Journal of Ethnopharmacology 127 (2010) 424–432

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

The protective effects of *Acanthopanax senticosus* Harms aqueous extracts against oxidative stress: Role of Nrf2 and antioxidant enzymes

X. Wang, C.X. Hai*, X. Liang, S.X. Yu, W. Zhang, Y.L. Li

Department of Toxicology, Faculty of Preventive Medicine, Fourth Military Medical University, Xi'an 710032, Shaanxi, PR China

ARTICLE INFO

Article history:
Received 13 November 2008
Received in revised form
25 September 2009
Accepted 16 October 2009
Available online 24 October 2009

Keywords:
Acanthopanax senticosus Harms
Oxidative stress
Tert-butyl hydroperoxide
Antioxidant enzyme
Antioxidant activity
Nrf2

ABSTRACT

Ethnopharmacological relevance: Acanthopanax senticosus (Rupr.et Maxim.) Harms, classified into the family of Araliaceae, is used in a variety of diseases in traditional Chinese system of medicine including hypertension, ischemic heart disease and hepatitis.
Materials and methods: Different doses (75 mg/kg, 150 mg/kg and 300 mg/kg) of aqueous extracts of *Acanthopanax senticosus* Harms were evaluated for the antioxidant activity against oxidative stress in mice induced by tert-butyl hydroperoxide (t-BHP) through observating histopathology of the liver and detecting antioxidant enzyme activity, concentration of antioxidant, and related gene and protein expression.
Results: Acanthopanax senticosus Harms aqueous extracts (ASE) attenuated the morphological injury of liver induced by t-BHP and increased the activity of antioxidant enzymes and the ratio of GSH/GSSG in serum and liver homogenates. Medium and high doses of ASE also elevated the gene expression of NF-E2-related factor-2 (Nrf2), but not CuZnSOD, MnSOD, catalase (CAT), glutathione peroxidase (GPx) and GCLC. Protein expression results showed that Nrf2 and the antioxidant enzymes were all increased significantly by medium and high doses of ASE.
Conclusion: The present results indicated that ASE protect against oxidative stress which may be generated via the induction of Nrf2 and related antioxidant enzymes.

1. Introduction

In recent years, there has been a global trend toward the use of natural substances existed in fruits, vegetables, oilseeds, and herbs as antioxidants and functional nutriments. *Acanthopanax senticosus* (Rupr.et Maxim.) Harms (AS) is a medicinal herb with a long history of use and it is also known botanically as *Eleutherococcus senticosus*. It has been constantly spread and cultivated in North Asia. Several parts of this kind of plant have been used for the treatment of various diseases such as rheumatism, hypertension, gastric ulcer, ischemic heart disease and hepatitis (Fujikawa et al., 1996; Lin and Huang, 2000; Yi et al., 2001). AS inhibited tumor necrosis factor-α production in a concentration-dependent manner and it indicates that AS may possess effective anti-anaphylactic activity (Yi et al., 2001). Ethanol-insoluble component of a water extract from *Acanthopanax koreanum* has a protective effect against the induction of fulminant hepatitis in mice by d-galactosamine and lipopolysaccharide (Nan et al., 2004). The studies of Fujikawa et al. (2005) demonstrated that AS prevented bradykinesia and catalepsy reaction in the MPTP-induced model of PD. Acanthoic acid (AA), a pimaradiene diterpene isolated from Acanthopanax koreanum may inhibit the production of inflammatory mediators through inhibition of ERK phosphorylation and NF-κB activation pathway in human mast cells (Kang et al., 2006). Several in vitro studies have described the different effects of AS components. A recent study showed that (+)-syringaresinol-di-O-β-d-glucoside, a phenolic compound from AS, modulated the inflammatory process involved in arthritis by suppressing various gene expression through inhibiting AP-1 and/or NF-κB activities (Yamazakia et al., 2007). In vitro experiments have shown that AE-7-CII (a compound with proven antioxidant activities, isolated and purified from the extract of Acanthopanax) exerted a more profound scavenging effect than that any of the other natural antioxidants did. It seemed that the compound of AE-7-CII purified from AS possessed an antioxidative profile that identified it as both an inhibitor and scavenger of free radicals (Juna et al., 2007). AS were used to be as a treatment for a series of diseases which partially attributed to its antioxidant capacity. Some in vitro experiments have demonstrated that AS has certain antioxidant ability (Jhon et al., 2008). However, there were few evidences that AS may play antioxidant

Abbreviations: ROS, reactive oxygen species; AS, *Acanthopanax senticosus* Harms; ASE, AS aqueous extracts; t-BHP, tert-butyl hydroperoxide; GSH, glutathione; GSSG, glutathione (oxidized form); CAT, catalase; GPx, glutathione peroxidase; GCL, glutamate cysteine ligase; GCLC, catalytic subunit of GCL; γ-GCS, γ-glytamyl cysteine synthetase; Nrf2, NF-E2-related factor-2; T-AOC, total antioxidant competence; MDA, malondialdehyde.

* Corresponding author. Tel.: +86 2983374879; fax: +86 2984774879.
E-mail addresses: wangxin8309@yahoo.cn (X. Wang), cx-hai@fmmu.edu.cn (C.X. Hai).

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doi:10.1016/j.jep.2009.10.022

activities in vivo and the molecular mechanisms were not well understood.

As an oxidant, tert-butyl hydroperoxide (t-BHP) is usually used to induce DNA damage, cell apoptosis and aging (Kim et al., 1998; Dumont et al., 2000), and is especially widely used to induce oxidative stress in rat hepatocytes (Gebhardt, 1997; Lin et al., 2000; Wang et al., 2000). Being a short chain analog of lipid hydroperoxide, t-BHP is metabolized into free radical intermediates by the cytochrome P450 system in hepatocytes, which in turn, initiates lipid peroxidation, glutathione depletion and cell damage (Minotti et al., 1986; Naohiko et al., 1989; Martin et al., 2001).

Taking into account all these facts, we designed to test if the aqueous extracts of AS could protect mice exposed to t-BHP from oxidative damage. Specifically, we were interested in the possible relationship between ASE and redox-regulation of Nrf2 and the expression and function of the antioxidant enzyme systems.

2. Methods and materials

2.1. Chemicals and reagents

Nitroblue tetrazolium (NBT), tert-butyl hydroperoxide (t-BHP), thiobarbituric acid (TBA), glutathione (GSH), glutathione (oxidized form) (GSSG), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma. Acanthopanax senticosus Harms were purchased locally (the traditional Chinese medicine market of Xi'an in Shaanxi Province, China). All other chemicals and solvents were of analytical grade with highest purity commercially available.

2.2. Preparation of aqueous extract of Acanthopanax senticosus Harms

The fresh stem bark of AS used in this study was identified by Dr. Miaomiao Xi (Department of Pharmacy, Fourth Military Medical University, Xi'an, China). A voucher specimen (FMMU TOX0701) has been deposited in the herbarium of the Department of Toxicology, Faculty of Preventive Medicine, Fourth Military Medical University (China). It has been well documented that AS contains several kinds of glucoside eleutheroside including A, B, B1, C, D, E, F, and G and polysaccharides. And eleutheroside B and E are the main active components of glucoside eleutheroside. The stem bark of AS used in this experiment has previously been titolated also by Dr. Miaomiao Xi that it has plenty of eleutheroside A, B, C, D, and E, especially a high percentage of eleutheroside E. Total glucosides and polysaccharides in dried stem bark are about 0.6–0.9% and 2.3–5.7%, respectively.

After being washed and open-air dried, 500 g of AS stem bark was immersed in cold water for 12 h, and then was added to distilled water and boiled for 3 h under reflux. The filtrate was freeze-dried, and the resulting powder, weighing 48 g (9.6%, w/w), was used as the crude aqueous extract of the herb. The aqueous extract was stored at 4 °C and diluted in saline when used.

2.3. Animals and treatment

Forty male Kunming mice 6–8 weeks old (18–22 g) were provided by the Experimental Animal Center of the Fourth Military Medical University. They were maintained in a standard environmental condition and fed with a standard pellet diet and water ad libitum. Mice were housed in stainless steel cages in a room kept at 22 ± 1 °C with a 12-h light/12-h dark cycle. The animals were treated according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication #85-23, revised in 1985) and their experimental use was approved by the animal Ethics Committee of the University.

Mice were randomly allocated into the following groups: (A) normal + saline group ($n = 8$); (B) t-BHP (0.8 mmol/kg) + saline group ($n = 8$); (C) t-BHP (0.8 mmol/kg) + 75 mg/kg ASE group ($n = 8$); (D) t-BHP (0.8 mmol/kg) + 150 mg/kg ASE group ($n = 8$); (E) t-BHP (0.8 mmol/kg) + 300 mg/kg ASE group ($n = 8$). Mice were injected intraperitoneally with 0.8 mmol/kg t-BHP once every 2 days for 1 month (saline was used as substitute in the control group). And then intragastric administration of 75 mg/kg/day, 150 mg/kg/day, and 300 mg/kg/day ASE were given to the (C), (D) and (E) group for 1 week (saline was used as substitute in the (A) and (B) group). Then the mice were killed by paralysing and the blood samples and part of livers were collected for the assays of SOD, CAT, GPx, GSH, GSSG according to the procedures described below. Part of livers was excised from the animals for the pathological histology. For Western immunoblot and RT-PCR analysis, part of livers from mice was quickly frozen and then stored at −80 °C until use.

2.4. Determination of SOD activity

SOD activity was assayed based on reduction of nitroblue tetrazolium (NBT) by $O_2^{\bullet -}$ produced by hydroxylamine hydrochloride autoxidation as described (Kono, 1978). The formazan, which is formed subsequent to electron transfer to NBT was spectrophotometrically determined at 560 nm. The assay monitored the inhibition of the rate of oxidation of NBT using hydroxylamine hydrochloride as the electron donor. After addition of hydroxylamine hydrochloride, the change in absorbance, $A(x)$, is measured optically at 560 nm. To this reaction mixture, sample was added and again a change in absorbance $A(y)$ was recorded at 560 nm. The difference in the rate is compared and expressed as percent inhibition:

$$\%\,\text{inhibition} = \frac{A(x) - A(y)}{A(x)} \times 100$$

One unit (U) of SOD is defined as the amount of protein that inhibits 50% of the rate of NBT reduction.

2.5. Determination of catalase activity

The activity of catalase was determined by a commercial kit (Nanjing Jiancheng Company, China). Ammonium molybdate may end the decomposition reaction of H_2O_2 catalyzed by catalase. The surplus H_2O_2 may have an interaction with ammonium molybdate generating a kind of comoles compound (peroxomolybdic acid complex) with a distinctive colour. The absorbance was measured optically at 405 nm, where it had its maximum absorbance. One unit of enzyme is defined as the amount of enzyme required to breakdown 1 μmol H_2O_2 per second.

2.6. Determination of glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was assayed by spectrophotometry (Paglia and Valentine, 1967). Glutathione peroxidase may catalyze the reaction of GSH and hydroperoxides. The activity of the enzyme could be evaluated by the consumption of GSH. The reaction was started by addition of 400 μl sample dilution. GSH may react with 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) forming a yellow product. The absorbance was measured optically at 422 nm.

2.7. Determination of GSH and GSSG concentration

GSH and GSSG contents of the samples were determined by the method of Hissin and Hilf (1976). When the pH is 8 or 12, o-phthaldialdehyde (OPT) may react with GSH or GSSG, respectively, generating fluorescent materials. For GSH, in brief, 10%

trichloroacetic acid was added to the sample. After centrifugation (5000 × *g*, 10 min), OPT was added to the supernatant and incubated for 40 min at room temperature. Fluorescence at 425 nm was determined with the excitation at 350 nm. For GSSG, fluorescence at 422 nm was determined with the excitation at 338 nm.

2.8. Determination of lipid peroxides concentrations

Levels of MDA were determined as described (Wills, 1966) with the colorimetric absorption of the TBA-MDA chromophore used to determine the index of lipid peroxidation.

2.9. Total antioxidant activity

The total antioxidant activity was determined by a commercial kit (Nanjing Jiancheng Company, China). Numerous antioxidants in organisms could reduce the Fe^{3+} to Fe^{2+}. Fe^{2+} could interact with phenanthroline when pH is in the range of 3–9, generating phenanthroline ferrous complex ($(C_{12}H_8N_2)_3Fe$) which is salmon pink. The absorbance was measured optically at 520 nm. One unit of enzyme is defined as the 0.01 increase of the system absorbance. The results from this kit do not distinguish between lipid- and water-soluble antioxidants but rather provide an estimate of total antioxidant capacity.

Enzyme activity unit was defined as U/mg-protein or U/g protein (in the tissue homogenates) or U/ml (in the serum sample). The GSH and GSSG contents were defined as mmol/L or mmol/g-protein, and MDA content were defined as μmol/L or μmol/g-protein. The protein concentrations of tissue homogenates were determined according to Lowry et al. (1951) using bovine serum albumin as a standard.

2.10. Reverse transcriptase-polymerase chain reaction analysis (RT-PCR)

RT-PCR was conducted as described previously (Choi et al., 2002). The liver tissue mRNA was isolated from the liver using trizol-reagent (Invitrogen, USA) and the quality of isolated RNA was checked by 1.2% formaldehyde agarose gel electrophoresis. For reverse transcriptase-polymerase chain reaction (RT-PCR), primers for Nrf2, CuZnSOD, MnSOD, CAT, GPx1, GCLC and β-actin were designed with the aid of Clone Manager software. The RT-PCR reaction used a template cDNA followed by PCR amplification with Taq DNA polymerase in the same tube. PCR products were analyzed by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light. Densitometric analysis of three different observations was performed using band 'n' map software (BIO-RAD, USA). The quantity of each transcript was calculated according to the instrument manual, and normalized to the amount of a β-actin, housekeeping gene. The sequences of each primer used in this study are shown in Table 1.

2.11. Western blotting

Western blot analysis was performed as follows. Briefly, to obtain total proteins, tissues were lysed for 30 min with a buffer containing 10 mM Tris-Cl, 100 mM NaCl, 1 mM EDTA, 100 μg/ml PMSF and 1 μg/ml Aprotinin. The lysates were centrifuged at 20,000 × *g* for 20 min, and the supernatants were added with same volume of 2× SDS buffer which contain 100 mM Tris–HCl, 200 mM DTT, 4% SDS, 0.2% Bromphenol Blue and 20% glycerine. Then the mixture was boiled for 10 min and centrifuged at 10,000 × *g* for 10 min. The supernatants were used for immunoblotting. Protein extractions were separated by using SDS-PAGE on 10% polyacrylamide gels, and transferred to nitrocellulose membranes (Millipore, USA). After blocking for 1 h with 8% skimmed milk in TBS-T buffer (10 mM Tris, 150 mM NaCl, and 0.1% Tween-20), the membrane was incubated with primary antibodies against Nrf2, CuZnSOD, MnSOD, CAT, GPx1, γ-GCS and β-actin for 1 h at 37 °C. Specific antibody binding was detected by horseradish peroxidase-conjugated secondary antibodies and visualized using enhanced chemiluminescence detection reagent (Santa Cruz). The band densities were quantified from three different observations using an image analyzer Quantity One System (BIO-RAD, USA). All protein quantifications were adjusted for the corresponding β-actin level, which was not consistently changed by the different treatment conditions.

Table 1
Sequences of primers for the genes studied in the text.

Gene	Sense primer (5′–3′)	Anti-sense primer (5′–3′)
Nrf2	CCATTTACGGAGACCCAC	TGAGCGGCAACTTTATTC
CuZnSOD	TCCGTCGGCTTCTCGTCT	TCTGCTCGAAGTGGATGGTTCCCTG
MnSOD	ATCCACTTCGAGCAGAAG	TTCCACCTTTGCCCAAGT
CAT	CAGCTCCGCAATCCTACACC	CAGCGTTGATTACAGGTGATCC
GPx1	GGGACTACACCGAGATGAAC	TCCGCAGGAAGGTAAAGA
GCLC	AGGAGCTTCGGGACTGTATT	TGGGCTTCAATGTCAGGGAT
β-actin	GTGGGCCGCTCTAGGCACCA	CGGTTGGCCTTAGGGTTCAGG

2.12. Statistical analysis

All values in the figures and text were expressed as means ± SD. In the experiments, the figures shown about RT-PCR and Western blots were representative of at least three experiments independently. The results were analyzed by one-way ANOVA followed by a SNK-q test for multiple comparisons. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software. A *p*-value less than 0.05 was considered significant.

3. Results

3.1. Histopathology of the liver

Histopathological studies showed that t-BHP in (B) group, compared to that in (A), induced disordered arrangement of hepatocytes, degeneration in hepatocytes and hepatic cords, dissolved cytoplasm, punctiform necrosis, congestion in central vein, and infiltration of lymphocytes (Fig. 1B). According to microscopic examinations, severe hepatic lesions induced by t-BHP were remarkably and dose-dependently reduced by the administration of ASE in (C)–(E). The injury of liver was gradually relieved and reversed with the increasing of dose of ASE. ASE at 300 mg/kg significantly improved the histopathology of the liver (shown in Fig. 1C–E).

3.2. Effects of ASE on the antioxidant activity of serum

To evaluate the protective effect of ASE on the system oxidative damage in mice induced by t-BHP, we determined the primary antioxidant enzymes in serum. Likely to histopathology of the liver, t-BHP significantly increased the concentrations of malondialdehyde (MDA) (Fig. 2A), which was product of lipid peroxidation, compared to that in (A). And the concentrations of MDA were dose-dependently reduced by the administration of ASE in (C)–(E). As shown in Fig. 2B, t-BHP markedly decreased the activity of serum SOD compared with that of control group. Different doses of ASE showed certain effect of increasing the activity of serum SOD, and ASE at 300 mg/kg showed the highest increase ($p < 0.05$ compared with that of t-BHP group). Surprisingly, t-BHP remarkably elevated the activity of serum CAT (>3 folds), though the results of the ASE administered groups were similar with that of the control group (Fig. 2C). t-BHP significantly decreased the activity of GPx in comparison with that of the control group, and ASE at 300 mg/kg

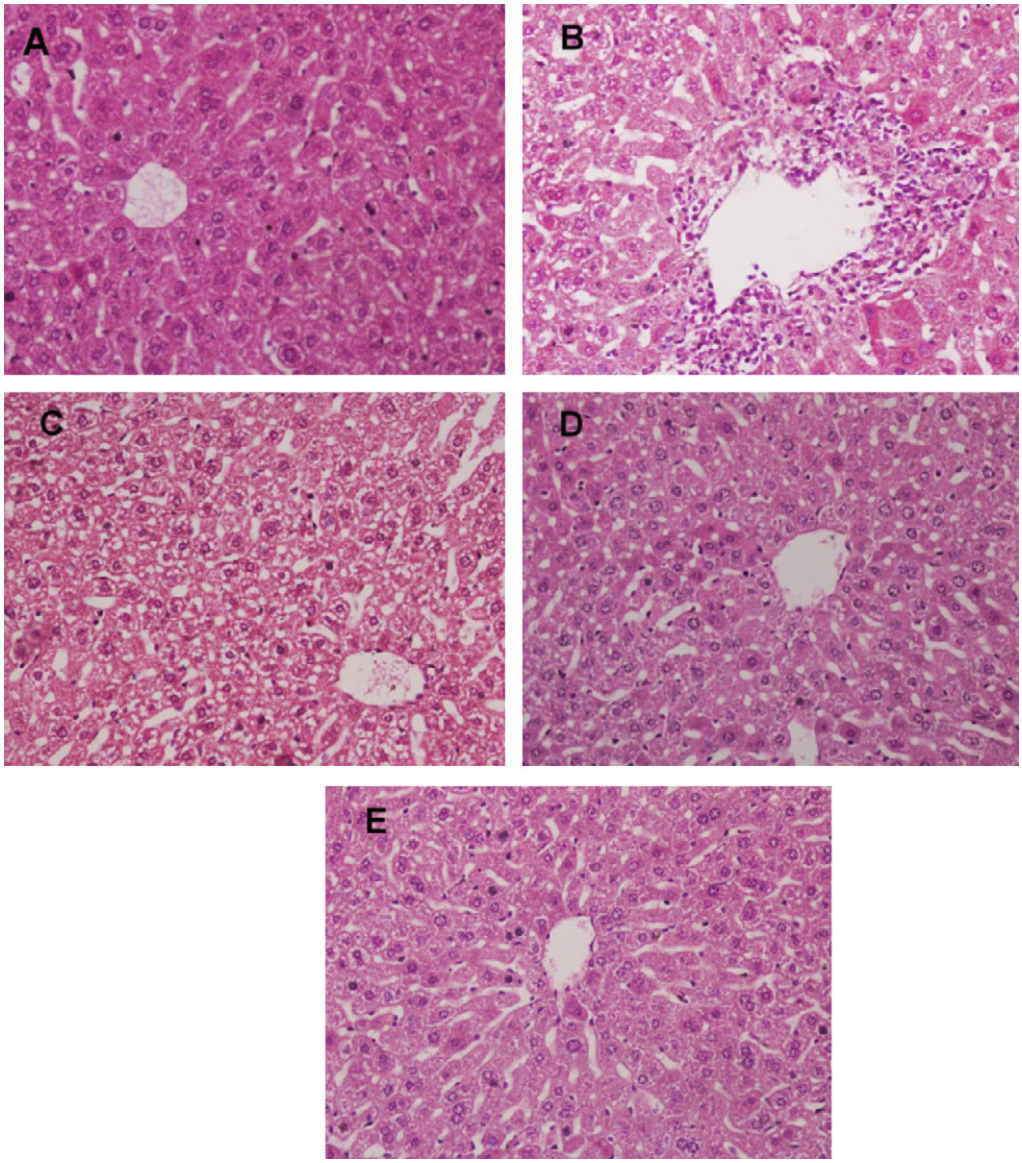

Fig. 1. Effects of ASE treatment on t-BHP-induced liver damage in mice. (A) Liver from mice treated with saline; (B) liver from mice treated with t-BHP; (C) liver from mice treated with t-BHP plus ASE (75 mg/kg); (D) liver from mice treated with t-BHP plus ASE (150 mg/kg); (E) liver from mice treated with t-BHP plus ASE (300 mg/kg). Hematoxylin/eosin staining; magnification 400×.

significantly improved the oxidative stress (compared to that of (B) group) (Fig. 2D). As shown in Fig. 2E, t-BHP markedly decreased the content of serum GSH, which was reversed by ASE in a dose-dependent manner. And the content of GSH in the ASE treating group was significantly higher than that of control group. In contrast, t-BHP markedly increased the content of serum GSSG, and ASE quenches the GSSG from high content to normal level without significant differences between the three doses (Fig. 2F). So by determination of the content of GSH and GSSG, we could calculate the ratios of GSH/GSSG. As shown in Fig. 2G, the ratio was significantly decreased in the t-BHP group, and ASE stimulated its elevation in a dose-dependent manner. And the situation was the same to the T-AOC (Fig. 2H)

3.3. Effects of ASE on the antioxidant activity of liver

The liver tissue was used to determine the protective effect of ASE. Similar to that of serum, the level of MDA was increased remarkably by t-BHP compared with that of control group. Different doses of ASE significantly decreased the content of MDA in a dose-dependent manner compared with that of (B) group (Fig. 3A). Compared with that of the control group, t-BHP markedly decreased the activities of SOD, CAT, GPx and the T-AOC, and ASE showed an increase of these enzymic activities and action of the T-AOC in different degrees (Fig. 3B–D and H). As shown in Fig. 3E, t-BHP significantly decreased the content of GSH, while administration of ASE markedly increased the GSH concentrations. In determining the concentration of GSSG, t-BHP markedly elevated the GSSG content, and ASE reversed this increase in a dose-dependent manner (Fig. 3F). So, Fig. 3G showed that, the ratio of GSH/GSSG of the t-BHP group was the lowest ($p < 0.05$), and it raised as the doses of ASE increased in a dose-dependent manner.

3.4. Expression of RT-PCR analysis

Transcripts corresponding to enzymes of the antioxidant system (CuZnSOD, MnSOD, CAT, GPx1) were increased in mRNA isolated from the liver of t-BHP and 75 mg/kg of ASE-treated mice relative to control animals. But 150 mg/kg and 300 mg/kg of ASE

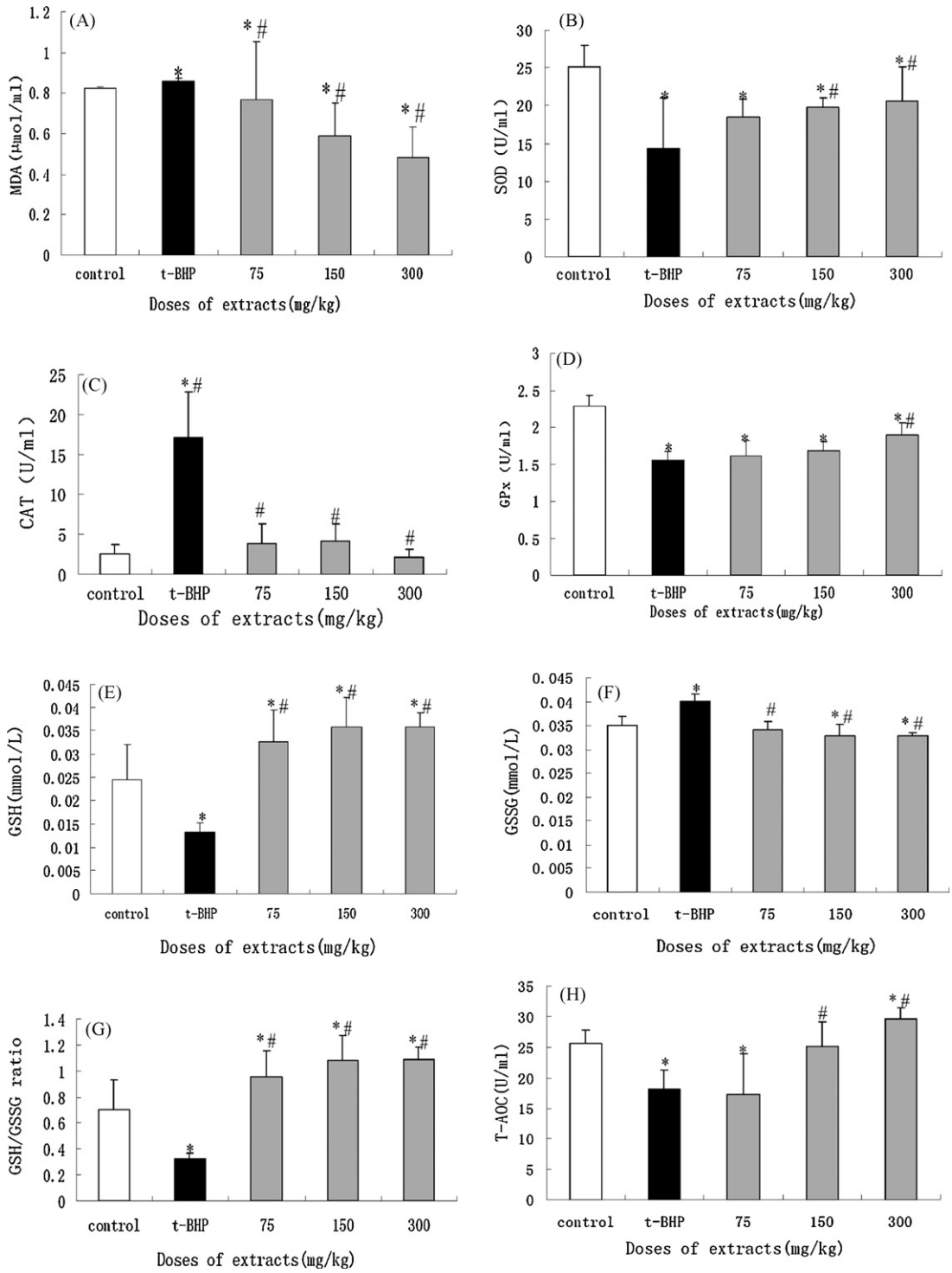

Fig. 2. Effects of ASE on t-BHP-induced oxidative stress in serum. (A) Effects of ASE on lipid peroxidation ($F = 8.00$); (B) effects of ASE on SOD activity ($F = 7.49$); (C) effects of ASE on CAT activity ($F = 34.22$); (D) effects of ASE on GPx activity ($F = 28.46$); (E) effects of ASE on GSH concentrations ($F = 23.53$); (F) effects of ASE on GSSG concentrations (24.68); (G) effects of ASE on the ratio of GSH/GSSG ($F = 104.63$); (H) effects of ASE on T-AOC ($F = 13.85$). Compared with control: $^{*}p < 0.05$; compared with (ii) group: $^{\#}p < 0.05$.

did not show a significant increase in these antioxidant enzymes mRNA (Fig. 4). Nrf2 mRNA isolated from the liver of t-BHP-treated mice was significantly increased (1.4-folds) relative to that of the control animals. Nrf2 mRNA was increased gradually in the ASE-treated groups in a dose-dependent manner (1.1-, 1.5-, and 2-folds) compared with that of control group (Fig. 4). The GCLC mRNA is increased significantly in the t-BHP-treated mice in comparison with that of the control group, the 75 mg/kg and 150 mg/kg of ASE make the GCLC mRNA lower than that of the t-BHP treated group with the 300 mg/kg group similar to the t-BHP treated group (Fig. 4).

3.5. Expression of Western blotting analysis

Nrf2 expression in the t-BHP treated group decreased significantly compared with that of the control group and also the expression of antioxidant enzymes of CuZnSOD, MnSOD, CAT, and GPx1 (Fig. 5). Overexpression of Nrf2 in the AS aqueous treated groups

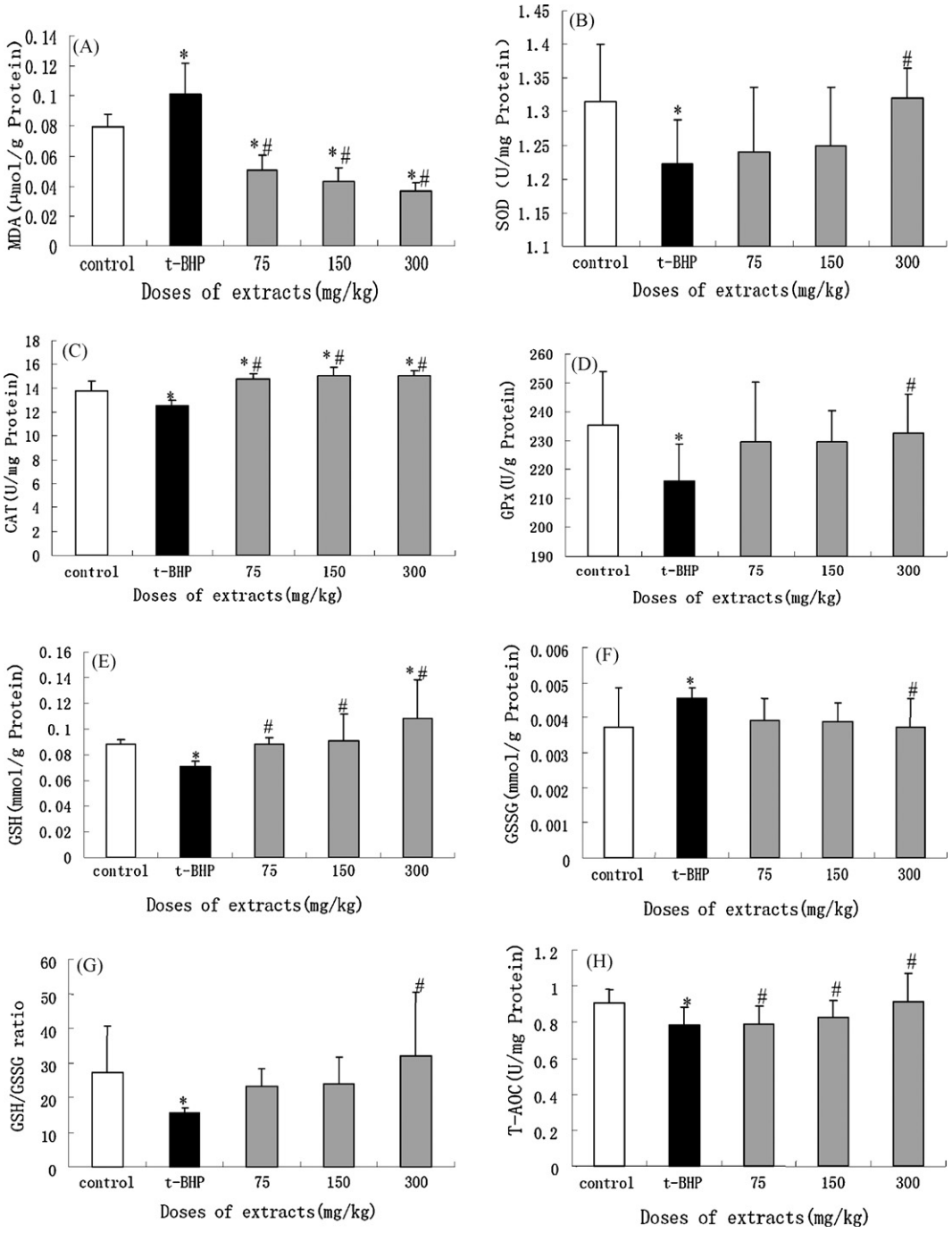

Fig. 3. Effects of ASE on t-BHP-induced oxidative stress in liver. (A) Effects of ASE on lipid peroxidation ($F = 3.34$); (B) effects of ASE on SOD activity ($F = 2.73$); (C) effects of ASE on CAT activity ($F = 29.91$); (D) effects of ASE on GPx activity ($F = 2.68$); (E) effects of ASE on GSH concentrations ($F = 4.89$); (F) effects of ASE on GSSG concentrations ($F = 2.72$); (G) effects of ASE on the ratio of GSH/GSSG ($F = 2.79$); (H) effects of ASE on T-AOC ($F = 2.71$). Compared with control: $^{*}p < 0.05$; compared with (ii) group: $^{\#}p < 0.05$.

induced an increase in antioxidant enzymes protein levels in the presence of ASE. And this increase was in a dose-dependent manner. An increase of γ-GCS expression was also observed in the ASE-treated groups in a dose-dependent manner (Fig. 5). These findings suggested a general correlation of the activities of ASE with nrf2 and antioxidant enzymes, and provided preliminary evidence for a response to oxidative stress at the level of liver protein expression.

4. Discussion

In recent years, much attention has been focused on the protective biochemical function of naturally present antioxidants in biological systems, and on the mechanisms of their action. *Acanthopanax senticosus* Harms, a traditional Chinese medicine was considered to play an important role on the treatment of a variety of diseases. However, the mechanism of the effect of ASE on antioxidant enzymes in mice has not been clearly clarified. The antioxidant activity of this herb extractant in vivo was determined in the present study.

Since tert-Butyl hydroperoxide (t-BHP) is widely used to induce oxidative stress in rat hepatocytes and can be metabolized into free radical intermediates by the cytochrome P450 system in hepatocytes (Kim et al., 2007), we use mice injected with t-BHP as a model of oxidative stress.

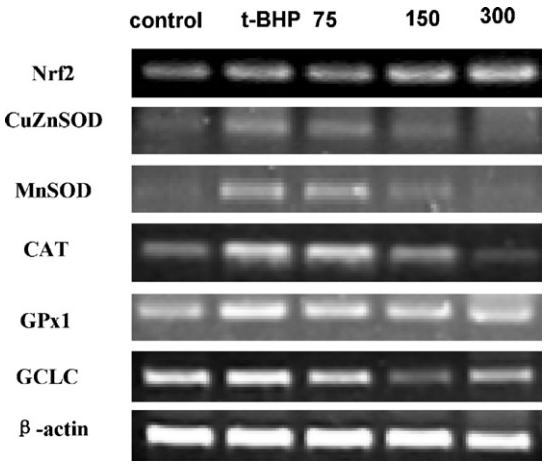

Fig. 4. Effects of ASE on mRNA expression of antioxidant enzymes and Nrf2 in the liver of mice. Each total of RNA preparation was reversely transcribed and the cDNA products were amplified by PCR using gene-specific primers. The bands correspond to Nrf2, CuZnSOD, MnSOD, CAT, GPx1, GCLC and β-actin of control, t-BHP, 75 mg/kg, 150 mg/kg and 300 mg/kg treated groups.

A significant damage of the liver tissue was observed after 1 month of exposure to t-BHP. As shown in Fig. 1B, t-BHP treated liver revealed disordered structures which were derangement of hepatocytes, degeneration in hepatocytes and hepatic cords, dissolved cytoplasm, punctiform necrosis, congestion in central vein, and infiltration of lymphocytes. The results indicated that the liver injuries of animals induced by t-BHP were closely related to oxidative stress. The animals treated with ASE showed lessened liver pathological injury in different degrees. As the doses of ASE increased, the injuries were improved. In the 300 mg/kg treated group, the histology of liver displayed no significant change compared with that of control group. These results indicated that ASE could effectively improve the histological damage of liver induced by t-BHP. MDA is an important product of lipid peroxidation, and the changes of the content of MDA indicate the degree of lipid peroxidation. MDA levels were increased markedly in the animals

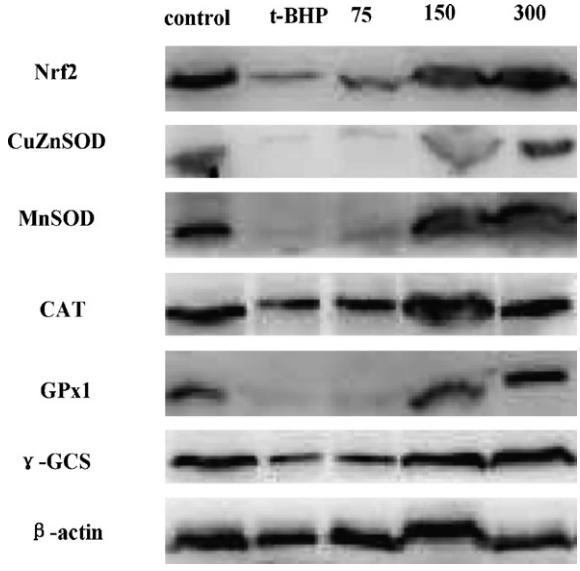

Fig. 5. Effects of ASE on protein expression of antioxidant enzymes and Nrf2 in the liver of mice. Fifty micrograms of proteins was electrophoresed on a 10% polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membranes. Protein expressions were detected by Western blot analysis. The bands correspond to Nrf2, CuZnSOD, MnSOD, CAT, GPx1, γ-GCS and β-actin of control, t-BHP, 75 mg/kg, 150 mg/kg and 300 mg/kg treated groups.

treated with t-BHP, and ASE decreased the MDA concentrations compared with that of control in a dose-dependent manner. It indicated that ASE may produce preventive effects even to normal animals.

In living organism, there is an intricate balance between production and destruction of reactive oxygen species (ROS). Excess ROS react with many biomolecules such as DNA (Marnett, 2000), lipids (Ylä-Herttuala, 1999), and proteins (Stadtman and Levine, 2000). The body has developed an antioxidant defence system against the harmful effects of ROS. Antioxidants are classified as chain breaking antioxidants, preventative antioxidants, and antioxidant enzymes that catalyze the breakdown of ROS (Maxwell, 1995). There are several enzymes that play critical roles on the removal of excess ROS in living organism. Among them, SOD, CAT, GPx are the most crucial enzymes in the cellular antioxidant system. The SOD that was demonstrated to have ROS-metabolizing activity can efficiently and specifically catalyze dismutation of $O_2^{\bullet -}$ to O_2 and H_2O_2 (McCord and Fridovich, 1969; McCord, 1993; Waddington et al., 2000). SOD is found in all the tissues and cells of the aerobic organisms. There are three types of SODs in mammalian cells, namely the cytosolic CuZnSOD, mitochondrial MnSOD, and extracellular CuZnSOD (Halliwell and Gutteridge, 1985). GPx catalyzes the decomposition of H_2O_2 and other peroxides (e.g., lipid peroxides in cell membranes) and then converts hydrogen peroxide into O_2 and H_2O, using reduced glutathione as substrate (Halliwell and Gutteridge, 1985; Cotgreave et al., 1992). And GPx1 is a major subtype of this enzyme. In fact, H_2O_2 reduction by GPx requires the recycling of oxidized glutathione by glutathione reductase, which is dependent on the supply of NADPH.

So, modulation of antioxidant enzymes such as SOD, CAT, GPx may be able to protect against oxidative stress. We detected the effect of ASE on these enzymes in mice pretreated with t-BHP in this paper. The augmented activities of SOD, CAT, GPx, T-AOC were observed in mice exposed to ASE after t-BHP treatment, and this could be responsible for the increased resistance to oxidative stress observed. But the activity of CAT in serum is an exception. As shown in Fig. 2C, a significant increase (>3 folds) of the activity of serum CAT in the t-BHP treated group was observed. It may be attributed to an instinct protective effect in response. In normal conditions, there is no CAT in extracellular fluid of human body, but under some unusual conditions, such as infection and stress, CAT would appear in serum. The higher activity of CAT could make the cells capable of tolerating higher H_2O_2 concentrations. We also observed the gene and protein expression of these antioxidants. As shown in Figs. 4 and 5, gene expression of CuZnSOD, MnSOD, CAT, GPx1 were upregulated significantly in the t-BHP and low dose of ASE group compared with that of control and this may be due to temporary and irritable protective effects of the organism. In contrast, Western blot analysis provided evidence for the determination of the activities of antioxidant enzymes. The upregulations of CuZnSOD, MnSOD, CAT, GPx1 were observed compared with t-BHP treated group. These results suggested that the antioxidant activities of ASE may be related to the increase of activity and protein expression of antioxidant enzymes.

Glutathione is a tripeptide, L-γ-glutamyl-L-cysteinyl-glycine, found in all mammalian tissues and especially highly concentrated in the liver. Glutathione exists in the thiol-reduced (GSH) and disulfide-oxidized (GSSG) forms (Kaplowitz et al., 1985). GSH is the predominant form, existing in millimolar concentrations in most cells (liver 5–10 mM). The GSSG content is less than 1% of GSH (Akerboom et al., 1982). A major function of GSH is detoxification of xenobiotics and/or their metabolites. All aerobic organisms are subject to a certain level of physiological oxidative stress from mitochondrial respiration. The intermediates that are formed, such as H_2O_2 and $O_2^{\bullet -}$, can lead to the production of toxic oxygen radicals that can cause lipid peroxidation and cell injury. To prevent

this, the endogenously produced hydrogen peroxide is reduced by GSH in the presence of selenium-dependent glutathione peroxidase. In the process, GSH is oxidized to GSSG, which in turn is reduced back to GSH by GSSG reductase at the expense of NADPH, forming a redox cycle (Shelly, 2008). GSH is synthesized in cytosol in virtually all cells (Meister and Anderson, 1983). The first step of GSH biosynthesis is considered rate-limiting and catalyzed by glutamate cysteine ligase-GCL (formerly γ-glytamyl cysteine synthetase, γ-GCS). γ-GCS is composed of a heavy or catalytic (GCLC, Mr ~73,000) and a light or modifier (GCLM, Mr ~30,000) subunit, which are encoded by different genes in species as divergent as humans and fruit flies. The second step in GSH synthesis is catalyzed by GSH synthase. Due to the fact that c-glutamylcysteine (product of γ-GCS) is present at exceedingly low concentrations when GSH synthetase is present, γ-GCS is considered rate-limiting (Dalton et al., 2004). GSH within cells protects against different ROS (Halliwell and Gutteridge, 2000). In addition to direct removal of ROS, GSH also control the redox state of the cell and regulate protein function through thiolation and dethiolation. It is well established that GSH, the most important biomolecule protecting against chemically induced cytotoxicity, can participate in the elimination of reactive intermediates by conjugation and hydroperoxide reduction, or of free radicals by direct quenching. There are two distinct pathways of t-BHP metabolism, one is via cytochrome P-450, and the other is by GPx which converts t-BHP to tert-butanol and oxidized GSH (Joyeux et al., 1990). So, we detected the contents of GSH and GSSG and the expression of GSH related enzyme (γ-GCS). In the present study, the ratios of GSH/GSSG were both decreased by t-BHP in the serum and liver. And the protective effects on GSH/GSSG against t-BHP showed a dose–effect relationship which indicated that the protective effects of ASE against oxidative stress attributed partly to the increase of GSH and the decrease of GSSG. The evidence of protein (not gene) expression of GCLC supported this deduction.

Recently, NF-E2-related factor-2(Nrf2), a member of the Cap-N-Collar transcription factor family, has been demonstrated to be a critical transcription factor that binds to the antioxidant response element(ARE) in the promoter region of a number of target genes, encoding for antioxidant enzymes in several types of cells and tissues (Nguyen et al., 2003; Kobayashi et al., 2004; Yu and Kensler, 2005). The transcription factor Nrf2 regulates the expression of many detoxifying genes (Itoh et al., 1997; Chan and Kwong, 2000; Thimmulappa et al., 2002; Kwak et al., 2003; Hu et al., 2006) such as SOD, CAT, GPx, γ-GCS. The protein products of these genes provide multiple layers of protection during cellular insults, collectively favoring cell survival (Osburn and Kensler, 2008). Nrf2 knockout mice (Nrf2−/−) possess much lower levels of Phase II detoxifying enzymes and are more susceptible to oxidative stress and carcinogen-induced tumorigenesis than wild-type animals (Leung et al., 2003). Nrf2 also plays a role in protecting liver, as evidenced by increased levels of lipid peroxidation and DNA damage in $Nrf2^{-/-}$ livers (Li et al., 2004). Activation of Nrf2, which controls constitutive and inducible expression of Phase II detoxifying genes, may be one of the protective mechanisms against xenobiotics (Iida et al., 2004). Although several chemopreventive compounds have been shown to induce Nrf2, antioxidant and detoxification genes (Zhang and Gordon, 2004; Choi and Kim, 2008), the effect of AS on Nrf2 expression in oxidative injury mice has not been considered prior to this study. Due to the strong dependency of Nrf2 in protection against both electrophilic and oxidative toxicities following environmental exposures, the study targeting the Nrf2 was conducted in the present study. As shown in our results, ASE induced the gene and protein expression of Nrf2 in different degrees. Although gene expression of antioxidant enzymes were not increased following the upregulation of Nrf2, on protein level, Nrf2 and antioxidant enzymes were upregulated in a similar tendency. This suggested that the antioxidant defense may be enhanced by ASE via activation of Nrf2 and then upregulation of expression of antioxidant enzymes. And the expression of antioxidant enzymes may be modulated post-transcriptionally.

In conclusion, the present results suggest that ASE induces antioxidant enzymes expression through the activation of Nrf2, and the increased antioxidant enzymes expression and activity induced by ASE could contribute to a defense mechanism against t-BHP-induced oxidative injury. Our results suggest that ASE could be beneficial in the treatment of several diseases associated with oxidative stress through the enhanced antioxidant defense. It has been well documented that *Acanthopanax senticosus* Harms contains eight kinds of glucoside such as elutheroside A, B, B1, C, D, E, F, and G. And eleutheroside B and E are the main active components of glucoside eleutheroside. The stem bark of AS used in this experiment has plenty of eleutheroside A, B, C, D, and E. Especially, it contains a high percentage of elutheroside E. However, which eleutheroside is the main active component that played the antioxidant activity is unknown. Further study is needed to identify the main active components of ASE that are responsible for the antioxidant activity.

Acknowledgements

This study was supported by the Natural Science Foundation of Shaanxi Province No. S2009JC1186 and the National Natural Science Foundation No. 30872135 and 30671788.

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