Protective Effect of *Allomyrina dichotoma* Larva Extract on *tert*-butyl Hydroperoxide-induced Oxidative Hepatotoxicity

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Abstract − **An extract of *Allomyrina dichotoma* larva (ADL), one of the insects used most frequently in traditional Chinese medicine for the treatment of liver diseases such as hepatocirrhosis and hepatofibrosis, was assessed for antioxidant bioactivity in this study. In the current work, we have investigated the protective effects of ADL extracts on *tert*-butyl hydroperoxide (*t*-BHP)-induced hepatotoxicity in cultured hepa1c1c7 cells and in the mouse liver. The treatment of the hepa1c1c7 cells with ADL extracts induced a significant reduction of *t*-BHP-induced oxidative injuries, as determined by cell cytotoxicity, lipid peroxidation (LPO) and reactive oxygen species contents, in a dose-dependent manner. Moreover, ADL extracts evidenced a protective effect against *t*-BHP-induced oxidative DNA damage, as revealed by the results of the Comet assay in hepa1c1c7 cells. ADL extracts also protected against hydroxyl radical-induced 2-deoxy-d-ribose degradation by ferric ion-nitrilotriacetic acid and H_2O_2. In addition, ADL extracts were shown to be able to quench 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals. Our *in vivo* study revealed that ADL extracts pretreatment applied prior to *t*-BHP administration significantly prevented an increase in the serum levels of hepatic enzyme markers and reduced LPO in the mouse liver in a dose-dependent manner. Taken together, these results suggest that the protective effects of ADL extracts against *t*-BHP-induced hepatotoxicity may be attributable, at least in part, to its ability to scavenge free oxygen radicals, and to protect against DNA damage due to oxidative stress.**

Key words : *Allomyrina dichotoma* larva, *tert*-butyl hydroperoxide, DNA damage, oxidative stress

INTRODUCTION

tert-Butyl hydroperoxide (*t*-BHP), a short-chain lipid hydroperoxide analogue, has been previously employed as a model to investigate the mechanism of cell injury initiated by acute oxidative stress (Jenner and Olanow 1998). This *t*-BHP can also be metabolized to free radical intermediates by cytochrome P450 in the hepatocytes (Minotti *et al.* 1986), which in turn may initiate lipid peroxidation, which affects cell integrity, and result in cell injury in hepatocyte cultures

and livers (Martin *et al.* 2001). Additionally, a number of pro-oxidant drugs, including *t*-BHP, have been implicated in the production of oxidative stress and cell injury resulting from the intracellular production of reactive oxygen species (ROS) in a variety of cell types (Goya *et al.* 2007; Kanupriya *et al.* 2007). Normally produced ROS are scavenged by endogenous antioxidants, which exist in abundance in the liver tissue (Yu 1994). However, liver damage can occur when large acute doses of or chronic exposure to toxic substances overpower the hepatic antioxidant defense system (Fruehauf and Meyskens 2007). These excess ROS interact readily with cellular macromolecules and structures, resulting in changes in membrane permeability, the activa-

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tion of proteases and nucleases, and altered gene expression in many tissues (Wu *et al.* 1999; Srinivasan *et al.* 2006; Fruehauf and Meyskens 2007). This is one of the principal reasons that the liver is a target for toxic substances.

Allomyrina dichotoma larva (ADL) have been extensively employed in traditional Chinese medicine for the treatment of liver diseases, including hepatocirrhosis, hepatofibrosis, liver cancer, etc (Miyanoshita *et al.* 1996; Sagisaka *et al.* 2001). ADL, the Asian beetle, has been shown to harbor an antineoplastic agent similar to lectin, and the substance responsible for this biological activity was detected principally in the larvae of the beetle (Yoshikawa *et al.* 1999). However, it remains to be determined whether ADL can prevent or alleviate liver injury induced by oxidative stress; no unequivocal evidence has been found to resolve this question. Therefore, we attempted to assess the protective effects of ADL on *t*-BHP-induced oxidative hepatotoxicity and DNA dama-ge, and also elucidated the mechanism(s) underlying its protective effects in hepa1c1c7 cells and the rat liver.

MATERIALS AND METHODS

1. Chemicals

Chemicals and cell culture materials were acquired from the following sources: *tert*-butyl hydroperoxide (*t*-BHP), thiobarbituric acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2-deoxy-D-ribose and aspartate aminotransferase (AST), and alanine aminotransferase (ALT) diagnostic kits from Sigma Co.; lactate dehydrogenase (LDH) and WST-1 assay kit from Roche Co.; the Biotrin OxyDNA assay kit for the detection of oxidative DNA damage from Biotrin International Ltd; 5-chloromethl-2′7′-dichorodihdydro-fluorescein diacetate ethyl ester (CM-DCFDA) was obtained from Molecular Probes; Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, neomycin, glutamine, and fetal bovine serum (FBS) were acquired from Life Technologies, Inc.; all other chemicals used were of the highest available commercial grade.

2. Preparation of ADL extracts

The *Allomyrina dichotoma* larva (ADL) extracts were obtained from Kachi-maeul Co., Chonnam, South Korea. The ADL were prepared using the method described else-

where, and their compositions were published previously (Yoshikawa *et al.* 1999).

3. Cell culture and treatment

The hepa1c1c7 cell was obtained from the American Type Culture Collection (Bethesda, MA). The cells were maintained in DMEM, 10% fetal bovine serum, 2 mM glutamine, and 1% penicillin/streptomycin in 75 cm^2 culture bottles in a 5% CO_2 atmosphere at 37°C. The cells were then pretreated with ADL extracts for 6 h prior to the addition of *t*-BHP (250 μM). The control cells were treated with DMSO, the final concentration of which never exceeded 0.1%, and this concentration exerted no noticeable effects on the assay system.

4. Cell toxicity assay

Cell toxicity was assessed by means of the WST-1 assay and by measuring the release of LDH. The level of LDH release in the supernatants was measured using a LDH kit. After the supernatant was removed for LDH determination, the cells were used for the WST-1 assay. The WST-1 and LDH assay were conducted in accordance with the manufacturer's instructions.

5. Lipid peroxidation assay

Malondialdehyde (MDA), the lipid peroxidation product in the cells, was assayed by a thiobarbituric acid fluorometric method using 1,1,3,3-tetramethoxy-propane as the standard. Control tests indicated that ADL extracts did not interfere with lipid peroxidation assays. The protein concentration was determined by the Bradford method, using bovine serum albumin as the standard.

6. Measurement of intracellular ROS production

The fluorescent probe, 5-chloromethly-2′7′-dichlorodihydrofluorescein diacetate ethyl ester (CM-DCFDA), was used to monitor the intracellular generation of reactive oxygen species by *t*-BHP, as previously described (Lee *et al.* 2004).

7. Comet assay

Oxidative DNA damage was evaluated via a Comet assay. Culture medium was a pirated from the hepa1c1c7 cell monolayer and the cells were exposed to different concentrat-

ions of ADL extracts and t-BHP in PBS for 20 min on ice. Following exposure to the oxidant, the cells were washed twice with ice-cold PBS. The cells were then detached from the culture dishes and processed for the Comet assay as previously described (Lee and Jeong 2007).

8. Measurement of 2-deoxy-d-ribose degradation

To prepare the Fe^{3+}-nitrilotriacetic acid (NTA), $FeCl_3$ was dissolved in 20 mM phosphate buffer to Fe^{3+} to an NTA molar ratio of 1 : 1, and the pH was adjusted to 7.4. A reaction mixture containing 2.8 mM 2-deoxy-d-ribose, 50 mM KH_2PO_4-KOH buffer at pH 7.4, 1 mM H_2O_2, and 30 mM Fe^{3+}-NTA was prepared. The mixture was then incubated for 1 hour with or without ADL extracts at 37°C. The degradation of 2-deoxy-d-ribose was measured by determining the formation of the thiobarbituric acid-reactive material as described previously (You *et al.* 2002).

9. Assay of free radical-quenching activity

The free radical-quenching capacity of ADL was tested by a method involving the bleaching of stable DPPH (Lee *et al.* 2004).

10. Animal treatment and hepatotoxicity assessment

SD rats (200 ± 5 g) were used for the experiments. The mice were allowed free access to Purina Rodent Chow and tap water, maintained in a controlled environment at 21 ± 2°C and 50 ± 5% relative humidity with a 12 h : 12 h dark/light photocycle, and acclimatized for at least l week before use. To study its protective effects against t-BHP-induced hepatotoxicity, ADL extracts in saline was intragastrically administered at $100 \sim 400$ mg kg^{-1} once daily for three consecutive days. Three hours after the final treatment, the rats were treated with t-BHP (2 mmol, intraperitoneally, 100: l dissolved in saline). 24 hours after the administration of t-BHP, the rats were anesthetized with CO_2, blood was removed via cardiac puncture to determine serum ALT and AST activity, after which the animals were sacrificed by cervical dislocation. After bleeding, the livers were weighed and a thin slice preserved in a buffered formalin solution to obtain the histological sections. The remaining livers were quickly frozen in dry ice/methanol and stored at -70°C for

lipid peroxidation analysis. Hepatotoxicity was assessed by quantifying the serum activities of ALT and AST and hepatic lipid peroxidation. The serum ALT and AST activities were measured using a spectrophotometric diagnostic kit.

11. Statistical analysis

All experiments were repeated at least three times. Results are expressed as the means $\pm$ SD. ANOVA was utilized to evaluate the difference between multiple groups. If significance was observed between groups, the Student's 't' test was used to compare the means of two specific groups, with $p < 0.05$ considered statistically significant.

RESULTS

1. Effect of ADL extracts on the t-BHP-induced cytotoxicity

The protective effects of ADL extracts against t-BHP-induced hepatotoxicity in hep1c1c7 cells were quantified via LDH and WST-1 assays. ADL, which is nontoxic even at high concentrations (400 μg mL^{-1}), afforded full protection from cell injury, as shown by the WST-1 assay (Fig. 1A). To quantify more precisely the protective effects of ADL extracts toward t-BHP, we also conducted LDH assays, and the results of those assays were consistent with those obtained from the WST-1 assay. The addition of ADL extracts to the cells effectively protected the cells against the cytotoxicity induced by t-BHP, as shown by the leakage of LDH (Fig. 1B).

2. Effects of ADL extracts on t-BHP-induced lipid peroxidation and intracellular ROS production

ADL extracts alone induced no change in the degree of MDA formation as compared to the untreated controls. However, 6 hours of exposure to t-BHP alone increased the amount of cell-associated MDA in hepa1c1c7 cells, and the presence of ADL extracts significantly prevented t-BHP-induced MDA production in a dose-dependent manner (Fig. 1C). In order to confirm that ADL extracts reduces t-BHP-induced oxidative stress in hepa1c1c7 cells, intracellular ROS production was evaluated by monitoring 5-chloromethyl-2′ 7′-dichlorodihydrofluorescein diacetate ethyl ester (CM-DCFDA) fluorescence. Rapid increases in intracellular
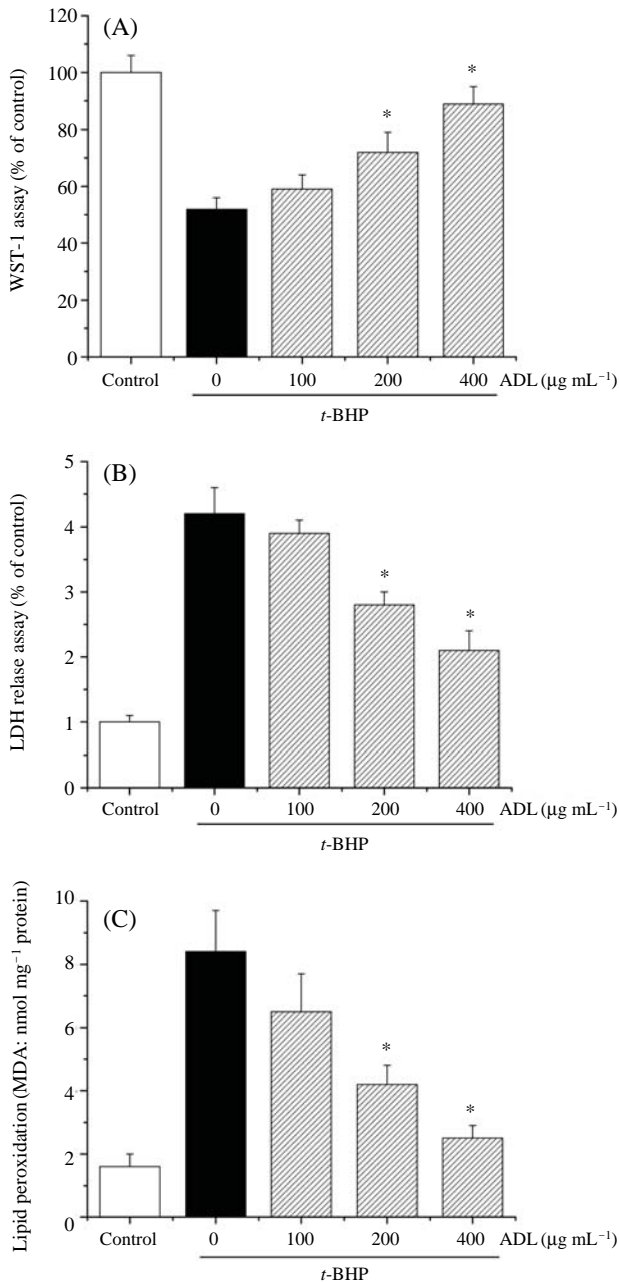
Fig. 1. Cytoprotective effects of ADL extracts on *t*-BHP-induced oxidative hepatotoxicity in hepa1c1c7 cells. The cells were plated on 24-well plates and various concentrations of ADL extracts were applied (250 μM) for 24 h. Cell viability was estimated by (A) WST-1 assay and (B) LDH release assay. (C) Lipid peroxidation was evaluated by malondialdehyde (MDA) formation as described in the Materials and Methods section. Each bar represents the mean ± SD calculated from three independent experiments. *Significantly different from control at $p < 0.05$.

oxidant levels were noted in the cells after *t*-BHP treatment, but the oxidant burden observed after *t*-BHP exposure was reduced in the presence of ADL extracts in a dose-dependent
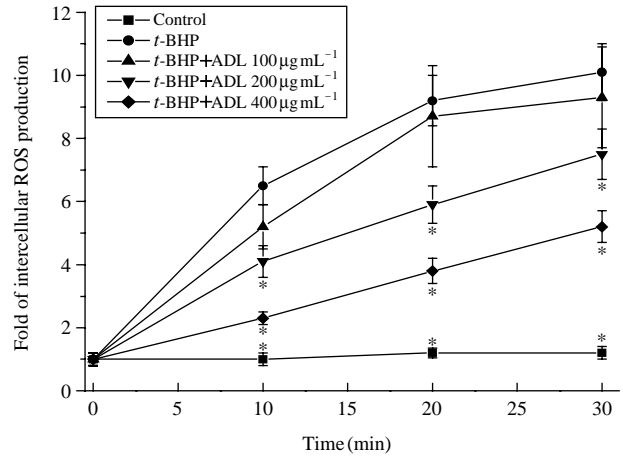

Fig. 2. Effects of ADL extracts on *t*-BHP-induced cellular ROS production. The Hepa1c1c7 cells were treated with ADL extracts and *t*-BHP (250 μM) and the intracellular ROS was measured by monitoring fluorescence increases for 30 min. *$p < 0.05$, significantly different from *t*-BHP alone.

manner (Fig. 2). These results show that ADL extracts were able to inhibit membrane lipid peroxidation via the suppression of intracellular ROS triggered by the injurious peroxy-radicals generated from *t*-BHP.

3. Effects of ADL extracts on *t*-BHP-induced oxidative DNA damage

The Comet assay is used to detect single- and double-stranded DNA breaks in naked supercoiled DNA. As *t*-BHP increased DNA damage via a mechanism that involved intracellular ROS production, *t*-BHP treatment applied for 20 minutes to the hepa1c1c7 cells caused a four-fold increase in the tail moments as compared to the control cells. However, the tail moment increase caused by *t*-BHP-induced oxidative DNA damage was reduced in the presence of ADL extracts in a dose-dependent manner (Fig. 3). These results indicated that the increase in intracellular ROS production might mediate *t*-BHP-induced DNA damage to the cells.

4. Free radical-quenching activity of ADL extracts on the DPPH

In order to determine whether the protection afforded to the hepatocytes by ADL extracts against *t*-BHP-induced oxidative cytotoxicity and DNA damage might be the consequence of the free radical-quenching capacity of ADL extracts, the bleaching of DPPH radical scavenging activity by
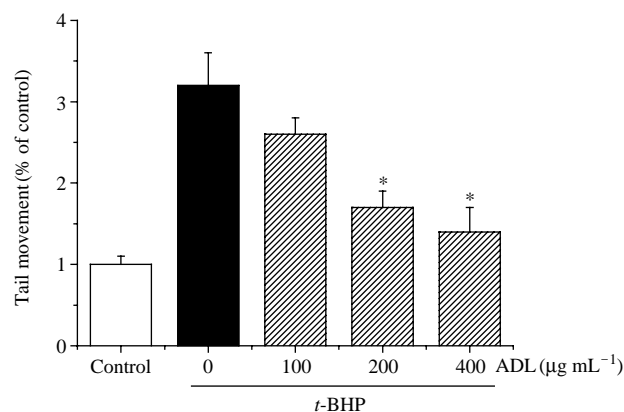
Fig. 3. Effects of ADL extracts on t-BHP-induced oxidative DNA damage. The Hepa1c1c7 cells were treated with ADL extracts and t-BHP (250 μM) and oxidative DNA damage was evaluated using the Comet assay by measurements of fluorescence intensity. $*p < 0.05$ significantly different from t-BHP alone.

ADL extracts were measured. The results, which are summarized in Fig. 4A, showed that ADL extracts were able to quench the DPPH free radicals in a dose-dependent manner.

5. Effects of ADL extracts on hydroxyl radical-induced oxidative 2-deoxy-d-ribose degradation

To further assess the inhibitory effects of ADL extracts on oxidative DNA damage, Fe^{3+}-NTA/H_2O_2-induced 2-deoxy-d-ribose degradation was assayed. The presence of ADL extracts were also determined to markedly inhibit the 2-deoxy-d-ribose degradation induced by Fe^{3+}-NTA/H_2O_2 in a dose-dependent manner, thereby indicating that ADL extracts are potent scavengers of the hydroxyl radical, and confer protection against Fe^{3+}-NTA/H_2O_2-mediated DNA damage (Fig. 4B). Therefore, these results show that ADL extracts have free radical-quenching activity.

6. Effect of ADL extracts on t-BHP-induced hepatotoxicity in rats

The hepatic enzymes, AST and ALT, were used as biochemical markers for early acute hepatic damage (Liu $et\ al.$ 2002). A single dose of t-BHP (2 mmol kg^{-1}) induced hepatotoxicity in the rats, as indicated by the increase in their ALT and AST serum levels following t-BHP administration and ADL extracts pretreatment prevented the t-BHP-induced elevation of ALT and AST serum levels in a dose-dependent manner (Table 1). In a manner which was consistent with
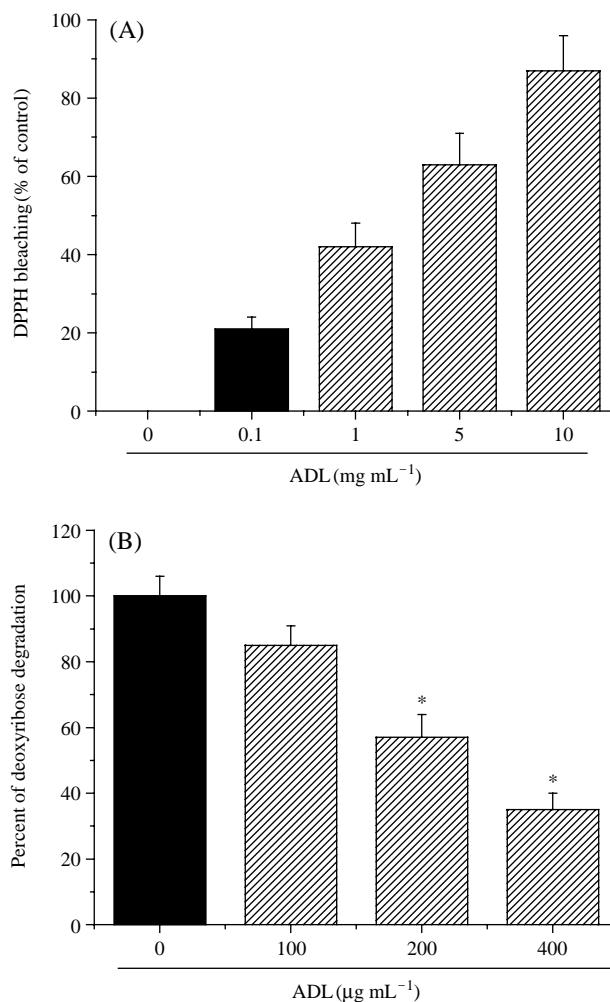

Fig. 4. Effects of ADL extracts on free radical quenching activity and 2deoxy-D-ribose degradation. (A) Radical quenching activity was measured by DPPH. ADL extracts were mixed with DPPH (10 mM, 30 μL) in methanol (3 mL) as described in the Materials and Methods section. (B) 2-deoxy-D-ribose degradation was measured as described in the Materials and Methods section and the percentage of inhibition was calculated from the difference in the presence and absence of ADL extracts. $*p < 0.05$, significantly different from Fe^{3+} NTA/t-BHP alone.

the effect on the serum levels of ALT and AST, pretreatment with ADL extracts caused a significant reduction in t-BHP-induced lipid peroxidation in the liver (Table 1).

DISCUSSION

Liver injury induced by t-BHP is the best-characterized system of acute hepatic damage, and is a commonly utilized model for the screening of the hepatoprotective activities of

Table 1. Dose-dependent effects of ADL extracts on *t*-BHP-induced hepatotoxicity in rats

Treatment	Serum ALT (Karmen Units mL^{-1})	Serum AST (Karmen Units mL^{-1})	Hepatic lipid peroxidation (MDA, nmole g^{-1} liver wt.)
Control	12±1*	56±14*	2.2±0.32*
t-BHP	172±44	270±52	5.6±0.72
ADL 100+*t*-BHP	153±12	241±21	4.9±0.42
ADL 200+*t*-BHP	97±10*	152±27*	3.5±0.42*
ADL 400+*t*-BHP	55±5*	97±8*	2.9±0.33*

Rats were pretreated with ADL extracts (100, 200 or 400 mg kg^{-1}, i.g.) once daily for 3 consecutive days. Control mice were given saline. Three hours after the final treatment, mice were treated with *t*-BHP (2 mmol, i.p.). Hepatotoxicity was determined 18 h later by quantifying the serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and hepatic lipid peroxidation. Each value represents the mean±SD of six mice. *Significantly different from *t*-BHP at $p < 0.05$.

drugs (Yen *et al.* 2004). The principal objective of this study was to evaluate the ability of ADL extracts to affect cellular and DNA damage in hepa1c1c7 cells, as well as hepatotoxicity in mice associated with *t*-BHP-induced oxidative stress. In the present study, ADL extracts effectively protected against *t*-BHP-induced hepatoxicity in hepa1c1c7 cells, as shown by measurements of cell viability and malondialdehyde (MDA) formation. MDA is the major oxidative degradation product of membrane unsaturated fatty acids, and is considered to be an indirect measure of ROS generation (Blair 2001). Thus, we assessed CM-DCFDA fluorescent changes after *t*-BHP treatment as an additional sensitive means of intracellular ROS detection. It was observed that the levels of ROS in ADL extracts-treated cells were lower than in control cells following exposure to *t*-BHP. Moreover, it has been shown that increasing cellular ROS can break DNA strands within the cell (Termini 2000). DNA damage was assessed by the comet assay, a procedure which detects DNA strand breaks in individual cells, resulting from events including the direct scission of the DNA backbone by free radical attack (Johnson and Loo 2000). Thus, we determined that *t*-BHP exposure induced a greater level of DNA damage in the control cells than in the ADL extracts-treated cells. Taken together, these data not only confirm the cytoprotective and anti-lipid oxidation activities of ADL extracts, but also support the proposed functions of ADL extracts as a scavenger of radicals, which ultimately attenuates the associated cytotoxicity and genotoxicity. Consistent with our data, we suggest that ADL extracts are an effective ROS scavenger, which can protect cellular DNA against oxidative damage. However, the exact mechanisms underlying the antioxidant effects of ADL extracts remain unclear. Therefore, we attempted to determine whether the protection afforded to cells by ADL extracts against *t*-BHP-induced

oxidative hepatotoxicity and DNA damage might be a consequence of the free radical-quenching capacity of ADL. DNA is a very sensitive target of hydroxyl radicals, and it has been demonstrated that in the presence of $Fe^{3+}NTA/H_2O_2$ hydroxyl radicals are produced (Lee and Jeong 2007). It has also been previously shown that $Fe^{3+}NTA/H_2O_2$ are capable of inducing 2-deoxy-D-ribose degradation (Lee *et al.* 2004). In the present data, ADL extracts inhibited the 2-deoxy-d-ribose degradation induced by Fe^{3+}-NTA/H_2O_2, and this result indicates that ADL extracts are a potent scavenger of the hydroxyl radical. Additionally, ADL extracts also evidence a DPPH queuing effect. Overall, these data indicate that ADL extracts are an effective scavenger of ROS, and can protect cellular DNA against oxidative damage.

The results of our *in vivo* study also indicated that oral ADL extracts pretreatment significantly lowered *t*-BHP-induced serum levels of the hepatic enzyme markers (ALT and AST) and reduced oxidative stress in the liver, as determined by our evaluation of lipid peroxidation.

In conclusion, the present study showed that ADL extracts protected against *t*-BHP-induced oxidative hepatotoxicity in Hep1c1c7 cells and in the mouse liver. Therefore, this study provides biological evidence that supports the notion that ADL extracts may prove useful for the treatment of liver disorders in living systems.

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