Original Article

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Protection of *Angelica sinensis* (Oliv) Diels against hepatotoxicity induced by *Dioscorea bulbifera* L. and its mechanism

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Summary ***Dioscorea bulbifera* L., a traditionally used medicinal plant in China, is reported to induce hepatotoxicity. The present study is designed to investigate the protection of an ethanol extract of *Angelica sinensis* (Oliv) Diels (AE) against an ethyl acetate fraction of *D. bulbifera* (EF)-induced liver injury and its engaged mechanism. High performance liquid chromatography (HPLC) analysis showed that the amount of diosbulbin B in EF was 16.03% and ferulic acid in AE was 0.18%. EF (350 mg/kg) increased serum alanine/aspartate aminotransferase (ALT/AST), alkaline phosphatase (ALP) activities and total bilirubin (TB) amount, while AE inhibited such an increase. Liver histological evaluation showed that AE prevented development of severe hepatic lesions induced by EF. Further results showed that EF decreased the expression of Bcl-2 and induced the cleaved activation of caspase-9 and -3, and all those effects were reversed by AE. AE also reversed EF-induced decreased expression of the inhibitor of kappa B (IκB), superoxide dismutase (SOD), and glutathione peroxidase (GPx). Taken together, our results demonstrate that AE can prevent EF-induced hepatotoxicity via preventing apoptosis, meanwhile IκB, SOD, and GPx may be involved in such protection.**

Keywords: *Dioscorea bulbifera* L., *Angelica sinensis* (Oliv) Diels, hepatotoxicity, apoptosis, oxidative stress injury

1. Introduction

Dioscorea bulbifera L. is a medicinal plant distributed in the tropics and the subtropical zone of Asia. It is used in folk medicine to treat thyroid disease (especially goiter) and cancer, as well as some other syndromes involving "toxic heat," such as sore throat, struma (*1,2*). In spite of its unique clinical benefit, its potential toxicity has been an increasing concern to clinical doctors. *D. bulbifera* has been documented to cause intoxication and is lethal in animals and humans (*3-5*). It has been elucidated that the main toxic target organ of *D. bulbifera* is liver, and its exposure could lead to severe liver damage, including liver swelling, fatty degeneration, even animal death (*3-5*). Intake of *D. bulbifera* has also been associated with high occurrence of jaundice or toxic hepatitis in humans (*3*). It is reported that diterpene lactones are the main hepatotoxins existing in the rhizome of *D. bulbifera* (*6,7*). So far, some structural analogues of diterpene lactones have been identified in *D. bulbifera* and of which the amount of diosbulbin B is the highest (*8-10*).

Danggui, the dried root of *Angelica sinensis* (Oliv) Diels, is a commonly used medicinal herb. It can enrich blood, promote blood circulation and clinically is used to treat blood deficiency patterns and menstrual disorders such as dysmenorrhea and irregular menstrual cycles (*11,12*). In recent years, pharmacological research has focused on elucidating the antioxidative, anti-cancer and neuroprotective properties of *A. sinensis* (*13-15*). In addition, *A. sinensis* also showed a hepato-protective effect, especially its active component ferulic acid (*16-18*). In the present study, we investigated the protection

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of the ethanol extract of *A. sinensis* (AE) against *D. bulbifera* (EF)-induced liver injury and its engaged mechanism.

2. Materials and Methods

2.1. *Drugs and reagents*

The rhizomes of *D. bulbifera* were collected in Qingyang, Anhui Province and authenticated by Prof. Shou-Jin Liu (Anhui College of Traditional Chinese Medicine, Anhui, China). The radixes of *A. sinensis* were purchased from Yanghetang drugstore (Shanghai) and authenticated by Associate Prof. Li-Hong Wu (Shanghai University of Traditional Chinese Medicine, Shanghai, China). The identified specimens were deposited in the herbarium of Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine. Diosbulbin B and ferulic acid were purchased from Shanghai Tauto Biotech Co., Ltd. Methanol and acetonitrile (HPLC Grade) were purchased from Fisher Chemical. Antibodies against Bcl-2, caspase-9, caspase-3, IκB and β-actin were all purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal antibody against superoxide dismutase (SOD) was purchased from Abcam (Cambridge, MA, USA). Mouse monoclonal antibody against glutathione peroxidase (GPx) was purchased from Santa Cruz (Santa Cruz, CA, USA). Peroxidase-conjugated goat anti-Rabbit IgG (H + L) and anti-mouse IgG (H + L) were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Pre-stained protein marker was purchased from Fermentas (Burlington, Ontario, Canada). Nitrocellulose membranes were purchased from Bio-Rad (Hercules, CA, USA) and an enhanced chemiluminescence detection system was obtained from Millipore Corporation (Billerica, MA, USA).

2.2. *Experimental animals*

Specific pathogen free male ICR mice (18-22 g body weight) were purchased from the Chinese Academy of Sciences. The mice were fed with a standard laboratory diet and given free access to tap water, kept in a controlled room temperature (22 ± 1°C), humidity (65 ± 5%), and a 12:12-h light/dark cycle. All mice receive humane care in compliance with the institutional animal care guidelines approved by the Experimental Animal Ethical Committee of Shanghai University of Traditional Chinese Medicine.

2.3. *Samples and preparation of extracts*

Preparation of ethyl acetate fraction from *D. bulbifera* (EF): The powder was soaked in 80% ethanol (w/v = 1:10) for 2 h at room temperature. The macerated plant material was extracted at 70-80°C and refluxed for 2 h, and then filtered through a cheese cloth. The residue was extracted as described previously (*19*). The combined filtrates were then evaporated to be free of alcohol using a rotary evaporator under reduced pressure at 50°C. The extracts were then partitioned with ethyl acetate to yield an ethyl acetate solution. After evaporation of solvents, an ethyl acetate fraction was achieved and stored in 70% ethanol.

Preparation of ethanol extract of *A. sinensis* (AE): The powder was soaked in 70% ethanol for 2 h at room temperature, and then percolated slowly until percolating liquid was colorless. Extracted liquid was evaporated using a rotary evaporator under reduced pressure at 50°C and adjusted to 1 mg/mL of raw material with 70% ethanol.

2.4. *HPLC analysis*

Analysis was carried out on a HPLC instrument (Agilent) consisting of an autosampler, quaternary pump, column heater compartment and diode array detection (DAD) with an on-line degasser. Chromatographic separation was achieved on an ultimate ™XB-C 18 column for diosbulbin B and CAPCELL PAK C18 column for ferulic acid (4.6 × 250 mm, 5 μm). The mobile phase consisted of acetonitrile and water (v/v = 25:75) for diosbulbin B or acetonitrile and 0.085% phosphoric acid (v/v = 17:83) for ferulic acid with a flow rate of 1.0 mL/min. Column temperature was set at 25°C and detection was carried out by UV absorbance at 210 nm for diosbulbin B and 323 nm for ferulic acid.

To obtain a calibration curve for diosbulbin B or ferulic acid, standard solutions were prepared in a range of 7.8-250 μg/mL for diosbulbin B and 2.7-173.6 μg/mL for ferulic acid on a column, and a 10 μL aliquot was injected. The samples were prepared and an aliquot (10 μL) each was injected into the above system respectively. The contents of diosbulbin B and ferulic acid in the respective extract were obtained using the calibration curves.

2.5. *Treatment protocol*

Mice were orally pre-administered AE suspended in 0.5% sodium carboxyl methyl cellulose (CMC-Na) (180, 360, or 720 mg/kg per day) for 6 consecutive days. Four hours after the treatment with AE on the last day, a single dose of EF (350 mg/kg, suspended in 0.5% CMC-Na) was administered orally. 0.5% CMC-Na was used as vehicle control. Twenty-four hours later, blood samples were collected by extirpating the eyeball. Mice were then sacrificed; and their livers were removed for further research.

2.6. *Serum biomarkers for liver injury*

Blood samples obtained from mice of all groups were

allowed to coagulate for 2 h on ice. Serum was then isolated following centrifugation at 840 × g for 15 min. Serum alanine/aspartate aminotransferase (ALT/AST), alkaline phosphatase (ALP) activities and total bilirubin (TB) amounts were measured with kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions.

2.7. *Histological examination*

Portions of the liver were fixed in 10% formalin and embedded in paraffin. Samples were subsequently sectioned five micrometers thick and stained with hematoxylin-eosin (HE) for further histological analysis.

2.8. *Western blot analysis*

Liver tissue (approximately 25 mg) was homogenized in 0.5 mL, ice-cold lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 0.5% NP-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A and incubated on ice for 10 min. The liver homogenate was centrifuged at 10,000 × g for 20 min at 4°C and protein concentration of the resulting supernatant was determined by the Bradford method. Two samples of each group were subjected to SDS-PAGE and then electrophoretically transferred onto a nitrocellulose membrane. Nonspecific binding was blocked for 1 h with 5% non-fat milk in Tris-buffered saline Tween-20 (TBST) (10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween-20), and then blots were incubated with primary antibody and secondary antibody. Immunoblots were visualized using a chemiluminescent reagent. The densities of the bands were normalized using a corresponding β-actin density as an internal control.

2.9. *Statistical analysis*

All experimental data were expressed as mean ± standard error (S.E.). Significant differences were determined by One-Way ANOVA. $p < 0.05$ was considered as a statistically significant difference.

3. Results

3.1. *HPLC analysis of plant extract*

Our previous study showed that diosbulbin B is the main hepatotoxic chemical compound in *D. bulbifera* (*19*). Ferulic acid is the main active component in *A. sinensis*. Measurement of diosbulbin B and ferulic acid were used to control the quality of EF and AE extracts in the present study, respectively. The chemical structures of diosbulbin B and ferulic acid are shown in Figure 1A and Figure 2A. As shown in Figures 1B-1D and Figures 2B-2D, the amount of diosbulbin B in EF was 16.03% and ferulic acid in AE was 0.18% as determined by HPLC-DAD.

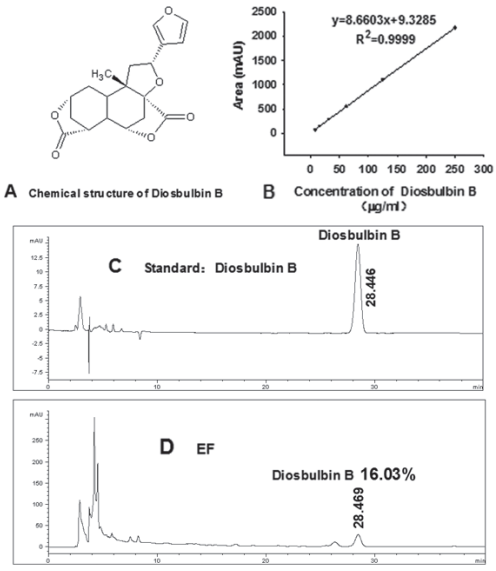

Figure 1. Chemical structure of diosbulbin B and the amount of diosbulbin B in EF. (A) Chemical structure of diosbulbin B. **(B)** Calibration curve of diosbulbin B. **(C)** HPLC chromatogram of diosbulbin B. **(D)** HPLC chromatogram of EF.

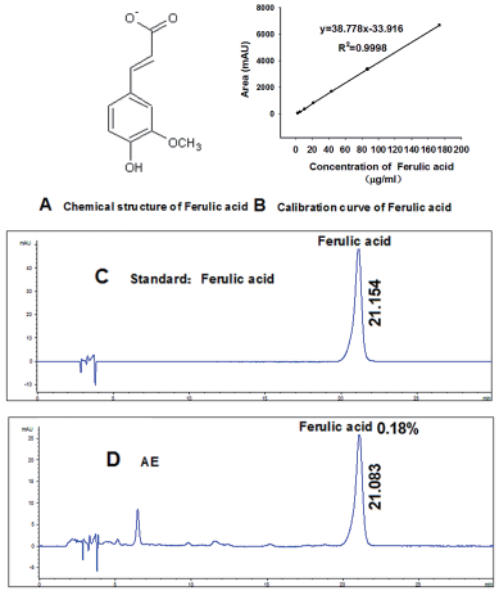

Figure 2. Chemical structure of ferulic acid and the amount of ferulic acid in AE. (A) Chemical structure of ferulic acid. **(B)** Calibration curve of ferulic acid. **(C)** HPLC chromatogram of ferulic acid. **(D)** HPLC chromatogram of AE.

3.2. *AE reversed EF-induced increase of ALT, AST, and ALP activities and TB amount*

As shown in Figure 3, EF (350 mg/kg) increased serum ALT, AST, and ALP activities and TB amount as compared with control, while pretreatment with 360 mg/kg, 720 mg/kg of AE could obviously inhibit

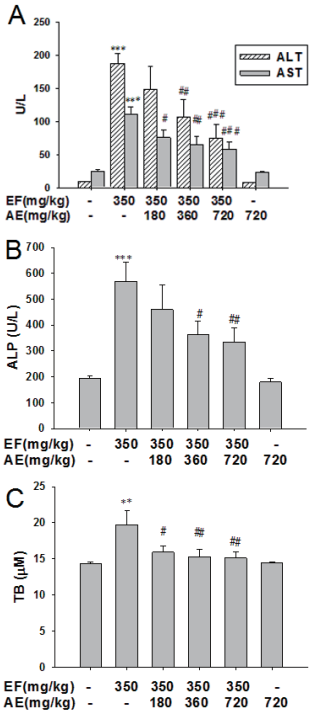

Figure 3. Serum ALT, AST, and ALP activities and TB amount in EF-treated mice with or without preadministration of AE. (A) Serum ALT and AST activities. **(B)** Serum ALP activity. **(C)** Serum TB amount. Data are shown as mean ± S.E. (n = 7-12). ** $p < 0.01$, *** $p < 0.001$ *versus* control group, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, *versus* EF-treated group.

such an EF-induced increase. In contrast, there were no significant differences in the activities of ALT, AST, and ALP and level of TB in mice treated with AE (720 mg/kg) alone when compared with control.

3.3. *Histological analysis of liver tissue*

The livers from control mice appear normal (Figure 4a). In contrast to control mice, the mice treated with EF exhibited severe liver damage, indicated by intrahepatic hemorrhage, extensive infiltration of lymphocytes into liver tissue and serious destruction of liver structure (Figure 4b). Interestingly, AE strongly prevented the development of severe hepatic lesions induced by EF, indicated by decreased intrahepatic hemorrhage and infiltration of lymphocytes, and little destruction of liver structure (Figure 4c). In addition, there was not much difference between control and AE-treated groups (Figure 4d).

3.4. *AE prevented EF-induced liver apoptosis*

Bcl-2, a membrane protein, can prevent the release of pro-apoptotic factors from the mitochondrial outer membrane and thus suppress apoptosis (*20*). As shown in Figures 5A and 5B, EF treatment (350 mg/kg) resulted in a marked decrease in the level of Bcl-2, whereas AE pretreatment reversed such a decrease.

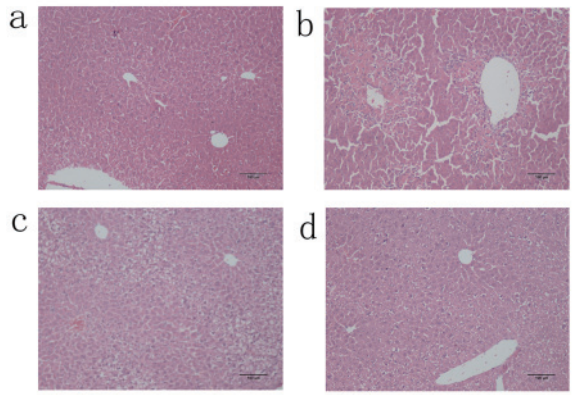

Figure 4. Liver histological evaluation of EF-treated mice with or without preadministration of AE. Liver sections were stained with hematoxylin-eosin. **(a)** Control group, **(b)** EF only group, **(c)** EF + AE (720 mg/kg) group, **(d)** AE (720 mg/kg) group. Magnification, ×100.

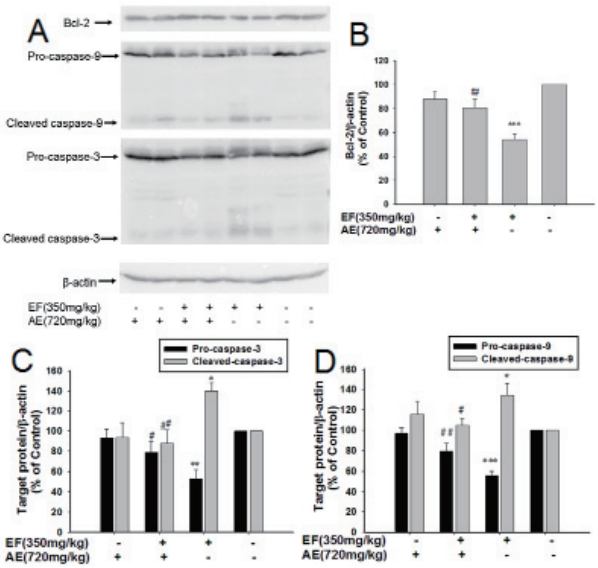

Figure 5. The expression of Bcl-2, and the activation of caspase-9 and caspase-3 in livers of EF-treated mice with or without preadministration of AE. (A) Protein extracts from liver tissue were analyzed by SDS-PAGE and immunoblotted using Bcl-2, caspase-9, capase-3 and β-actin antibodies, respectively. The result represents one of three separate experiments, and the level of β-actin was used as loading control. The bands of Bcl-2 **(B)**, Pro- and Cleaved caspase-3 **(C)** and Pro- and Cleaved caspase-9 **(D)** were normalized to basal β-actin expression and the vehicle control is set as 100%. Data are shown as means ± S.E., and the results represent three separate experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *versus* control, # $p < 0.05$, ## $p < 0.01$ *versus* EF-treated group.

Our results in Figures 5A, 5C, and 5D indicated that the expression of pro-caspase-9 and -3 were decreased due to EF administration, while the cleaved fragments of caspase-9 and -3 were increased significantly. In contrast, the decreased levels of pro-caspase-9 and -3 were increased and the elevated fragments of caspase-9 and -3 were markedly decreased in the livers of mice pretreated with AE.

3.5. *AE reversed EF-changed expression of SOD, GPx, IκB*

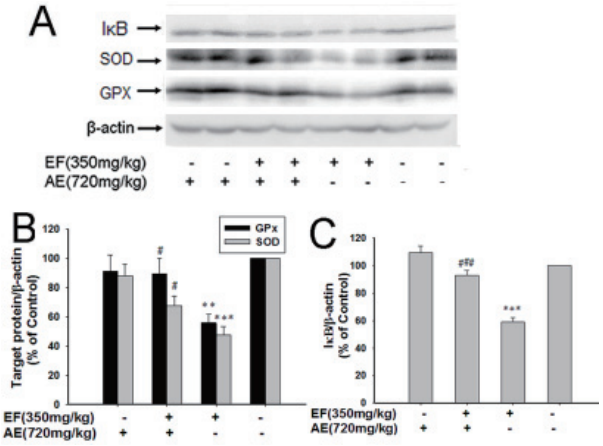

Figure 6. The expression of IκB, SOD, and GPx in livers of EF-treated mice with or without preadministration of AE. (A) Protein extracts from liver tissue were analyzed by SDS-PAGE and immunoblotted using IκB, SOD, GPx, and β-actin antibodies, respectively. The result represents one of three separate experiments, and the level of β-actin was used as loading control. The bands of SOD and GPx **(B)**, IκB **(C)** were normalized to basal β-actin expression and the vehicle control is set as 100%. Data are shown as means ± S.E., and the results represent three separate experiments. ** $p < 0.01$, *** $p < 0.001$ *versus* control, # $p < 0.05$, ### $p < 0.001$ *versus* EF-treated group.

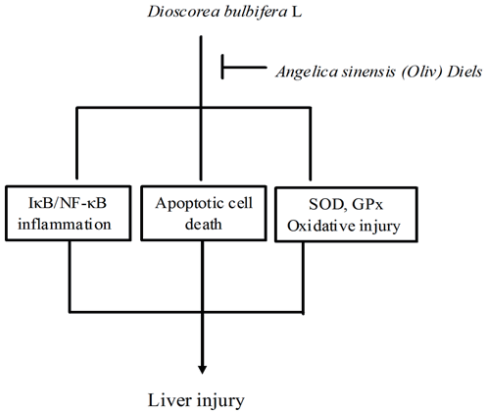

Figure 7. Schematic diagram of mechanism involved in EF-induced liver injury and AE's target(s) of protective action.

As shown in Figures 6A and 6C, EF decreased IκB expression as compared to control, while such a decrease was reversed in mice pretreated with AE. In addition, we also detected the expression of two antioxidant-related enzymes SOD and GPx. The results in Figures 6A and 6B showed that the decreased expression of SOD and GPx induced by EF was reversed by the pretreatment with AE.

4. Discussion

The elevation of ALT, AST, ALP, and TB is the diagnostic indicator for acute liver injury (*21*). As shown in Figure 3, AE can prevent EF-induced liver injury, as indicated by the decreased ALT, AST, and ALP activities and the TB amounts which are increased after EF treatment. Histological analysis further confirmed the protection of AE against EF-induced liver injury (Figure 4). There are some reports about the protection of *A. sinensis* and its main compound ferulic acid against liver injury (*16-18*). In addition, our previous study demonstrated that ferulic acid can prevent diosbulbin B-induced liver injury in tumor-bearing mice (*22*). The present results demonstrate that *A. sinensis* prevented liver injury induced by *D. bulbifera*, and ferulic acid may be the active compound in *A. sinensis*.

Apoptosis is the process of programmed cell death which occurs during animal development and tissue homeostasis, and plays an important role in a variety of physiological and pathological processes (*23*). There are two major pathways during the initiation of apoptosis: the extrinsic pathway and the intrinsic pathway. The extrinsic pathway is related to death receptors, while the intrinsic pathway involves mitochondria: the release of pro-apoptotic factors from the mitochondrial outer membrane and subsequent activation of downstream caspase-9, finally resulting in activation of downstream executioner caspase-3 (*24,25*). Our results showed that EF down-regulated the expression of Bcl-2, pro-caspase-9, pro-caspase-3, while increased the expression of cleaved caspase-9 and caspase-3. In contrast, such phenomena were all reversed in the livers of mice pretreated with AE (Figure 5). All these results suggest that AE prevents EF-induced apoptotic liver injury.

Many studies have demonstrated that reactive oxygen species (ROS) play an important role in various hepatotoxin-induced liver injuries (*26-28*). The body has an effective defense mechanism to prevent and neutralize the free radical-induced damage. Hepatic cellular oxidative stress often takes place during the lack of antioxidants. SOD and GPx, two endogenous antioxidant enzymes, play important roles in the defense against ROS (29). SOD can efficiently and specifically catalyze dismutation of $O^{2\cdot-}$ to O_2 and H_2O_2, while GPx catalyzes the further decomposition of H_2O_2 using reduced glutathione as a substrate (*30,31*). The results show that EF down-regulated the expression of SOD and GPx, while AE prevented such a decrease. The results suggest that *A. sinensis* can elevate antioxidant capacity to counteract with *D. bulbifera*-induced liver injury.

IκB is an inhibitory protein, and it inhibits the transcription of nuclear factor κB (NF-κB), which plays a pivotal role in inflammatory and immune responses (*32-34*). There are already reports that immune dysfunction is involved in alcohol and various drug-induced liver injuries, of which IκB/NF-κB plays an important role (*35,36*). As shown in Figure 6C, AE can reverse EF-induced decrease of IκB expression. The decrease of IκB protein will lead to the activation of NF-κB, and our result indicates that the IκB/NF-κB signal pathway may also be involved in the protection

of *A. sinensis* against the hepatotoxicity induced by *D. bulbifera*.

In the present study we found that *A. sinensis* prevented the hepatotoxicity induced by *D. bulbifera via* inhibiting apoptosis, meanwhile two important antioxidant enzymes SOD and GPx, and IκB may be involved in such protection (Figure 7).

Acknowledgements

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