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Protective effect of 70% ethanolic extract of *Lindera obtusiloba* Blume on *tert*-butyl hydroperoxide-induced oxidative hepatotoxicity in rats

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ABSTRACT

Lindera obtusiloba Blume, a native plant of East Asia, has traditionally been used as a folk medicine for liver disease. We studied the *in vitro* antioxidant and *in vivo* hepatoprotective activities of a 70% ethanolic extract of *L. obtusiloba* (LOE) containing 62.9% quercitrin and 22.0% afzelin. LOE prevented *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative damage in HepG2 cells. Along with its high antioxidant potency *in vitro*, our animal study confirmed that pretreatment with LOE (500 or 2000 mg/kg) for 7 days prior to a single dose of *t*-BHP (i.p.: 0.5 mmol/kg) significantly lowered the serum levels of alanine and aspartate aminotransferases. In addition, glutathione levels were increased in the liver, and lipid peroxidation levels were decreased in a dose-dependent manner. The histopathological examinations of rat livers showed that LOE significantly reduced the incidence of liver lesions induced by *t*-BHP. Therefore, we concluded that LOE has merit as a potent candidate to protect the liver against oxidative damage.

1. Introduction

The liver is one of the largest internal organs in a living organism, and is absolutely crucial to life. It is also a silent organ, and harbors enormous regenerative capacity after injuries (Davis et al., 1998; Roskams, 2006). It plays a key role in food processing and in the process of detoxification. Alcohol consumption and cigarette smoking are the most widely known causes of liver cell damage (Pessione et al., 2001; Diehl, 1998). Malnutrition-defined by the lack of calories and protein, oxidative stress on the liver, toxic drugs or medicines, and viruses are also associated with the impairment of liver function. Furthermore, the production of oxidative stress induces reactive oxygen species (ROS). Unsaturated fatty acids rich in cell membranes are susceptible to lipid oxidation by ROS generated by cytochrome p450 metabolisms occurring in liver abundantly. The lipid oxidation products, including peroxides and aldehydes cause losses of cellular organelles, and also form covalent bonds with cellular macromolecules such as proteins resulting in liver oxidative damages. In addition, such as lipid oxidation products move to other organ tissue site of distant regions and can cause severe damage to cells or cell death (Lee et al., 2006; Wang et al., 2000). These results can induce various clinical conditions, such as liver disease, diabetes mellitus, a numbers cancer, diverse gastrointestinal tract inflammatory diseases and arteriosclerosis (Foo et al., 2011; Kim et al., 2012; Park and Oh, 2011).

tert-Butyl hydroperoxide (*t*-BHP) is often used as a model compound for hydroperoxides, which is metabolized in hepatocytes, and can be converted to toxic peroxyl and alkoxyl free radicals by cytochrome P-450 initiating lipid peroxidation, and thus leading to *t*-BHP-induced hepatic oxidative damage (Liu et al., 2002; Valentão et al., 2004; Valentová et al., 2007).

Especially, the antioxidant action of several natural substances display important roles in protecting cells against aging and cancer development by protecting lipid peroxidation induced cell damage. Our group has studied *Lindera obtusiloba* Blume as a plant candidate providing natural sources of antioxidants. *L. obtusiloba* is a native plant of Asia, has been used in traditional medicine for treatment of inflammation and for the improvement of blood circulation (Yook, 1989). The young buds and leaves of *L. obtusiloba* are traditionally taken as both food and tea. Obtusilactone derivatives (Niwa et al., 1975), flavonoid glycosides (Park et al., 1996), phytosterols (Komae and Hayashi, 1972), lignans (Kwon et al., 1999) and

Abbreviations: ALT, alkaline phosphatase; AST, aspartate aminotransferase; DM, dry matter; DPPH•, 2 2-diphenyl-1-picryl hydrazyl radical; FRAP, Ferric reducing/antioxidant power; GAE, gallic acid equivalent; GSH, reduced glutathione; LDH, lactate dehydrogenase; LOE, *Lindera obtusiloba* Blume extract; MDA, malondialdehyde; MTT, 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide; ROS, reactive oxygen species; *t*-BHP, *tert*-butyl hydroperoxide; TBARS, thiobarbituric acid reactive substances; TF, total flavonoid; TP, total polyphenol; QE, quercetin equivalent.

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butanolides (Kwon et al., 2000) have been reported as purified compounds from the stems and leaves of *L. obtusiloba*. Hong et al. (in press), Kwon et al. (2000) and Lee et al. (2010), have reported the antioxidant effect of *L. obtusiloba* extract (LOE). Despite these studies of antioxidants activity of LOE, there is unknown about the hepatoprotective properties *in vivo*.

The aim of the present investigation as we report for the first time, was to evaluate the antioxidant and hepatoprotective properties of ethanolic extract of *L. obtusiloba*, containing 62.9% quercitrin and 22.0% afzelin, against hepatotoxicity induced by *t*-BHP-induced oxidative stress *in vitro* and *in vivo*. *In vitro* of antioxidant activities were tested by total polyphenol and total flavonoid content, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, ferric reducing/antioxidant power (FRAP) and MTT assays. Also *in vivo* of hepatoprotective properties measured various biochemical parameter such as ALT, AST, MDA and GSH status, index of oxidative stress, and liver histopathology in rats.

2. Materials and methods

2.1. Materials

Minimum essential medium (MEM) was obtained from Gibco Life Technologies (Grand Island, NY, USA). *t*-BHP, 3-[4,5-dimethyl-thiazol-2-yl]-2,5-dephenyl tetrazolium bromide (MTT), reduced glutathione (GSH), thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane, DPPH, iron (II) sulfate heptahydrate, gallic acid, quercetin, streptomycin, penicillin, bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of the highest (HPLC/analytical) grade.

2.2. Preparation of 70% ehtanolic extract of LO

L. obtusiloba leaves were collected in Chunan, Chungchungnam-Do, Korea, and were identified by Dr. Kwon-Woo Park (College of Life Sciences, Korea University). Voucher specimens were deposited in the herbarium of the College of Life Sciences and Biotechnology, Korea University, register number KUST-2008. The dried *L. obtusiloba* leaves were ground with a mortar and pestle, and were then soaked three times in 70% ethanol (EtOH; 386.40 g/4000 mL) followed by refluxing for 3 h and cooling. The undissolved materials were removed by passing through a Whatman No. 41 filter paper (Clifton, NJ, USA). The solvent from the LOE was removed under reduced pressure, and the concentrate was then freeze-dried, yielding a final amount of exsiccated LOE of approximately 26.84 g.

2.3. HPLC determination of the extract

A high-pressure liquid chromatography apparatus (Varian Pro Star, Model 210; Palo Alto, CA, USA) with a diode array detector (Varian, Model 335) was used. Separation of the extract was carried out on a XTerra® C18 ODS2 column (3.96 × 150 mm; Waters, Milford, MA, USA) at room temperature. The compounds were separated with a linear solvent system of methanol in water from 20% to 100%, at flow rate of 1 mL/min with injection volume, 10 μL.

2.4. Measurement of antioxidant activities of LOE

2.4.1. Hepatocyte protective effect of LOE on cell

Hepatocyte protective effect of LOE was determined by using MTT and Thiobarbituric acid reacting substances (TBARs) assay as previously described in Lee et al. (2005) and Yang et al. (2012). The MTT assay is a quantitative count of viable cell numbers performed by determining the amount of violet formazan crystals produced by metabolic activity in treated versus control cells. The cells were seeded at a density of 1.8×10^5 cells/well in 24 well plates for 24 h, and after treatment with various concentrations of LOE and 0.7 mM *t*-BHP for 2 h at 37 °C, viable cells were stained with MTT. The media were then removed, and the formazen crystals produced in the wells were extracted by addition of 20% SDS. The absorbance was measured at 540 nm using a spectrophotometer (Biotek Instruments, Winooski, VT, USA).

The secondary products of lipid peroxidation, the major compound being malondialdehyde (MDA), are TBARs. The estimation of MDA content was performed by the thiobarbituric acid (TBA) assay method using freshly diluted 1,1,3,3-tetramethoxypropane as a standard. This method has been widely adopted as a sensitive assay for lipid peroxidation in cell.

2.4.2. Total polyphenol (TP) and total flavonoid (TF) content

TP and TF content was determined as described previously (Lee et al., 2005) using a modification of Folin–Ciocalteu reagent and 0.2% $AlCl3{\cdot}6H_2O$ solution introduced in our laboratory. TP absorbance was measured at 765 nm and TF was 367 nm. Gallic acid and quercitrin as the standard of TP and TF and result were expressing as gallic acid and quercitrin equivalents (GAE and QE) in 1 mg of extract.

2.4.3. DPPH• radical scavenging and FRAP assay

The scavenging activity of the extracts was estimated by using DPPH• as a free radical model, and a method adapted from Lee et al. (2005). Briefly, DPPH• was dissolved in ethanol to give a 100 μmol/L solution which was measured spectrophotometrically at 517 nm. The antioxidant activity was evaluated through the determination of EC_{50} values that reflect the concentration of sample required to scavenge 50% of DPPH•. FRAP assay was then conducted according to our in-house method (Lee et al., 2005). The FRAP reagent was 20 mM $FeCl_3{\cdot}6H_2O$ and determinations were carried out at 593 nm.

2.5. In vivo liver protective effects against oxidative stress by t-BHP

2.5.1. Animals and treatment

6–8 week old male Sprague–Dawley (SD) rats with a body weight of 200 ± 10 g were purchased from Samtako Bio Korea Co. (Gyeonggi-do, Korea), and allowed free access to a standard diet and tap water. Rats were housed in stainless steel cages in a room kept at 22 ± 1 °C with a 12-h light/12-h dark cycle. All animal handling was performed according to the instructions of the Committee for Ethical Usage of Experimental Animals in Korea University.

Rats were randomly allocated into 4 groups: (A) normal + saline group ($n = 5$); (B) *t*-BHP (0.5 mmol/kg) + saline group ($n = 5$); (C) *t*-BHP (0.5 mmol/kg) + 500 mg/kg LOE group ($n = 5$); (D) *t*-BHP (0.5 mmol/kg) + 2,000 mg/kg LOE group ($n = 5$). To investigate hepatoprotective activity against *t*-BHP-induced oxidative stress, LOE (500 or 2000 mg/kg) was administered to the animals daily, by gavage, for 7 consecutive days. On day 7, 0.5 mmol/kg of *t*-BHP was injected intraperitoneally (i.p.) into each animal (saline was used as substitute in the control group). 18 h later the rats were sacrificed by cervical dislocation and a blood sample was taken from the vena cava caudalis for AST and ALT assays. Immediately after blood collection, the livers were excised and rinsed in PBS. A small section of each liver was placed in 10% phosphate-buffered formalin to be used for histochemical analysis. A portion of the remaining liver was frozen in liquid nitrogen, and stored at −80 °C for biochemical analysis.

2.5.2. Hepatotoxicity assessment in serum

The hepatic enzymes, AST and ALT, were used as the markers for early acute hepatic damage. The serum activities of AST and ALT were determined by the colorimetric method (Reitman and Frankel, 1957).

2.5.3. Lipid peroxidation assay and reduced glutathione (GSH) assay of liver tissue

The intracellular GSH of the liver tissues was determined by HPLC as described by Reed et al. (1980). MDA, the lipid peroxidation product in the liver, was assayed using a thiobarbituric acid fluorometric method, with excitation at 515 nm and emission measured at 552 nm, using 1,1,3,3-tetramethoxypropane as the standard (Lee et al., 2000; Yang et al., 2012). The protein concentration was determined by the method proposed by Lowry et al. (1951); using BSA as the standard.

2.5.4. Pathological histology

Immediately after removal from the animals, the hepatic tissues were fixed in 10% buffered formaldehyde. The formaldehyde-fixed tissue samples were embedded in parafilm™, and 5 μm sections were cut, processed for histological examination according to conventional methods, and stained with hematoxylin and eosin (H&E).

2.6. Statistical analysis

The results obtained were expressed as mean ± standard deviation (SD). One-way ANOVA was used to assess significant differences among the treatment groups.

3. Results

3.1. HPLC fingerprint of the LOE

At present, there is no report on the appropriate marker and HPLC fingerprint pattern of LOE. As shown in Fig. 1, LOE had two single major peaks at 10.96 min and 11.97 min in HPLC analysis. The two main components were separated, and refined by the method described in our Korean Patent No. 10–1081601(http://kips.korea.ac.kr). Finally, LC/MS and NMR analysis confirmed that the two components were quercetin-3-*O*-α-rhamnoside