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Hepatoprotective and Antioxidative Effects of *Alisma orientale*

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Abstract – The rhizome of *Alisma orientale* Juzep (Alismataceae) has been used as a crude drug for diabetes, edema, inflammation and urinary disturbances in oriental medicine. Recent animal studies have shown that the extract of *Alisma orientale* rhizome (AOR) can potently lower high levels of serum lipids and improve insulin resistance, which are usually detected in patients and animals with non-alcoholic fatty liver disease. So, we studied the antioxidative effects of AOR extracts and fraction *in vitro* and their protective effects against acute hepatotoxicity induced by CCl_4 *in vivo*.. We then investigated the effects of each fraction on hepatotoxicity induced by *t*ert-butyl hydroperoxide (*t*-BHP). DAOR (dichloromethane fraction of the *Alisma orientale* rhizome) scavenged free radicals and superoxide anions. DAOR protected against CCl_4 induced hepatotoxicity. DAOR had hepatoprotective and antioxidative effects against *t*-BHP-induced hepatotoxicity in HepG2 cells and in rats.
Keywords – *Alisma orientale*, hepatotoxicity, alisol B acetate, antioxidative effect, CCl_4, *t*-BHP

Introduction

All organisms are exposed to reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anions (O_2^-) and hydroxyl radicals (OH^-). ROS not only act as chemical oxidants, but also, they act as subcellular messengers in such complex cellular processes as mitogenic signal transduction, gene expression, and regulation of cell proliferation. However, high levels of ROS production may lead to oxidative stress, loss of cell functions, and ultimately apoptosis or necrosis (Noh *et al.*, 2010). Clinical and experimental studies suggest that oxidative stress is involved in the pathogenesis of a high number of diseases such as inflammation, aging and cancer.

A number of pro-oxidants like *tert*-butyl hydroperoxide (*t*-BHP) have been implicated in oxidative stress and cell injury that results from intracellular production of ROS (Williams *et al*., 2000). *t*-BHP is widely used to investigate the mechanism of cell injury initiated by oxidative stress (Rush *et al.*, 1985). It can be metabolized to free radical intermediates by cytochrome P-450 in hepatocytes, which in turn can initiate lipid peroxidation, affect cell integrity and mediate DNA damage (Minott *et al.*, 1986).

Because ROS formation is a naturally occurring process, mammalian cells have developed several protective mechanisms to prevent excessive ROS formation and to detoxify ROS. These mechanisms employ chemical antioxidants as well as protective enzymes. Antioxidants, including vitamins, flavonoids and polyphenols, are an important aspect of health maintenance based on their modulation of oxidative processes in the body (Morton *et al.*, 2000).

The *Alisma orientale* rhizome (AOR) is a member of family Alismataceae and has been used as a crude drug for diabetes, edema, inflammation and urinary disturbances in oriental medicine (Zhu *et al.*, 1998). The most effective components in AOR are terpenoids, especially a series of triterpenoids such as alisol A, B and its monoacetates, and sesquiterpenoids such as alismols A, B and C (Peng *et al.*, 2002). These components of AOR exhibit diverse biological activities. These include inhibition of lipopolysaccharide-induced expression of inducible nitric oxide synthase and nitric oxide production (Matsuda *et al.*, 1999), inhibition of complementary activity (Lee *et al.*, 2003; Matsuda *et al.*, 1998) and antibody-mediated allergic reactions (Kubo *et al.*, 1997), and induction of cell death in hepatoma and leukemia cells (Chou *et al.*, 2003; Chen *et al.*, 2001).

The present study was performed to evaluate the antioxidative effects of the AOR fraction on hepatic

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injury induced by several oxidative stressors such as *t*-BHP and CCl_4, through several biochemical assays *in vitro* and *in vivo*.

Materials and Methods

Materials – The air-dried *Alisma orientale* rhizome produced in Yeosu province (Korea) was purchased from Kyung Hee Herb Pharm. (Wonju, Korea). The human hepatoblastoma cell line (HepG2) was obtained from the Korean Cell Line Bank (Seoul, Korea).

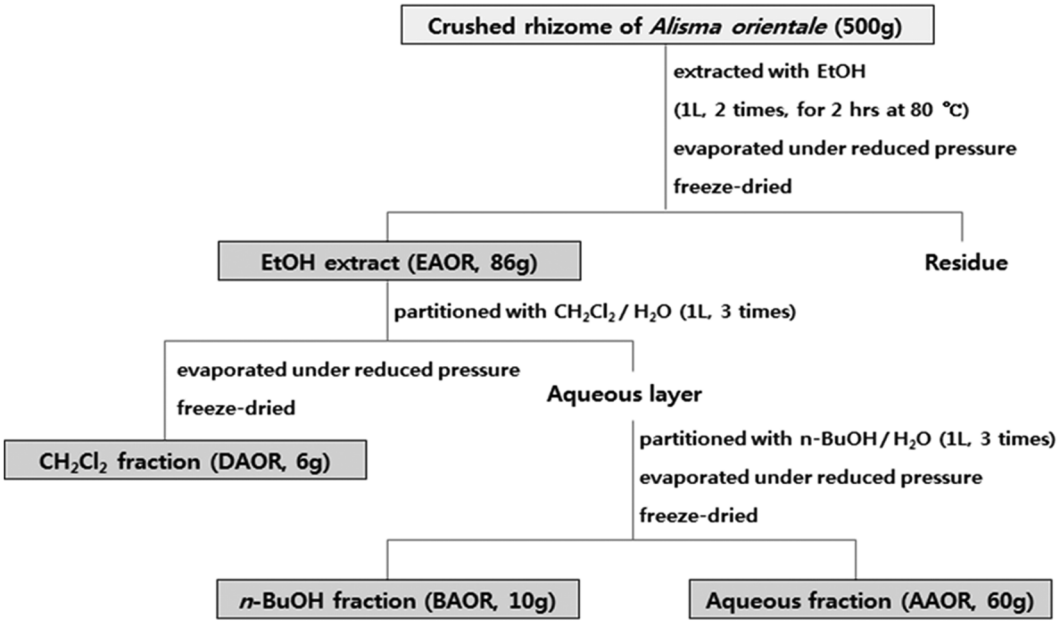
Alisol B acetate was obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Fetal bovine serum (FBS), minimum essential medium (MEM) and trypsin-EDTA were purchased from Gibco BRL (Grand Island, N.Y., U.S.A). Thiazolyl blue tetrazolium bromide (MTT), dimethylsulfoxide (DMSO), 1, 1-diphenyl-2-picrylhydrazyl and *tert*-butyl hydroperoxide were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A). Ethanol, dichloromethane and *n*-butanol for extraction were purchased from Duksan Pure Chemicals Co., Ltd. (Ansan, Korea).

Animals – Five-week-old male Sprague-Dawley (SD) rats were obtained from Samtako (Seoul, Korea). The animals were allowed tap water *ad libitum*. The rats were maintained in a controlled environment at 21 ± 2 ºC and 50 ± 5% relative humidity with a 12 h dark/light cycle and acclimatized for at least one week prior to use.

Extraction of *Alisma orientale* – Crushed AOR (500 g) was extracted twice with 1 L of 80% ethanol for 2 h at 80 ºC. This extract was evaporated under reduced pressure and then freeze-dried to acquire an ethanol extract (EAOR). The extraction rate was 19.6%. The ethanol extract was partitioned into a dichloromethane-H_2O mixture (1 L × 3 times), and the H_2O soluble portion was further partitioned into an *n*-butanol-H_2O mixture (1 L × 3 times). To remove the solvent from each fraction, it was evaporated under reduced pressure and freeze-dried. We then obtained a dichloromethane fraction (DAOR, 6 g), an *n*-butanol fraction (BAOR, 10 g) and an aqueous fraction (AAOR, 60 g) (scheme 1.).

Determination of alisol B acetate – Each AOR fraction was assayed to determine alisol B acetate content by HPLC. The HPLC system consisted of an Alliance 2690 Separation Module, a Waters 996 Photodiode Array Detector operated at 203 nm and a Millenium^{32} Chromatography Manager Version 3.2. Chromatographic analysis was performed on a Nucleosil C_{18} (Waters Corporation, Milford, MA, USA) column (4.0 mm × 250 mm I.D, 5 μm). The mobile phase was acetonitrile-water (80:20, *v/v*) with a flow rate of 1 ml/min.

Determination of polyphenols – The most common method for the quantification of phenolic content in plant products is based on the reaction of phenols with a colorimetric reagent such as Folin-Ciocalteu. This method relies on the reaction of phosphomolybdate and phosphotungstate with phenolic and polyphenolic compounds. A calibration curve was compiled with gallic acid



Scheme 1. Procedure for extraction and fractionation of AOR.

and absorbance was measured at 725 nm (Ramirez-Sanchez *et al.*, 2010).

Antioxidative activity *in vitro* – The scavenging effect of the samples on DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radicals was investigated *in vitro*, according to the procedure described by Blois (1958). The degree of decoloration indicated the scavenging efficiency of the added substances. DPPH solution was added to samples which were then incubated for 30 min and absorbance was measured at 520 nm. The EC_{50} denotes the concentration of the sample required to scavenge 50% of the total DPPH free radicals.

Superoxide anion was generated by xanthine and xanthine oxidase (XOD), as described by Oberley and Spitz (1984). The XOD concentration was adjusted as the rate of nitroblue tetrazolium reduction at 560 nm on a spectrometer, according to the procedure described by Elliott (1992). The EC_{50} shows the concentration of the sample required to scavenge superoxide anion 50%.

Hepatotoxicity assessments – Rats were divided into six groups and each group consisted of eight animals. Group 1 and 2 served as the normal group and the control group, respectively. Group 3, 4, 5 and 6 served as the experimental groups, and EAOR, DAOR, BAOR and AAOR were orally administered, respectively (1 g/kg) for three consecutive days. Three hours after final administration, animals of group 2, 3, 4, 5 and 6 were orally administered 1 ml/kg of carbon tetrachloride (20% CCl_4 in olive oil) to induce hepatotoxicity, while group 1 was supplied the same volume of olive oil. After 24 h, rats were sacrificed by overdose of diethyl ether and blood was collected into heparinized tubes. Serum biochemistry (Glutamic Oxaloacetic Transaminase [GOT], Glutamic Pyruvic Transaminase [GPT]) was assayed according to the manufacturer's instructions (YD Diagnostics, Yong-In, Korea), and analyzed using an automatic chemistry analyzer (Prime, BPC biosed., Italy).

Meanwhile, to study the protective effects of AOR against *t*-BHP-induced hepatotoxicity, DAOR (1 g/kg) was orally administered once daily for three consecutive days. Three hours after the final administration, the animals were treated with *t*-BHP (0.2 mmol/kg, i.p., 100: 1 dissolved in saline). Twenty-four hours after *t*-BHP administration, the rats were anesthetized with diethyl ether. Then, blood was collected into heparinized tubes to determine serum GOT, GPT and LDH activities. After blood was collected, livers were weighed and sliced. Sliced liver sections were frozen quickly in dry ice and stored at −70 °C to determine lipid peroxidation (LPO) levels. Hepatotoxicities were assessed by quantifying hepatic LPO levels. Levels of hepatic LPO were measured using the fluorometric methods described below.

Cell culture and cell viability assays – A human hepatoblastoma cell line (HepG2) was maintained in minimum essential medium (MEM) containing 100 U/ml penicillin and 100 μg/ml streptomycin supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere with 5% CO_2 at 37 °C. Cell viability detected as mitochondrial activity was quantified by measuring dehydrogenase activity retained in the cultured cells, using MTT assays. HepG2 cells were seeded into 96 well plates at a density of 2×10^4 cells/well. After an overnight incubation, the cells were treated with various concentrations of samples for 2 h, and then the cells were co-incubated with *t*-BHP (0.3 mM) for 24 h. Cell viability was determined by MTT assays (Chen *et al.*, 2006).

LPO assays in cell and liver tissue – Malondialdehyde (MDA : the lipid peroxidation product) was assayed using a thiobarbituric acid fluorometric method with 1, 1, 3, 3-tetraethoxypropane as the standard (Hwang *et al.*, 2008). Protein concentrations were determined using the Bradford method with bovine serum albumin as the standard (Bradford *et al.*, 1976).

Statistical analysis – All values are expressed as mean ± S.D or standard error of the mean (SEM). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls multiple comparison tests or an unpaired Student *t*-test. Statistical significance was accepted for *P* values less than 0.05.

Results and Discussion

Previous studies have reported that natural products, especially herbal plants, have beneficial effects on cancer, metabolic diseases, allergy, ischemia and inflammation, which seem to be related to their antioxidative potentials (Kapha *et al.*, 2007; Balssano *et al.*, 2009). The *Alisma orientale* Juzep rhizome (AOR) has been used as a crude drug for diabetes, edema, inflammation and urinary disturbances in oriental medicine. In a recent study, it was observed that AOR possesses potent effects in lowering high levels of serum lipids and improving insulin resistance, which are usually present in patients and animals with non-alcoholic fatty liver disease. So, we studied the antioxidative effects of AOR and its fractions *in vitro*. Also, we evaluated the antioxidative effects of the fractions in *t*-BHP-treated HepG2 cells, and CCl_4- or *t*-BHP-treated rats.

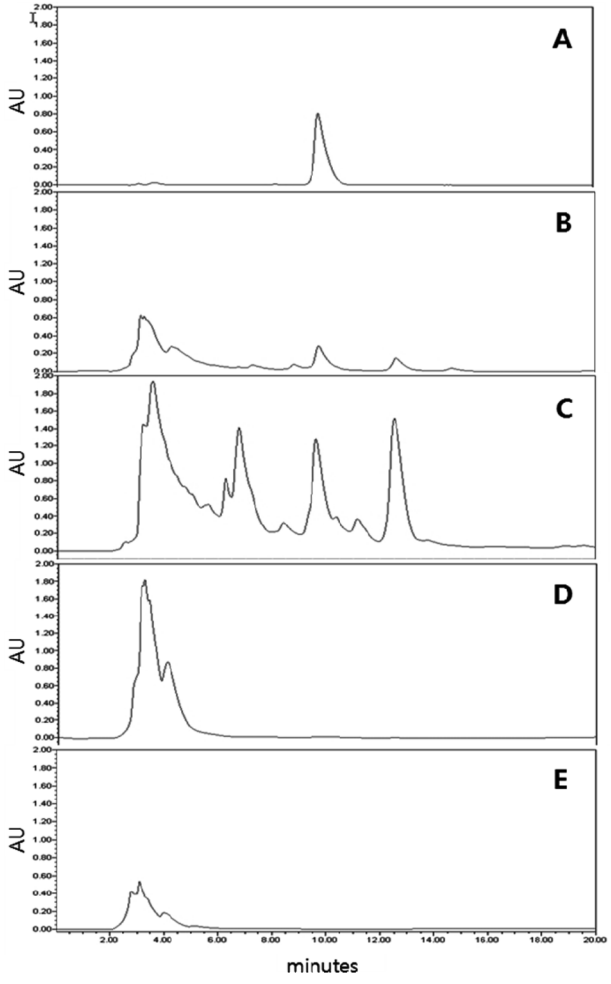


Fig. 1. Chromatograms of alisol B acetate and each fraction of AOR.
A; alisol B acetate, B; ethanol extract of AOR (EAOR), C; dichloromethane fraction of AOR (DAOR), D; *n*-butanol fraction of AOR (BAOR), E; Aqueous layer (AAOR).

First, AOR was extracted with ethanol (EAOR). And the EAOR was partitioned into dichloromethane (DAOR), *n*-butanol (BAOR) and aqueous fractions (AAOR) (Scheme 1.). Each fraction of AOR was assayed for alisol B acetate content, and total polyphenol content. The alisol B acetate contents of EAOR and DAOR were 0.82% and 3.43%; BAOR and AAOR were not detected. Therefore, we inferred that most of the alisol B acetate in AOR had moved from EAOR to DAOR. The chromatograms are shown in Fig. 1. Total polyphenol contents were expressed as gallic acid equivalents (mg/g of sample), and were abundant in DAOR and BAOR (20 ± 0.282 and 25 ± 0.401 mg/g). The results are shown in Fig. 2.

Also, by measuring the scavenging effects on DPPH and superoxide anion radicals, the antioxidative effects of

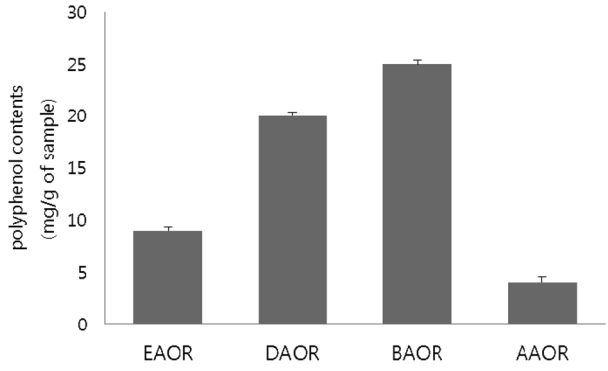


Fig. 2. Total polyphenol contents in each fraction of AOR.
The total polyphenol content measurement method is based on the reaction of phenols with Folin-Ciocalteu reagent. Absorbance was measured at 725 nm. Total polyphenol content is expressed as gallic acid equivalents (mg/g of sample).
EAOR; ethanol extract of AOR, DAOR; dichloromethane fraction of AOR, BAOR; *n*-butanol fraction of AOR, AAOR; Aqueous layer

Table 1. Antioxidative effects of AOR extract and its fractions on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging

Groups	EC_{50} (mg/ml)*
EAOR	0.798
DAOR	0.511
BAOR	0.559
AAOR	0.986
Vitamin C	0.001

*Effective concentration 50 (EC_{50}) : amount of antioxidant needed to decrease the initial DPPH concentration by 50%

each fraction were compared. All fractions of AOR showed dose-dependent scavenging effects against DPPH radicals. Among these fractions, the scavenging effect of DAOR was the greatest. DAOR scavenged as much as 91.4% of DPPH radicals at 1 mg/ml (Table 1). EAOR, DAOR and BAOR showed dose-dependent inhibitory effects against XOD. The scavenging effect of superoxide anion was strong for DAOR, too. The EC_{50} value was 0.878 mg/ml. AAOR had little scavenging effect against superoxide anions (Table 2). As a result of these experiments, we could assume that AOR is useful in the prevention of various forms of hepatic damage that are induced by oxidative stress.

To confirm the antioxidative defense system of AOR *in vivo*, hepatotoxicity markers were measured in a CCl_4-induced liver injury model. The antioxidative activity or the inhibition of the generation of free radicals is important in the protection against CCl_4-induced hepatotoxicity (Castro *et al.*, 1974). CCl_4 administration sharply increased serum biochemical markers such as

Table 2. Antioxidative effects of AOR extract and its fractions on superoxide anion radical scavenging

Groups	EC_{50} (mg/ml) *
EAOR	4.982
DAOR	0.878
BAOR	1.239
AAOR	>5
Vitamin C	0.008

*Effective concentration 50 (EC_{50}) : amount of antioxidant needed to decrease the initial superoxide anion concentration by 50%

Table 3. Effects of AOR extract and its fractions on CCl_4 induced hepatotoxicity in rats

	GOT (IU/L)	GPT (IU/L)
Normal	84.2 ± 4.3	48.1 ± 8.3
Control	473.4 ± 25.1[a]	890.0 ± 49.1[a]
EAOR	256.6 ± 37.3[b]	603.8 ± 76.2[b]
DAOR	282.7 ± 26.6[b]	419.3 ± 52.7[b]
BAOR	253.0 ± 34.3[b]	653.6 ± 79.4[b]
AAOR	369.7 ± 42.8[b]	697.8 ± 94.2[b]

Rats were pretreated with EAOR, DAOR, BAOR and AAOR (1 g/kg). Three hours after the final administration of samples, rats were treated with CCl_4 (1 ml/kg). Hepatotoxicity markers were determined 24 h later by quantifying the serum activities of GOP and GPT. Data are expressed as mean ± SEM for eight rats.
[a] $P < 0.05$ compared with normal group. [b] $P < 0.05$ compared with control group.

GOT and GPT, which are attributed to cellular leakage and damage to the integrity of hepatocyte membranes (Yokogawa *et al.*, 2004). In our experiment, administration of CCl_4 increased GOT and GPT levels ($P < 0.05$). Meanwhile, pre-treatment with each fraction of AOR significantly decreased serum GOT and GPT levels ($P < 0.05$, Table 3). In particular, DAOR was the most effective in reducing hepatotoxicity markers.

Based on the above results, we studied the antioxidative effects of DAOR in *t*-BHP-treated HepG2 cells and rats. *t*-BHP has often been used as a model to investigate the mechanism of cell injury initiated by acute oxidative stress (Rush *et al.*, 1985). *t*-BHP can be metabolized to free radical intermediates by cytochrome P450 (in hepatocytes) or hemoglobin (in erythrocytes), which can subsequently initiate lipid peroxidation and affect cell integrity (Hogberg *et al.*, 1975). On the other hand, as a biomarker for LPO, hepatic LPO was determined by measuring MDA concentrations in *t*-BHP-treated HepG2 cells and liver tissues. *t*-BHP (2 mmol/kg) treatment elevated serum GOT, GPT and LDH levels in rats, and increased MDA formation in the liver. Pre-treatment with DAOR significantly reduced serum GOT,

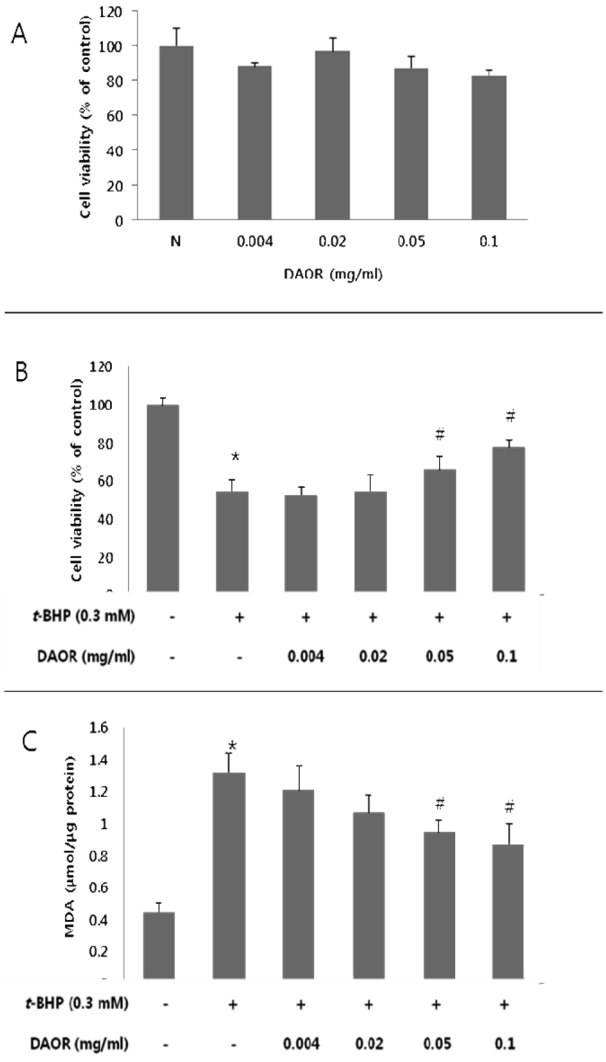


Fig. 3. Protective effects of DAOR against *t*-BHP-induced cell death and LPO formation.

HepG2 cells were treated with DAOR in the absence or presence of *t*-BHP (0.3 mM) for 24 h, and cell viability was determined by MTT assays (A and B). HepG2 cells were pre-treated with DAOR for 2 h, and were then co-incubated with *t*-BHP (0.3 mM) for 3 h to measure LPO (C).
*$P < 0.05$ compared with normal group, #$P < 0.05$ compared with control group

GPT and LDH levels. Also, DAOR significantly reduced *t*-BHP-induced LPO in liver ($P < 0.05$, Table 4).

On the other hand, DAOR had no cytotoxic effect at 0.1 mg/ml, and prevented *t*-BHP-induced cell death in HepG2 cells (Fig. 3A, 3B.). Also, DAOR treatment significantly and dose-dependently reduced *t*-BHP-induced MDA formation (Fig. 3C.).

In this study, pre-treatment with DAOR protected rats from hepatic damage and oxidative stress caused by *t*-BHP and CCl_4. Also, DAOR prevented *t*-BHP-induced

Table 4. Effects of DAOR on *t*-BHP induced hepatotoxicity in rats

	GOT (IU/L)	GPT (IU/L)	LDH (IU/L)	MDA (nmol/g liver)
Normal	107.6 ± 4.7	26.8 ± 2.2	321.5 ± 47.7	4.1 ± 0.2
Control	351.1 ± 36.8[a]	116.2 ± 12.2[a]	734.5 ± 82.2[a]	6.2 ± 0.4[a]
DAOR	184.8 ± 36.4[b]	63.2 ± 4.4[b]	386.1 ± 62.2[b]	4.7 ± 0.3[b]

Rats were pretreated with DAOR (1 g/kg). Three hours after the final administration, the rats were treated with *t*-BHP (0.2 mmol/kg, i.p.). Hepatotoxicity was determined 24 h later by quantifying the serum activities of GOP, GPT and LDH, and MDA formation in liver tissues. Data are expressed as mean ± SEM for eight rats.
[a] $P < 0.05$ compared with normal group. [b] $P < 0.05$ compared with control group.

cell death and LPO formation in HepG2 cells. Therefore, DAOR has the ability to protect against damage due to oxidative stressors including *t*-BHP and CCl_4. DAOR might be useful as a natural ingredient for the prevention of oxidative damage in liver cells and tissues.

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