction of *Trapa japonica* pericarp and its hepatic protective effects *in vitro* and *in vivo*

Yon-Suk Kim[a], Eun-Kyung Kim[a,e], Jin-Woo Hwang[a], Il-Bok Seo[b], Jae-Hyuk Jang[c], Sangkeun Son[c], Jae-Hyun Jeong[d], Sang-Ho Moon[e], Byong-Tae Jeon[e], Pyo-Jam Park[a,e*]

[a]Department of Biotechnology, Konkuk University, Chungju, 380-701, Korea

[b]Department of Anatomy, College of Oriental Medicine, Semyung University, Jecheon, 390-711, Korea

[c]Chemical Biology Research Center, Korea Research Institute of Bioscience and Biotechnology, Ochang, 363-883, Korea

[d]Department of Food Science and Technology, Korea National University of Transportation, Chungju, 380-702, Korea

[e]Nokyong Research Center, Konkuk University, Chungju, 380-701, Korea

[*]Corresponding author; Park, P. J., Tel: +82-43-840-3588; Fax: +82-43-852-3616; E-mail address: parkpj@kku.ac.kr

Abstract

Ethanolic extract of *Trapa japonica* pericarp (TJP) and its various fractions were evaluated for their antioxidant potential. The ethyl acetate fraction (EF) from TJP exhibited significant antioxidant and protective effects against *tert*-butylhydroperoxide (*t*-BHP)-induced oxidative damage *in vitro* and *in vivo*. *In vitro* experimental results showed that the EF suppressed *t*-BHP-induced damage in Chang cells by inhibiting reactive oxygen species generation and regulating the mitochondrial membrane potential. Furthermore, western blot analysis showed that the EF effectively inhibited *t*-BHP-induced apoptosis by suppressing caspase-3, caspase-7, caspase-8, and caspase-9. *In vivo* study, the EF significantly prevented serum increases in glutamate oxaloacetate transaminase and glutamate pyruvate transaminase and hepatic malondialdehyde levels caused by *t*-BHP. Furthermore, the EF markedly increased hepatic superoxide dismutase, catalase, and glutathione levels. Histopathological examinations further confirmed that the EF could protect the liver from *t*-BHP-induced oxidative injury. These findings indicate that the EF could be developed as a therapy or to prevent hepatic injury.

Keywords : *Trapa japonica*, *tert*-butylhydroperoxide, 3,9-dihydroxy-dibenzo[b,d]pyran-6-one, reactive oxygen species

Introduction

Oxidative stress is defined as an imbalance between antioxidants and free radicals, which can potentially lead to cellular damage.[1] Oxidative stress is induced by reactive oxygen species (ROS), such as superoxide anions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$).[2-3] Cellular ROS play important roles in regulating various physiological functions, and they represent an essential part of aerobic metabolism.[4] Excessive ROS generation disrupts the cellular antioxidant defense system and may lead to oxidative stress.[5] It has been reported that the deleterious effects of ROS on human cells may result in oxidative stress-induced programmed cell death or apoptosis.[6] Antioxidants are capable of preventing oxidative damage. Natural antioxidants have been widely used as replacements for conventional synthetic antioxidants in food and food supplements because natural products are considered safer and effective.[7]

In Asia, the fruit of *T. japonica* has been eaten by steaming and boiling. The pericarp of *T. japonica* is considered to be the waste and generally discarded. However, the husk of water chestnut contains many dietary fibers[8] and phenolic compounds such as eugeniin, 1,2,3,6-tetra-O-galloyl-beta-D-glucopyranose (TGG), trapain, ellagic acid, eugeniin and gallic acid. [9-10] *tert*-Butylhydroperoxide (*t*-BHP)-induced oxidative stress and hepatotoxicity are associated with an imbalance in the oxidant/antioxidant system of the liver.[11-12]

The aim of the present study was to investigate the hepatoprotective and antioxidant activities of *Trapa japonica* pericarp-derived fractions against *t*-BHP-induced oxidative damage *in vitro* and *in vivo*. To confirm *t*-BHP-induced apoptosis and to study the anti-apoptotic effect of the ethyl acetate fraction (EF) of *T. japonica* pericarp, we performed immunoblot analyses of Bax, Bcl-2, caspase-3, caspase-7, caspase-8, and caspase-9. Additionally, we confirmed the protective effect of the EF on lipid peroxidation, glutathione content, and antioxidant enzymes in male imprinting control region (ICR) mice that have sustained *t*-BHP-induced acute oxidative damage.

Materials and Methods

Materials

Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin were purchased from Hyclone (Logan, Utah, USA). *N*-Acetyl-L-cysteine (NAC), silymarin, *t*-BHP, 2′,7-dichlorodihydrofluorescein diacetate (DCFH-DA), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), and rhodamine 123 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hoechst 33342 was purchased from Invitrogen. All other reagents were of the highest grade available commercially. Dried *T. japonica* was obtained from Jecheon herbal medicine mall (Jecheon,

Korea) in January 2014. The sample was authenticated by Prof. K.H. Leem (Department of Herbology, Semyung University) and voucher specimen (KUB13-20) was deposited in the Konkuk University Herbarium. The pericarp collected from *T. japonica* was ground into a fine powder for ease of extraction.

Preparation of *T. japonica* pericarp fractionation

Ethanol extraction of the dried *T. japonica* pericarp (TJP; 6 kg) was carried out in duplicate using 70% ethanol under reflux for 1 day. The total filtrate was concentrated under reduced pressure to produce the ethanolic extract (257.9 g, 4.29% yield). Ethanolic extract (100 g) was suspended in water and partitioned with hexane (2 L, thrice), chloroform (2 L, thrice), ethyl acetate (2 L, thrice), *n*-butanol (2 L, thrice), and residual aqueous fraction successively, to remove solvent and produce hexane (HF, 4.117 g, 4.11% yield), chloroform (CF, 2.146 g, 2.14% yield), ethyl acetate (EF, 49.653 g, 49.65% yield), *n*-butanol (BF, 19.95 g, 19.95% yield), and aqueous (AF, 9.685 g, 9.68% yield) fractions. Each fraction was collected, dried, and stored at 4°C.

Isolation and characterization of compounds from EF

The Inspire C18 column (250 mm × 21.2 mm, 10 μm) was operated in the HPLC (ultimate 3000,

Thermo Scientific) system using an injection volume of 1.0 mL at a flow rate of 5 mL/min and a detection wavelength of 254 nm. The mobile phases were 0.1% acetic acid in water (A) and methanol (B), and the gradient condition were 0-55 min, 0-100 B%, and 55-60 min, 100 B%. We isolated 4 fractions (labeled as EF1-EF4) from the EF according to their respective peaks.

Liquid chromatography-mass spectrometry (LC-MS) was performed using an LTQ XL linear ion trap (Thermo Scientific, USA) equipped with an electrospray ionization (ESI) source that was coupled to a rapid separation LC (RSLC; ultimate 3000, Thermo Scientific) system (ESI-LC-MS) using an HSS T3 column (Waters, UK) (2.1 × 150 mm; 2.5 μm particle size). The linear gradient of the binary solvent system consisted of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile) at a flow rate of 0.3 mL/min. A linear gradient was initiated with 5% B and linearly increased to 100% at 0-15 min. The ESI (negative ion) parameters for the EF4 were: source voltage (+5 kV), entrance capillary voltage (+18 V), entrance capillary temperature (275ºC), and tube lens voltage (+120 V). The scan range was fixed from m/z 50 to 1500. The data-dependent mass spectrometry experiments were controlled using the menu driven software provided with the Xcalibur system (version 2.2 SP1.48; Thermo Scientific).

***In vitro* study**

DPPH radical scavenging activity

The DPPH radical scavenging activity was measured using an ESR spectrometer (JES-FA machine; JOEL, Tokyo, Japan) according to the technique described by Kim et al.[13] Sixty microliters of each sample (or ethanol itself as control) was added to 60 μL of DPPH (60 μM) in ethanol. After 10 s of vigorous mixing, the solutions were transferred to Teflon capillary tubes and fitted into the cavity of the ESR spectrometer. The spin adducts were determined by the ESR spectrometer exactly 2 min later under the following measurement conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2G, microwave power 5 mW, gain 6.3×10^5, and temperature 298 K.

ABTS radical scavenging activity

For ABTS assay, the procedure followed the method of Erkan et al.[14] with some modifications. The stock solutions included 7.4 mM ABTS cation solution and 2.6 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 14 h at R.T in the dark. The mixture was diluted that its absorbance was adjusted to 1.5 ± 0.02 at 734 nm. To determine the scavenging activity, 0.9 mL of ABTS cation solution was mixed with 0.1 mL of extracts and the absorbance was measured at 734 nm after 6 min of reaction at R.T, using ethanol as a control. The antioxidant activities of samples were expressed by Trolox equivalents antioxidant capacity (TEAC), as mM Trolox

109 eq./mg extract.

110

111 **Ferric reducing antioxidant power (FRAP) assay**

112 FRAP assay was carried out by the method of Benzie and Strain [15] with minor modification. The
113 method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to
114 the ferrous form (Fe^{2+}-TPTZ). To conduct the assay, a 3 mL aliquot of a FRAP reagent, a
115 mixture of 0.3 M acetate buffer, 10 mM TPTZ in 40 mM HCl, and 20 mM ferric chloride
116 (10:1:1 v/v/v), were combined with 1 mL of samples. To determine the antioxidant capacity of
117 the samples, the absorbance values were compared with those obtained from the standard curves
118 of $FeSO_4$ (0-5 mM). The antioxidant capacity values were expressed as mM $FeSO_4$ equivalents
119 in mg extract (mM $FeSO_4$ eq./mg extract).

120

121 **Cell culture**

122 Chang liver cells purchased from American Type Culture Collection (ATCC CCL-13^{TM}) were cultured at
123 37°C in humidified 5% CO_2, 95% air mixture in DMEM supplemented with heat-inactivated 10%
124 FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Adherent cells were detached by trypsin-
125 ethylenediaminetetracetic acid (EDTA) and plated onto 6-, 48-, or 96-well plates at 70-80% confluence.

126

MTT assay

The cell viability was estimated by MTT assay. Chang liver cells were seeded in 48-well plates at a concentration of 7.0×10^3 cells/well. After 20 h, the cells were treated with various concentrations of samples and incubated in a humidified incubator at 37°C for 1 h. Then, *t*-BHP was added to a final concentration of 100 μM and incubated for 24 h. Thereafter, 100 μL of MTT stock solution (0.5 mg/mL) was added and incubated for 4 h. Then, the supernatants were aspirated, and the formazan crystals in each well were dissolved in 150 μL of dimethyl sulfoxide (DMSO). Absorbance was measured by microplate reader (SpectraMax M2/M2e, Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 540 nm. Relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The optical density of the formazan formed in the control cells was considered as 100% viability.

Lactate dehydrogenase (LDH) release assay

The activity of LDH released into the culture medium through damaged membranes was measured using the LDH cytotoxicity assay kit (BioVision, CA, USA). Briefly, Chang liver cells were seeded in 96-well plates at a density of 4.0×10^3 cells/well. After 20 h, the cells were treated with various concentrations of the EF and incubated in a humidified incubator at 37°C for 1 h. Then, *t*-BHP was added to a final concentration of 100 μM and incubated for 24 h. After

incubation, 10 μL of supernatant was added to 100 μL of LDH reaction mix and incubated for 30 min. Absorbance was measured at 450 nm using a microplate reader.

Cell cycle analysis by flow cytometry

Cells were plated in 6-well plates (2×10^4 cells/well) with 2 mL of culture medium for 24 h. They were pretreated with the EF for 1 h before exposure to 100 μM *t*-BHP for 24 h. For flow cytometry cell cycle analysis, cells were harvested and washed twice with PBS buffer (pH 7.4). After fixing in 80% ethanol overnight, cells were washed twice and resuspended in PBS buffer containing 50 μg/mL PI and 5 μg/mL ribonuclease A for DNA staining. Cells were then analyzed by flow cytometry (FACSCalibur, Becton & Dickinson Co., Franklin Lakes, NJ, USA). At least 10,000 events were evaluated.

Reactive oxygen species (ROS) measurement

Intracellular formation of ROS was assessed as described previously using the oxidation sensitive dye DCFH-DA.[16] Chang liver cells were seeded in 6-well plates (2.0×10^4 cells/well). Cells were pretreated with the EF and incubated for 1 h, followed by 100 μM *t*-BHP treatment for 24 h. Next, cells were incubated with DCFH-DA (5 μg/mL) for 30 min at 37 ºC in the dark. Non-fluorescent cell permeable DCFH-DA dye freely penetrates into cells and is hydrolyzed by

intracellular esterase to 2'7-dichlorofluorescin (DCFH), which is trapped inside the cell. The formation of 2'7-dichlorofluorescin (DCF) due to the oxidation of DCFH in the presence of ROS was analyzed by flow cytometry.

Measurement of mitochondrial membrane potential (MMP)

Mitochondrial membrane potential was monitored by the fluorescent dye rhodamine 123, which is a cell permeable cationic dye that preferentially enters the mitochondria with highly negative membrane potentials.[17] Depolarization of MMP results in the loss of rhodamine 123 from the mitochondria and a decrease in the intracellular fluorescence intensity. After 24 h treatment with *t*-BHP and pretreatment with the EF for 1 h, rhodamine 123 (10 μM) was added for 30 min at 37°C in the dark. Cells were harvested, washed with PBS, and the MMP was measured by flow cytometry.

Apoptosis assay

Cell apoptosis was measured by Annexin V/PI staining. Briefly, after treatment, cells were washed twice with PBS and incubated in 100 μL of binding buffer containing 5 μL of Annexin V-FITC and 5 μL of PI in the dark for 15 min at room temperature. The stained samples were added to 400 μL of binding buffer and then analyzed by a FACSCalibur flow cytometer and

quantified using Cell Quest software.

Western blot analysis

Total cell extracts were prepared using PRO-PREP protein extraction solution (iNtRON Biotechnology). Briefly, after treatment with the various concentrations of the EF, cells were harvested, washed once with PBS, and gently lysed for 30 min in 100 μL of ice-cold PRO-PREP lysis buffer. Lysates were centrifuged at 13,000 ×g at 4°C for 10 min. Supernatants were collected, and protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Samples were stored at -80°C unless immediately used for western blot analysis. Proteins were separated on a sodium dodecyl sulfate (SDS)-polyacrylamide gel, transferred onto polyvinylidene fluoride membranes (PVDF), blocked with 5% skim milk, and incubated at 4°C overnight with primary antibodies. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies. Chemiluminescence was detected using western ECL substrate, and membranes were subjected to direct densitometry analysis. Images were captured using a charge-coupled device camera system (LAS-3000; Fujifilm, Tokyo, Japan). Band intensity was normalized with respect to β-actin levels as an internal control.

In vivo study

Animals

Male, 6-week-old ICR mice were purchased from OrientBio Co. (Sungnam, Korea) and were housed with free access to a standard rodent diet (Samyang Feed Co., Ltd., Incheon, Korea) and tap water. Mice were housed in humidity- (55 ± 5%) and temperature-controlled (23 ± 1ºC) rooms with a 12-h/12-h light/dark cycle for at least 1 week before experimentation. The license number for using experimental animals was KU14102. All animal care and studies were approved by the Institutional Animal Care and Use Committee of Konkuk University in accordance with the principles and guidelines of the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals.

t-BHP-induced oxidative toxicity

The mice were divided into five groups (n=5). Group I was the control, and they received oral doses of vehicle for 5 days. Group II was the toxic group, which received oral doses of vehicle for 5 days. Group III received the EF at 50 mg/kg for 5 days, Group IV received the EF at 100 mg/kg for 5 days, and Group V received silymarin at 100 mg/kg for 5 days. On the fifth day, all the animal groups except Group I were injected with 2 mmol/kg *t*-BHP intraperitoneally, and 18 h later, the mice were euthanized. Blood samples were collected from the heart for the glutamate

oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) assays. After blood collection, the livers were removed, rinsed with PBS, and stored at -80 ºC for biochemical analysis.

Preparation of liver homogenates

Following euthanasia, the liver of each mouse was removed and frozen immediately in dry ice. The frozen tissues were stored at -80 ºC until further use. Liver homogenates were prepared with 50 mM cold potassium phosphate buffer (pH 7.4). The resulting suspension was centrifuged at 12,000 ×g for 15 min, and the supernatant was collected for further analysis. The cell-free supernatant was used to measure enzyme activity and lipid peroxidation.

Determination of antioxidant enzyme activity and lipid peroxidation levels

The activity of superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) was determined using commercial reagent kits obtained from Cayman Chemical Company (Ann Arbor, MI, USA), according to the instruction manuals. Malondialdehyde (MDA), an end product of lipid peroxidation, was estimated in liver homogenates. The assay involves a reaction between MDA and thiobarbituric acid (TBA), which produces a pink colored MDA-TBA complex.[18] Sample (100 μL) and 200 μL of 1.0 M phosphoric acid were incubated for 15 min on

ice. After centrifugation, 200 μL of supernatant and 200 μL of 0.67% TBA standard solution were mixed. The tubes were kept on a boiling water bath for 10 min and then cooled under tap water. Supernatant absorbance was read at 532 nm. The standard used for plotting the calibration curve was 1,1,3,3-tetramethoxypropane. The results were expressed as nmol/mg protein.

Trolox equivalent antioxidant capacity (TEAC) assay

Seven millimolar 2,2´-azinobis-(3-ethyl-benzothiazoline-6-sulfonate) (ABTS) was reacted with 2.45 mM potassium persulfate solution overnight in the dark to generate ABTS cation radical. Later, ABTS cation solution was diluted with ethanol to an absorbance of 0.7 ± 0.05 at 734 nm. Serum (20 μL) was mixed with 1.0 mL of diluted ABTS cation solution. Absorbance was measured 6 min after the initial mixing at 734 nm. TEAC was expressed as μM Trolox equivalent/mg protein.

Statistical analysis

Data were assessed in triplicate and reported as mean ± standard deviation. Analysis of variance (ANOVA) and the Dunnett's test (GraphPad Prism 5) were used to identify significant differences between samples ($p < 0.05$).

253

254 **Results**

255 **DPPH radical scavenging activity**

256 The TJP extract and its fractions elicited high DPPH radical scavenging activity. IC_{50} values of
257 DPPH radical scavenging activity of EE and its fractions (HF, CF, EF, BF, AF) were 1.69 ±
258 0.01, 3.62 ± 0.02, 25.85 ± 0.25, 1.21 ± 0.01 0.01, 1.07 ± 0.01 and 3.28 ± 0.21 μg/mL,
259 respectively (Table 2). Especially, the ethanolic extract and its ethyl acetate and *n*-butanol
260 fraction demonstrated significant DPPH radical scavenging activity compared to the positive
261 control; vitamin C (IC_{50}, 3.11 ± 0.20 μg/mL).

262

263 **ABTS radical scavenging activity**

264 The antioxidant activity in TJP extract and its fractions were determined as Trolox equivalents
265 using the ABTS assay. TJP extract and its fractions were observed to have significant
266 antioxidant action. As depicted in Table 2, the TEAC value of the EF and BF exhibited a similar
267 TEAC value (1.523 ± 0.001, 1.525 ± 0.002 mM Trolox eq./mg extract) as compared to that of
268 vitamin C (1.528 ± 0.001 mM Trolox eq./mg extract).

269

270 **FRAP (ferric reducing antioxidant power) assay**

271 The FRAP assay has been commonly used for rapid evaluation of the total antioxidant capacity
272 of various potential antioxidants. The ferric reducing antioxidant power of TJP extract and its
273 fraction is shown in Table 2. The results displayed that EE (6.439 ± 0.200 mM $FeSO_4$ eq./mg
274 extract), EF (6.465 ± 0.173 mM $FeSO_4$ eq./mg extract) and BF (6.512 ± 0.207 mM $FeSO_4$
275 eq./mg extract) have a similar FRAP antioxidant activity as compared to vitamin C (6.779 ±
276 0.191 mM $FeSO_4$ eq./mg extract) used as a positive control. These values suggest that the
277 ethanolic extract of TJP, fraction of ethyl acetate and *n*-butanol had a strong ferric reducing
278 antioxidant power.
279
280 **Cell viability**
281 The cytotoxic and protective effects of the TJP ethanolic extract and its fractions on *t*-BHP-
282 induced oxidative stress in Chang cells were determined by MTT and LDH assays. In the MTT
283 assay, cell viability increased following pretreatment with the ethanolic extract of TJP as well as
284 its fractions, including HF, CF, EF, BF, and AF at 20 μg/mL, compared to cells treated with *t*-
285 BHP alone [50.15 ± 6.85% cell viability; Figure 2(A)]. The cell viability of the ethanolic
286 extracts from TJP and its fractions HF, CF, EF, BF, and AF in the presence of *t*-BHP was 73.56 ±
287 1.54%, 72.24 ± 0.88%, 46.12 ± 6.03%, 75.73 ± 0.93%, 65.81 ± 2.55%, and 52.24 ± 3.67%,
288 respectively. From the experiment dealing with antioxidant activity, it was evident that BF

289 demonstrated highest activity than other fractions and was then followed by EF for antioxidant
290 activity. However, for further experiments, EF was chosen over BF since the former had higher
291 protective effect. As shown in Figure 2(B), pretreatment with the EF dose-dependently
292 alleviated the *t*-BHP-induced reduction in cell viability. The EF showed a stronger protective
293 effect than the positive control, silymarin. Furthermore, *t*-BHP-treatment significantly increased
294 LDH release (3.8 ± 0.1-fold) into the culture medium, whereas the EF pretreatment (5-20
295 μg/mL) significantly diminished this release in a dose-dependent manner Figure 2(C). These
296 results clearly indicate that the EF confers a significant protective effect against *t*-BHP in Chang
297 cells.
298
299 **Identification of active compounds from EF**
300 Isolated EF was analyzed by LC-MS. A total of 16 different organic compounds were identified
301 in the EF based on LC retention time. The 16 chemical constituents (Table 1, Figure 1) are as
302 follows: gallic acid, digalloyl-glucoside, trigalloyl-glucoside, eugeniin, tetrahalloyl-glucoside,
303 and ellagic acid. In this study, five digalloyl-glucose and seven trigalloyl-glucoside were
304 detected in the EF from TJP. From the EF (100 mg), along with 16 known compounds, we have
305 discovered another bioactive ingredient by repeated column chromatography. We have named
306 this as compound 17 (3,9-dihydroxy-dibenzo[b,d]pyran-6-one) with a content of 1 mg. Shirataki

and Toda have reported the isolation of 3,9-dihydroxy-dibenzo[b,d]pyran-6-one from *Trapa natans* fruits, and it is believed to act as an antioxidant and inhibit lipid peroxidation.[19] Its structure was elucidated based on MS and various NMR spectroscopic data. Compound 17, the molecular formula: $C_{13}H_8O_4$, positive ESI-MS *m/z*: 229.1 $[M+H]^+$; ^{1}H NMR (700 MHz, CD_3OD) δ 8.02 (d, *J* = 8.8 Hz, 1H, H-1), 6.81 (dd, *J* = 8.8, 2.3 Hz, 1H, H-2), 7.23 (d, *J* = 2.2 Hz, 1H, H-4), 7.89 (d, *J* = 8.8 Hz, 1H, H-7), 6.80 (dd, *J* = 8.7, 2.4 Hz, 1H, H-8), 6.67 (d, *J* = 2.4 Hz, 1H, H-10); ^{13}C NMR (176 MHz, CD_3OD) δ 132.06 (C-1), 118.50 (C-2), 170.1 (C-3), 106.73 (C-4), 138.11 (C-5), 110.19 (C-6), 123.84 (C-7), 112.84 (C-8), 160.28 (C-9), 102.71 (C-10). 152.69 (C-11), 163.00 (C-13), 107.6 (C-14).

Cell cycle analysis

When cells were exposed to *t*-BHP, there was a distinct increase in the percentage of cells with sub-G1 DNA content (12.66%), which was considered an indicator of cell damage (Table 3). However, by pretreating cells with 5, 10, and 20 μg/mL EF, the percentage of cells in the sub-G1 phase decreased to 7.41%, 7.27%, and 5.95%, respectively. These results indicate that the EF protects cells from *t*-BHP-induced oxidative damage.

Apoptosis analysis

Apoptosis in Chang cells was tested by Annexin V/PI double staining. As shown in Figure 3A, exposure to *t*-BHP (100 μM) yielded a significant increase in Annexin V-positive cells (40.32%). However, the EF pretreatment (5, 10, and 20 μg/mL) significantly and dose-dependently reduced the number of Annexin V-positive cells to 16.86% (5 μg/mL), 14.52% (10 μg/mL), and 9.29% (20 μg/mL), respectively. These measurements indicate that the EF may protect against *t*-BHP-induced injury by inhibiting apoptosis.

Measurement of mitochondrial membrane potential (MMP)

We investigated the effect of EF on the MMP in *t*-BHP-treated Chang cells. As shown in Figure 3B, the MMP of cells incubated with *t*-BHP decreased 23.75% compared to untreated cells, indicating that *t*-BHP causes mitochondrial damage. However, the EF pretreatment significantly protected Chang cells by increasing the mitochondrial membrane potential of *t*-BHP-treated cells by 1.89% (5 μg/mL), 1.68% (10 μg/mL), 1.50% (20 μg/mL), and 3.73% (silymarin, 5 mM).

Intracellular ROS content

To confirm that the EF reduced *t*-BHP-induced oxidative stress in Chang cells, intracellular ROS production was assessed by monitoring DCFH-DA fluorescence. After *t*-BHP treatment, intracellular oxidant levels increased, but cells pretreated with the EF exhibited reduced ROS generation compared to *t*-BHP-stressed cells. After 24 h of 100 μM of *t*-BHP treatment, 72.22%

of cells were DCFH-DA fluorescent, while 28.71% (5 μg/mL), 14.88% (10 μg/mL), and 3.72% (20 μg/mL) of the EF pretreated cells were fluorescent. The positive control NAC (5 mM) exhibited a 34.22% decrease in ROS content Figure (3C). These results indicate that ROS production caused by *t*-BHP-induced oxidative damage reduces in a dose-dependent manner when Chang cells are treated with the EF. The EF was a stronger inhibitor of ROS production than NAC.

Western blot analysis

We hypothesized that the EF protects cells against *t*-BHP-induced oxidative stress by inhibiting apoptosis. Hence, we attempted to investigate the potential molecular mechanisms involved. We detected the expression of Bcl-2 (26 kDa), Bax (21 kDa), and the cleaved forms of caspase-3 (17,19 kDa), caspase-7 (18 kDa), caspase-8 (41, 43 kDa), and caspase-9 (37 kDa). We found that pretreatment with the EF (5, 10, and 20 μg/mL) can dose-dependently inhibit caspase-7, caspase-8, and caspase-9 protein expressions Figure (3D).

Pathological histology estimation

Treatment with *t*-BHP caused neutrophilic infiltration and cell death, which are the frequently and widely observed salient features on the surface of the liver, including focal necrosis,

362 inflammatory cell infiltration, and massive histological changes (Figure 4). However,
363 microscopic examination showed that the severe hepatic lesions induced by *t*-BHP were
364 markedly reduced by the EF administration (50 mg/kg and 100 mg/kg).
365
366 **GOT and GPT assay**
367 We investigated the effect of EF on *t*-BHP-induced changes on the hepatic enzymes GOT and
368 GPT. GOT and GPT are well known biomarkers for early acute hepatic damage. The *t*-BHP (2
369 mmol/kg) treated group showed a 2.2-fold increase in mice serum GOT levels and a 14.2-fold
370 increase in GPT levels, whereas pretreatment with 50 and 100 mg/kg EF significantly
371 suppressed *t*-BHP-induced acute hepatotoxicity reactions (Table 4).
372
373 **Measurement of antioxidant activity of hepatic enzymes, lipid peroxidation, and total**
374 **antioxidant activity**
375 We investigated the activity of relevant antioxidant enzymes during *t*-BHP-induced oxidative
376 stress. *t*-BHP treatment significantly decreased CAT and SOD activity compared to the control
377 group (Table 4). The EF-treated group recovered their enzyme activity, and the silymarin group
378 displayed significantly enhanced enzyme activity compared to the control group.
379 GSH is known to play a protective role against *t*-BHP-induced toxicity. While *t*-BHP

administration significantly reduced GSH levels, pretreatment with the EF significantly recovered GSH depletion by *t*-BHP. In terms of lipid peroxidation inhibition, we observed significantly lower MDA levels in the EF-treated groups (50 mg/kg, 100 mg/kg) than the only *t*-BHP treated group (Table 4). In addition, the group treated with 100 mg/kg silymarin had even lower MDA levels than the untreated group. The scavenging activity of the EF on $ABTS^{+}$ radicals generated by potassium persulfate was compared to a standard amount of Trolox. We found that 50 mg/kg EF (TEAC value 0.356) and 100 mg/kg EF (TEAC value 0.372) displayed a higher radical scavenging activity than the *t*-BHP-only treated group (TEAC value 0.337; Table 4). These results reveal that the EF has strong antioxidant activity.

Discussion

In this study, three antioxidant assays (DPPH, ABTS, and FRAP) were determined to obtain more precise evaluation of antioxidant activities of TJP extract and its various fractions. As mentioned in earlier reports,[20] we also used a *t*-BHP-based model to induce oxidative stress and subsequent cellular damage to investigated the protective effects of EF.

In this study, EF and BF were observed to have powerful antioxidant activities, comparable to vitamin C. Also, we found EF has more strong protective effect against *t*-BHP-induced oxidative stress in *in vitro* and *in vivo* studies. Interestingly, we also discovered a compound

known as 3,9-dihydroxy-dibenzo[b,d]pyran-6-one. This compound was also reported by Shirataki and colleagues and was obtained from *Trapa natans* fruit. This is of interest since, 3,9-dihydroxy-dibenzo[b,d]pyran-6-one is believed to act as an antioxidant and inhibit lipid peroxidation. [19]

The EF increased cell survival, as measured by the cellular metabolic activity (MTT assay) and plasma membrane integrity (LDH assay). We found that the EF protects against *t*-BHP-induced oxidative stress by measuring oxidative stress markers, such as the intracellular accumulation of ROS. In our results, the EF showed significant cytoprotective and antioxidant effects against *t*-BHP-induced oxidative stress through the inhibition of ROS production and mitochondrial damage. In many cases, ROS is an important component of cell death.[21-22] Thus, we hypothesized that the EF's protective effect might be mediated by inhibition of ROS-mediated apoptosis. To assess this possibility in Chang cells, cell cycle analysis was performed by flow cytometry. We found that *t*-BHP caused Chang cells to accumulate in sub-G1, but the EF pretreatment dose-dependently reduced the sub-G1 population.

Various regulatory proteins such as the Bcl-2 family have been implicated in the induction of the mitochondrial apoptotic signaling pathway.[23] We explored the signal pathway involved in the EF's protective effect against *t*-BHP-induced oxidative stress in Chang cells. We found that EF significantly reduced Bax and upregulated Bcl-2 in *t*-BHP-exposed Chang cells. In general,

apoptosis is regulated by an intrinsic mitochondria-mediated pathway and/or an extrinsic receptor-mediated pathway.[24] Both pathways are medicated by the activation of caspases, which are cysteine-aspartic proteases.[25] There are two types of apoptotic caspases, initiator caspases and effector caspases.[26] Initiator caspases (e.g., caspase-2, caspase-8, caspase-9, and caspase-10) cleave inactive pro-forms of effector caspases, thereby activating them.[27] Effector caspases (e.g., caspase-3, caspase-6, and caspase-7) in turn cleave other protein substrates within the cell to trigger the apoptotic process.[28] The EF pretreatment effectively reduced these *t*-BHP-induced intrinsic and extrinsic pro-apoptotic events. In our *in vivo* study, animals were pretreated with the EF over a 5-day period before being exposed to *t*-BHP to investigate the protective potential of the EF. Our results showed that *t*-BHP increased hepatic GOT, GPT, and MDA levels; however, pretreatment with the EF effectively decreased these phenomena. As measured by TBARS, the EF displayed significantly great inhibitory activity on lipid peroxidation. In addition, pretreatment with the EF (50 and 100 mg/kg) conferred significant protection against *t*-BHP-induced oxidative stress in male ICR mice. Based on histopathological analysis, the EF treatment showed normal morphology compared to the control group. Therefore, we consider the EF to be a good candidate material for the prevention or treatment of liver dysfunction.

Conclusion

We demonstrated that the EF increased cell viability by reducing the intracellular accumulation of ROS, which contributes to apoptosis, in Chang cells. The mechanism for the EF's protective and preventive effects is most likely mediated by the inhibition of apoptosis through the regulation of caspase signaling pathways. Mice pretreated with the EF exhibited hepatoprotective effects against liver damage caused by *t*-BHP-induced oxidative stress. Therefore, EF could be used as a source of natural antioxidants and may have potential application as a food supplement.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Figure captions

Figure 1. Chromatogram of the ethyl acetate fraction (EF) from the *Trapa japonica* pericarp obtained by an HPLC-PDA total scan. Numbers correspond to the main compounds detected and the isolated compounds (17) are reported.

Figure 2. Effects of the ethanolic extract from *Trapa japonica* pericarp and its fractions (hexane, chloroform, ethyl acetate, *n*-butanol and aqueous) in Chang cells. (A) Cell viability of the ethanolic extract from *Trapa japonica* pericarp and its fractions were measured in Chang cells. (B) The protective effects of the ethyl acetate fraction (EF) were determined by the mitochondrial tetrazolium (MTT) assay. (C) The LDH activity was measured as the LDH release from damaged membranes, as described in Section 2. Data are represented as mean ± SD and were analyzed using a one-way ANOVA followed by the Dunnett's test. $^{###}p < 0.001$ versus control, $^{***}p < 0.001$ versus *tert*-butylhydroperoxide.

Figure 3. Ethyl acetate fraction (EF) completely protects against *tert*-butylhydroperoxide (*t*-BHP)-treated cells. (A) Annexin V^+/PI^+ staining. Dot plots represent three independent experiments with similar results. (B) Mitochondrial membrane potential (MMP) measurement. (C) Intracellular reactive oxygen species (ROS) measurement. Values are expressed as the mean

± SD of three independent experiments. (D) Effect of the ethyl acetate fraction (EF) on the expression of various caspases in the apoptotic pathway in Chang cells. Western blot analysis for protein expression was represented for three independent experiments.

Figure 4. Effect of the ethyl acetate fraction (EF) on *tert*-butylhydroperoxide (*t*-BHP)-induced liver damage. Mice were pretreated with EF and silymarin once daily for five consecutive days. After the final treatment, mice were treated with *t*-BHP (2 mmol/kg, i.p.). (A, a) Control group (n = 5), (B, b) animals treated with *t*-BHP showed spotty liver cell death with neutrophilic infiltrates, (C, c) animals pretreated with 50 mg/kg EF and then *t*-BHP, (D, d) animals pretreated with 100 mg/kg EF and then *t*-BHP, (E, e) and animals pretreated with 100 mg/kg silymarin and then *t*-BHP. Hematoxylin & eosin staining; magnification 200×.

522 **Table 1. Characterization of compounds in EF by LC-MS**

Peak	R_t (min)	λ_{max} (nm)	[M-H]$^-$	Fragment (% intensity)	Assignment	Reference
1	2.39	215, 272	169	125 (100%), 91 (13%)	Gallic acid	Shindo *et al*. (2013)
2	2.82	211, 277	483	113 (9%), 91 (100%)	Digalloyl-glucoside	Shindo *et al*. (2013)
3	3.02	209, 278	483	91 (100%)	Digalloyl-glucoside	Shindo *et al*. (2013)
4	3.34	214, 276	483	113 (7%), 91 (100%)	Digalloyl-glucoside	Shindo *et al*. (2013)
5	3.65	208, 274	483	113 (7%), 91 (100%)	Digalloyl-glucoside	Shindo *et al*. (2013)
6	4.01	215, 276	483	113 (6%), 91 (100%)	Digalloyl-glucoside	Shindo *et al*. (2013)
7	4.37	216, 280	635	321 (13%), 91 (100%)	Trigalloyl-glucoside	Shindo *et al*. (2013)
8	4.90	215, 277	635	91 (100%)	Trigalloyl-glucoside	Shindo *et al*. (2013)
9	5.01	216, 275	635	392 (12%), 91 (100%)	Trigalloyl-glucoside	Shindo *et al*. (2013)
10	5.19	222, 278	635	465 (21%), 317 (17%), 240 (18%), 91 (100%)	Trigalloyl-glucoside	Shindo *et al*. (2013)
11	5.43	218, 277	635	317 (11%), 91 (100%)	Trigalloyl-glucoside	Shindo *et al*. (2013)
12	5.59	216, 277	635	91 (100%)	Trigalloyl-glucoside	Shindo *et al*. (2013)
13	5.86	216, 278	635	393 (31%), 317 (13%), 91 (100%)	Trigalloyl-glucoside	Shindo *et al*. (2013)
14	6.28	219, 277	937	787 (16%), 491 (16%), 479 (12%), 469 (10%), 468 (100%), 91 (30%)	Eugeniin	Shindo *et al*. (2013) Yasuda *et al*.(2014)
15	6.52	224, 230, 273, 298	787	393 (57%), 91 (100%)	Tetrahalloyl-glucoside	Shindo *et al*. (2013) Yasuda *et al*.(2014)
16	6.94	254, 366	301	113 (6%), 91 (100%)	Ellagic acid	Wang *et al*. (2011)

523

524

525 **Table 2. DPPH radical scavenging activity, values for ABTS radical scavenging and FRAP**
526 **of ethanolic extract from *Trapa japonica* pericarp and its fractions (hexane, chloroform,**
527 **ethyl acetate, *n*-butanol and aqueous)**

Sample	DPPH radical scavenging activity (IC_{50}, μg/mL)[a]	TEAC (mM Trolox eq./mg extract)[a]	FRAP (mM $FeSO_4$ eq./mg extract)[a]
EE	1.69 ± 0.01	1.521 ± 0.001	6.439 ± 0.200
HF	3.62 ± 0.02	1.268 ± 0.002	2.419 ± 0.089
CF	25.85 ± 0.25	1.053 ± 0.080	2.086 ± 0.023
EF	1.21 ± 0.01	1.523 ± 0.001	6.465 ± 0.173
BF	1.07 ± 0.01	1.525 ± 0.002	6.512 ± 0.207
AF	3.28 ± 0.21	1.519 ± 0.001	5.480 ± 0.115
Vitamin C	3.11 ± 0.20	1.528 ± 0.001	6.779 ± 0.191

528 TEAC (Trolox equivalent antioxidant capacity), FRAP (ferric reducing antioxidant power).
529 [a] Values represent means ± SD (n = 3).
530

531 **Table 3. Cell cycle analysis (%)**

Group	Sub-G1 phase	G1 phase	S phase	G2/M phase
Control	3.35 ± 0.21	43.71 ± 3.12	7.83 ± 1.26	34.86 ± 2.36
t-BHP (100 μM)	12.66 ± 2.23$^{###}$	25.71 ± 2.28	13.43 ± 2.13	38.90 ± 2.52
t-BHP + EF (5 μg/mL)	7.41 ± 1.01***	24.57 ± 2.13	8.65 ± 1.25	50.02 ± 4.21
t-BHP + EF (10 μg/mL)	7.27 ± 1.25***	27.5 ± 2.26	8.67 ± 2.32	44.36 ± 2.36
t-BHP + EF (20 μg/mL)	5.95 ± 0.78***	42.73 ± 3.26	12.68 ± 2.14	31.29 ± 2.54
t-BHP + silymarin (50 μg/mL)	6.37 ± 1.05***	23.37 ± 2.52	16.34 ± 2.42	47.85 ± 2.58

532 $^{###}p < 0.001$ versus control, $^{***}p < 0.001$ versus *tert*-butylhydroperoxide (*t*-BHP).

Table 4. Measurement of serum GOT, GPT, antioxidant activity of hepatic enzymes, lipid peroxidation and antioxidant activity

Group	GOT[a]	GPT[b]	SOD[c]	CAT[d]	Total GSH[e]	TBARS value[f]	TEAC[g]
Group I	36.75 ± 7.07	3.28 ± 1.07	26.08 ± 1.51	59.68 ± 8.46	21.69 ± 3.75	69.45 ± 15.64	0.368 ± 0.054
Group II	82.97 ± 3.39$^{\#}$	46.61 ± 4.53$^{\#}$	24.70 ± 2.09	42.10 ± 8.57$^{\#}$	17.65 ± 2.01$^{\#}$	97.52 ± 28.74$^{\#}$	0.337 ± 0.020$^{\#}$
Group III	74.53 ± 4.46*	40.50 ± 3.16*	25.56 ± 1.28	57.36 ± 5.52*	18.95 ± 3.13	80.05 ± 19.54*	0.356 ± 0.032*
Group IV	65.53 ± 3.54*	37.01 ± 5.20*	26.35 ± 1.71	57.64 ± 7.44*	19.82 ± 2.77*	75.22 ± 30.40*	0.372 ± 0.047*
Group V	64.64 ± 4.05*	34.07 ± 3.31*	26.73 ± 0.98	52.79 ± 9.49*	18.33 ± 1.38	65.63 ± 11.87*	0.355 ± 0.033*

Group I: Control group, Group II: animals treated with *tert*-butylhydroperoxide (*t*-BHP), Group III: animals pretreated with 50 mg/kg the ethyl acetate fraction (EF) and then *t*-BHP, Group IV: animals pretreated with 100 mg/kg EF and then *t*-BHP, Group V: animals pretreated with 100 mg/kg silymarin, and then *t*-BHP. [a]Glutamate oxaloacetate transaminase (GOT) levels were expressed as IU/L. [b]Glutamate pyruvate transaminase (GPT) levels were expressed as IU/L. [c]Superoxide dismutase activities were expressed as U/mL. [d]Catalase activities were expressed as nmol/min/mL. [e]Total glutathione levels were expressed as μM/mg protein. [f]Malondialdehyde levels were expressed as nmol/mg protein. [g]Trolox equivalent antioxidant capacities were expressed as mM Trolox equivalent/mg protein. Data are represented as mean ± SD (n=5) and were analyzed using a one-way ANOVA followed by the Dunnett's test. $^{\#}p < 0.05$ versus control, $^{*}p < 0.05$ versus *t*-BHP.

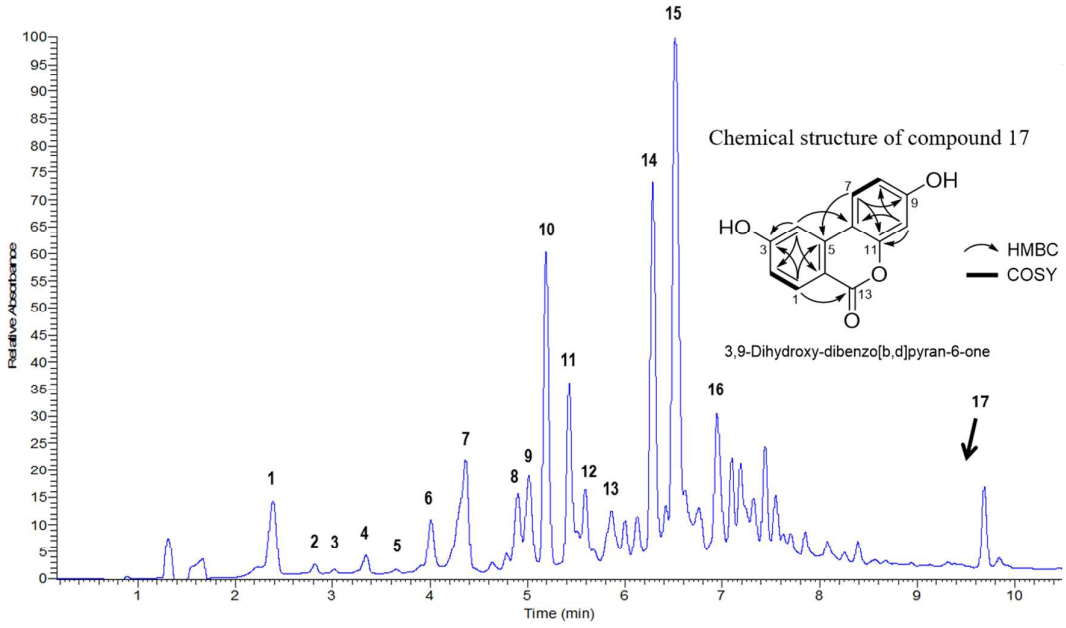
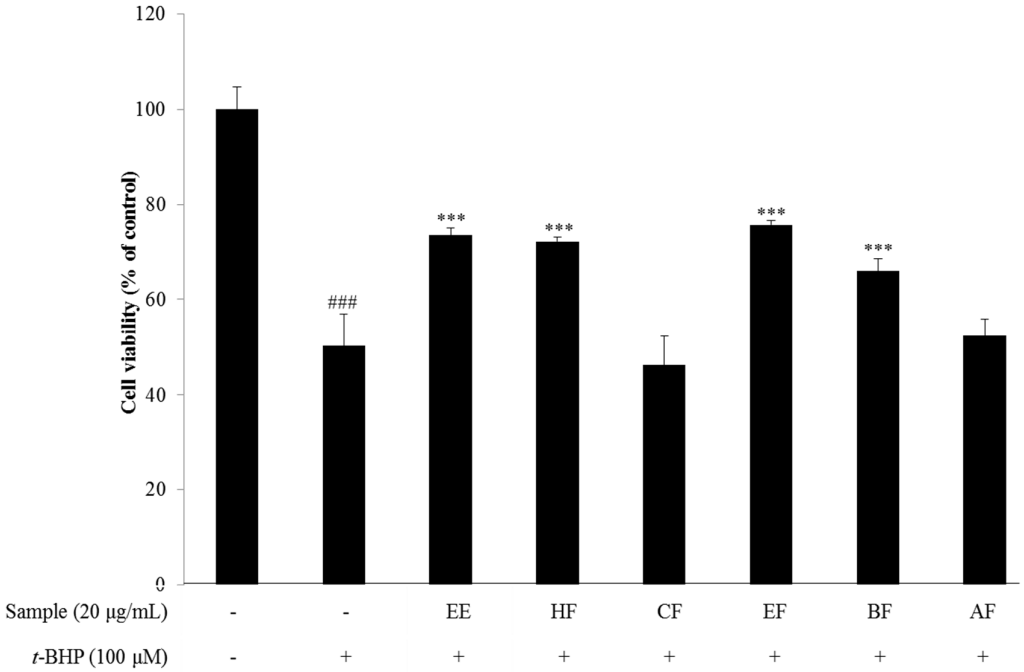


Figure 1. Kim et al



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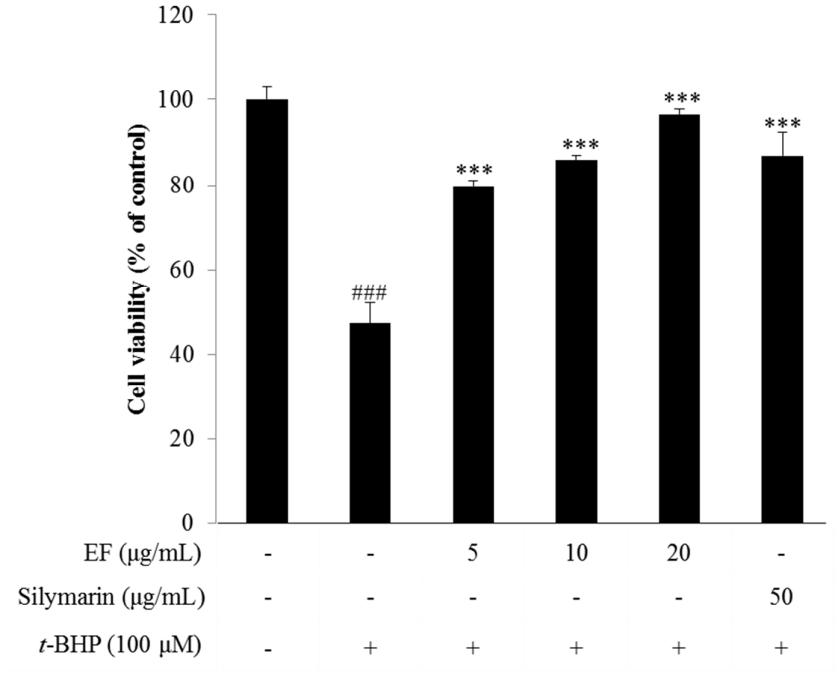

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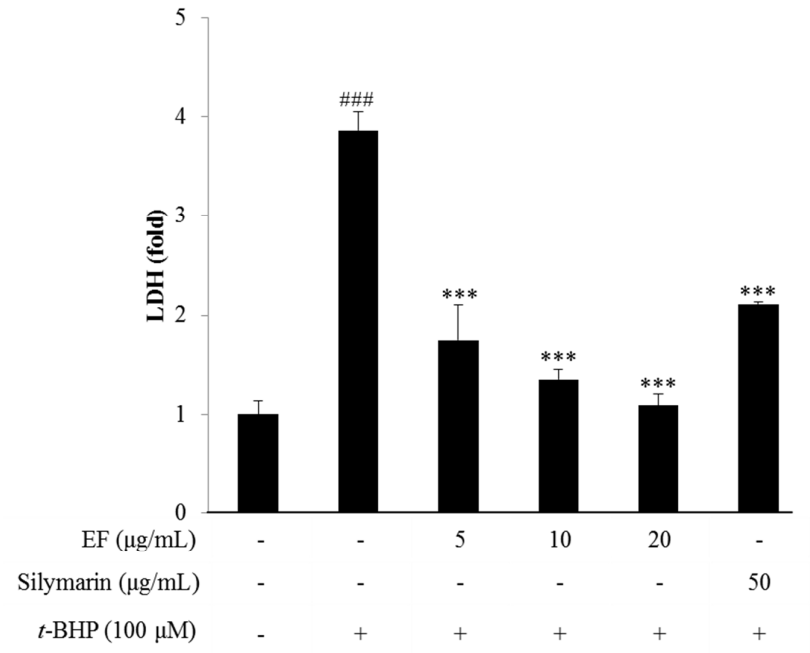

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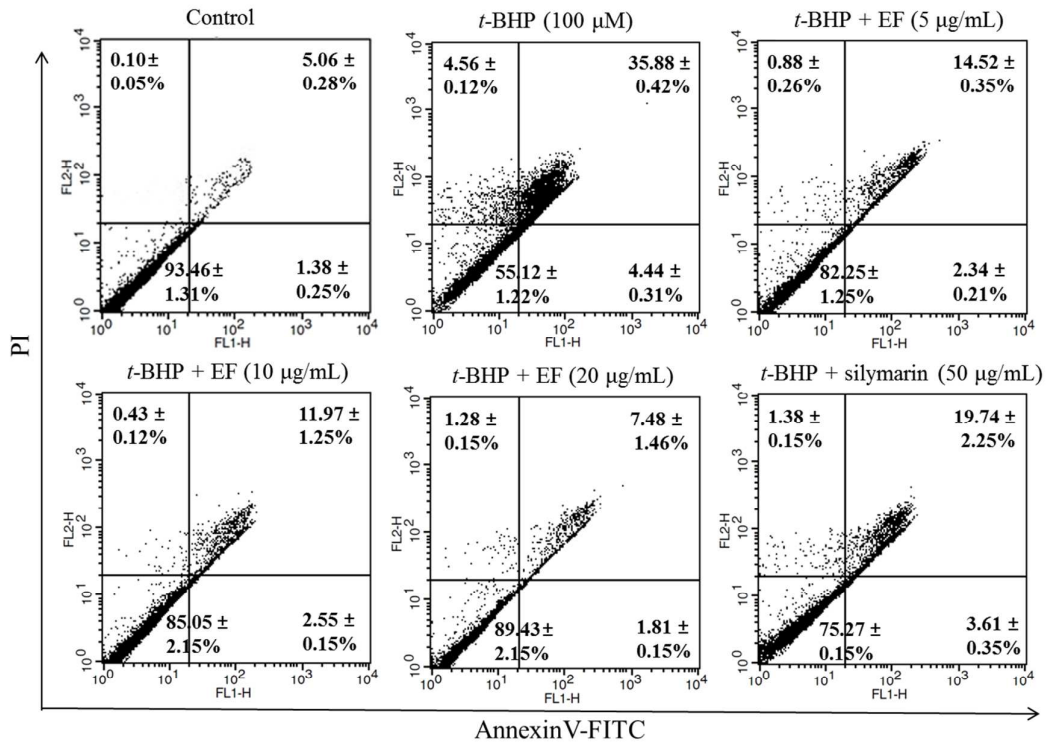

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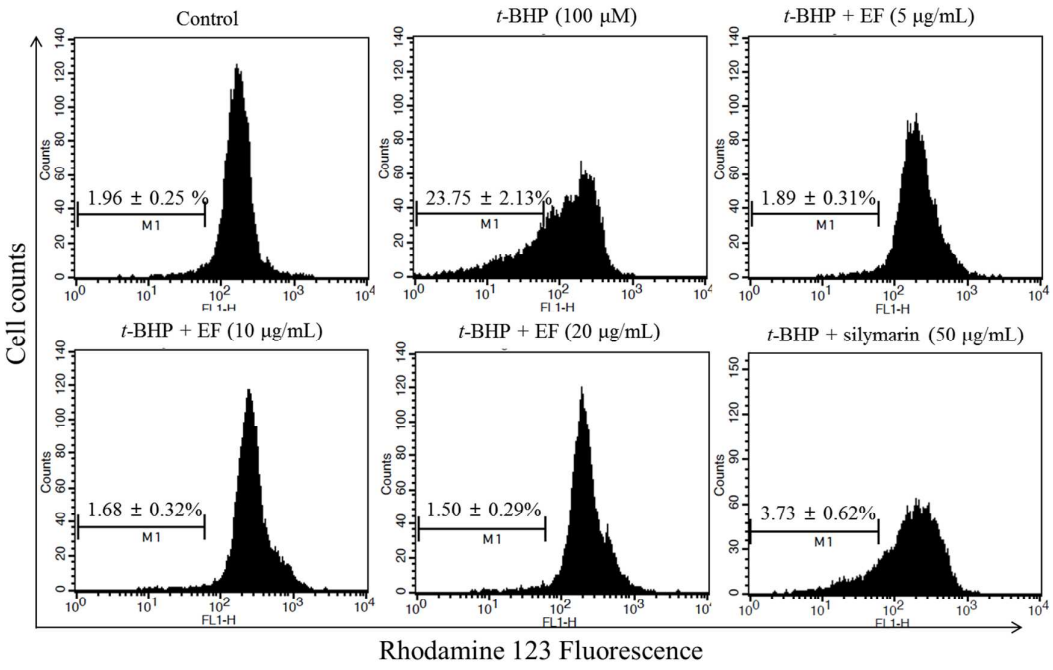

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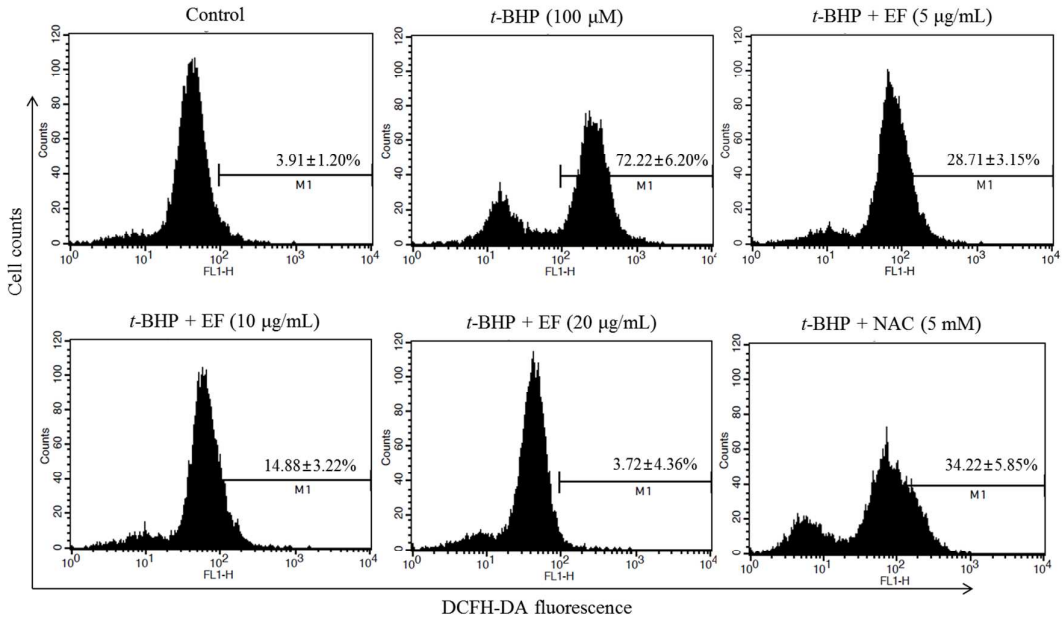

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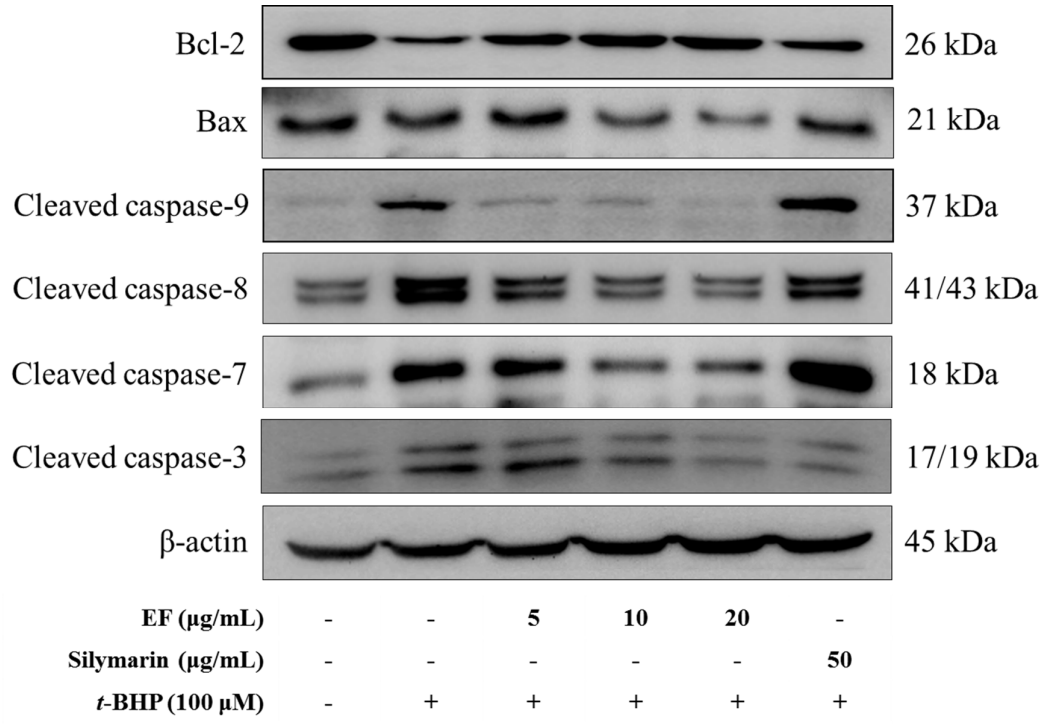

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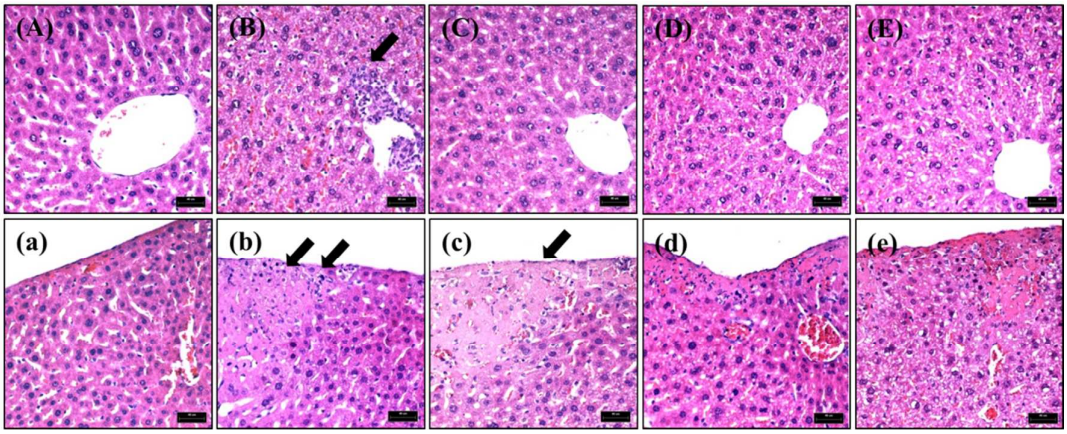

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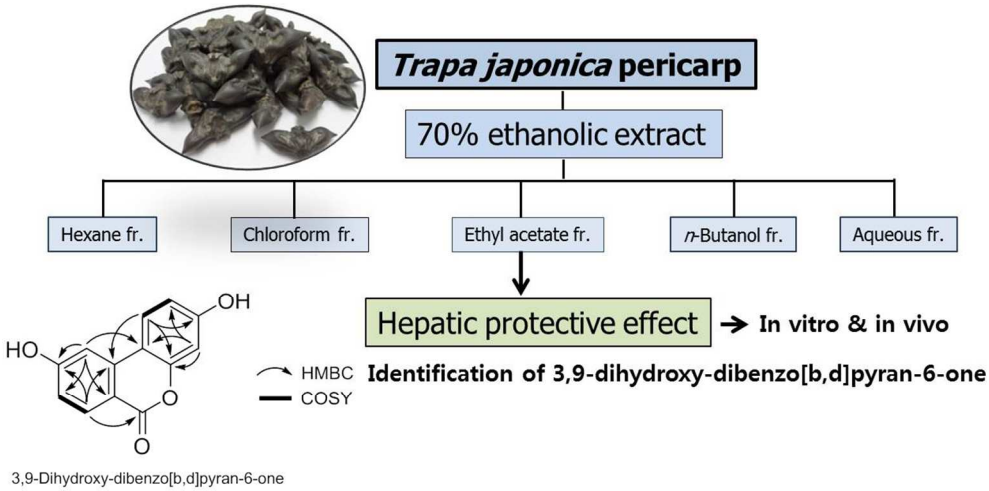

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