Sichuan pepper attenuates H_2O_2-induced apoptosis via antioxidant activity and up-regulating heme oxygenase-1 gene expression in primary rat hepatocytes

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Running title: Anti-apoptotic effect of Sichuan pepper

ABSTRACT

Sichuan pepper, a well-known traditional Chinese spice, is popularly used in Chinese cuisine. In this study the antioxidant activities of Sichuan pepper water extract (SP) were evaluated both chemically [in 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) assay] and biologically [in 2,2'-azobis-(2-amidinopropane) (AAPH)-induced haemolysis assay]. The protective effect of SP against H_2O_2-induced cytotoxicity in primary SD rat hepatocytes was investigated by using cell viability assay, nuclear staining, intracellular reactive oxygen species (ROS) measurement and flow cytometry. It was found to possess high total phenolic content as assessed by the Folin-Ciocalteu method. The anti-apoptotic effect of SP may be mediated, in part, by significantly inhibiting intracellular ROS generation and depletion of the mitochondrial membrane potential. SP was also found to enhance the cellular antioxidant defense system through up-regulating heme oxygenase-1 expression. SP is an effective antioxidant that can be consumed regularly as a functional food for the prevention of oxidative stress-related liver diseases.

PRACTICAL APPLICATIONS

The present study demonstrated that Sichuan pepper water extract (SP) could enhance the cellular antioxidant ability in hepatocytes. SP is an effective antioxidant that can

be consumed regularly as a functional food for the prevention of oxidative stress-related liver diseases.

KEYWORDS: *Zanthoxylum bungeanum* Maxim; reactive oxygen species; liver; Rutaceae

INTRODUCTION

Sichuan pepper is the ripe pericarp of the fruit of *Zanthoxylum bungeanum* Maxim, (Rutaceae), and is widely used in Asia as a spice. It is also used as a traditional medicinal herb which is officially listed in *China Pharmacopoeia* as 'Huajiao'. In China, Sichuan pepper is used in the treatment of "colds on stomach", abdomen pains, abdominal or epigastric pain, vomiting, diarrhea, enterozoic abdominalgia and ascariasis (National Commission of Chinese Pharmacopoeia 2010). It is also applied externally to treat eczema and pruritus. Essential oils (e.g. linalool and limonene) (Yang 2008) and alkylamides (e.g. α-hydroxysanshool) (Zhao *et al.* 2013) are major components in Sichuan pepper. Pharmacological research studies indicate that the extract of Sichuan pepper and its major compounds are effective for the treatments of inflammatory diseases (Tezuka *et al.* 2001), asthma (Tang *et al.* 2014) and Alzheimer's disease (Tang *et al.* 2013). In addition, the pericarp of Sichuan pepper is

also used as antimicrobial (Zhu *et al.* 2011) and cosmetic ingredients (Artaria *et al.* 2011).

Liver damage ranges from acute hepatitis to chronic liver diseases such as non-alcoholic fatty liver disease, hepatocellular carcinoma and viral hepatitis. All the processes involve hepatocyte, Kupffer, stellate, and endothelial cells. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) play an important role in the induction and progression of the above-mentioned liver diseases (Loguercio and Federico 2003, Simeonova *et al.* 2014). Hydrogen peroxide (H_2O_2), a representative membrane-permeable oxidant, is one of the major intracellular ROS. H_2O_2-induced intrinsic caspase-dependent apoptosis plays an important role in promoting hepatocytes injury. Furthermore, there is consistent evidence of enhanced production of free radicals and/or significant decrease in antioxidant defense in the liver diseases. Thus, many investigations aim at discovering the pathogenetic significance of therapeutic intervention that could restore the antioxidant potential of cells and, therefore, improve or prevent liver injuries.

Natural products are a good source of antioxidants (Chan *et al.* 2008). Sichuan pepper is a traditional medicinal herb and is commonly use in Chinese cuisine (Tang, Xie, Guan, Jin and Zhao 2014, Yang *et al.* 2014). It was shown to possess high antioxidant activity (Lu *et al.* 2011). For the close relationship between liver diseases

and pro-oxidant, it was postulated that Sichuan pepper with antioxidant property has hepatoprotective effect. The aim of the present study was to evaluate the hepatoprotective effect of Sichuan pepper water extract (SP) on H_2O_2-induced apoptosis in primary rat hepatocytes. To understand the molecular mechanisms involved, the gene expression of heme oxygenase-1 (HO-1) and intracellular ROS generation and mitochondrial membrane potential were also investigated. The present study demonstrated for the first time that SP possesses anti-apoptotic potential in primary rat hepatocytes.

MATERIALS AND METHODS

Chemicals and reagents

High glucose Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), phosphate buffer saline (PBS), trypsin, antibiotics and antimycotics were purchased from Gibco (Grand Island, NY, USA). H_2O_2 was obtained from Calbiochem (San Diego, CA, USA). Hoechst 33342, 2,2-azobis(2-methylpropionamidine) dihydrochloride (AAPH), 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) and Folin-Ciocalteu reagent were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetra

zolium (MTS) assay kit was purchased from Promega (Madison, WI, USA). All other chemicals used were of analytical grade.

Preparation of Sichuan pepper water extract

Sichuan pepper (10 g) was ground into fine powder and mixed with 100 ml distilled water. After shaking in a horizontal shaker at 37ºC and 300 rpm for 2 h, the solution was centrifuged at 3,000 rpm for 30 min. The supernatant was collected, and the residue was re-extracted for two more times with the same volume of distilled water. Finally, the pooled supernatant was lyophilized by a freeze-dryer (Labconco, Freezone 6). Since most antioxidants are heat labile and it was postulated that Sichuan pepper's hepatoprotective effect are closely related to its antioxidant property, extraction was done at 37ºC in water to avoid destroying those heat labile compounds. The extraction yield was 8.40% of the crude material**.** SP was stored at -20°C before use.

Chemical analysis of SP sample

Powder of SP (0.2 g) was accurately weighed and extracted with *n*-hexane (10 mL) for 30 min. The extraction was evaporated to dryness with a rotary evaporator. Finally, the residue was re-dissolved in methanol (1 mL) and filtrated through a 0.45 μm

syringe filter before HPLC-DAD analysis. Chromatographic analysis was carried out on a C18 column (250 mm × 4.6 mm, 5 μm) (Alltima, Grace) at 25°C using an Agilent 1100 liquid chromatography system equipped with a quaternary solvent deliver system, an autosampler and a DAD system. The detection wavelength was 210 nm. The gradient elution of the mobile phase consisting of (A) 0.1% (v/v) acetic acid and (B) acetonitrile is as follows: 60% (A) at 0 – 12 min; from 60% to 5% (A) at 12 – 23 min. The flow rate was 1.0 mL/min and aliquots of 5 μL were injected into HPLC analysis

To identify components in SP sample, an Agilent MSD Trap VL module mass spectrometer was connected to the Agilent 1100 HPLC instrument via an electrospray ionization ESI interface. The LC effluent was introduced into the ESI source in a postcolumn splitting 1:4. Ultra high-purity helium was used as the collision gas, while high-purity nitrogen was used as the nebulizing gas. The parameters in the positive ion mode were listed as follows: the capillary voltage 4000 kv; nebulizing gas at 15 psi, drying gas of 8.0 L/min at 220°C. Data acquisition was performed in the full-scan mode form m/z 100 – 1000 for mass spectrometer and with an accumulation time of 200 ms and 7 microscans averaged per recorded scan.

Total phenolic content assay

The total polyphenol quantification in SP was measured by Folin-Ciocateu method (Lee *et al.* 2012). In brief, the dried SP powder (0.1 g) was dissolved in 1 mL Milli-Q water. 100 µL of SP of appropriate concentrations were mixed with 500 µL of 2N Folin-Ciocalteau reagent (1:10 diluted with Milli-Q water) and 400 µL of Na_2CO_3 (75.05 g/L). The absorbance at 750 nm was measured after 2 h reaction at room temperature. The aqueous gallic acid solution was freshly prepared in a series of concentrations (10 – 80 µg/mL) to establish the standard curve. The total phenolic content in SP was expressed as gallic acid equivalents (GAE) per gram of sample used.

Free radical scavenging capacity

The antioxidant activities of SP were measured by the DPPH assay. According to the procedure described previously (Lee, Zhou, Or, Kwan and Yeung 2012), 50 µL of SP of different concentrations were added to 950 µL of DPPH (24 mg/L) in methanol. After incubation (1 h), the absorbance value of the sample (A_{sample}) was determined at 515 nm, while the absorbance value of a mixture of 950 µL of DPPH in methanol and 50 µL Milli-Q water was measured as control ($A_{control}$). The percentage free radical scavenging capacity (SR%) was calculated using the following equation: $SR\% = \left(1 - \frac{A_{Sample}}{A_{Control}}\right) \times 100\%$. The concentrations of SP for scavenging 50% free

radical were recorded as SC_{50}.

Inhibition of AAPH-induced hemolysis in human red blood cells

Free radicals generated by AAPH attack polyunsaturated fatty acids of the membrane of red blood cells (RBCs) to cause hemolysis. The inhibition of AAPH-induced hemolysis by SP was measured as described previously (Guo *et al.* 2008). Briefly, RBCs were collected from heparinized whole human blood (male, 30-40 years old; from Nanshan chronic Diseases Prevention & Cure hospital, Shenzhen, China) by centrifugation at 625 × *g* for 10 min, and re-suspended with PBS (20%, *v/v*). 600 μL of RBC suspension was mixed with 600 μL SP (0.3-0.5 mg/mL) or PBS. Then, the mixtures were incubated by rolling at 22 rpm and 37 °C for 10 min before adding 300 μL AAPH (400 mM in PBS). After incubation for 2 h, 100 μL of the mixtures were added into chilled PBS (1.25 mL) for dilution or into water (1.25 mL) to completely lyse the RBC. The supernatant was collected by centrifugation at 385 × *g* for 10 min, and its absorbance was measured at 540 nm.

Animals

Male Sprague Dawley rats (260 – 280 g) were supplied by the Laboratory Animal Service Centre, The Chinese University of Hong Kong (CUHK). Animals were

housed in a 12-h light–dark cycle holding room with free access to rat chow (Glen Forrest Stockfeeders, Australia) and tap water. All the experimental procedures had been approved by the Animal Experimentation Ethics Committee (CUHK) in accordance to the guidelines in Care and Use of Animals regulated by Department of Health (HKSAR).

Culture of primary rat hepatocytes

Primary rat hepatocytes were prepared by a two-step collagenase perfusion technique as described in our previous publication (Lee, Zhou, Or, Kwan and Yeung 2012). A batch of hepatocytes was used in subsequent experiments. Freshly isolated rat hepatocytes (2×10^5 cells/cm^2) were seeded for 3 h in a plating medium containing DMEM supplemented with 10% (*v/v*) FBS, 1% (*v/v*) antibiotics and antimycotics, and 100 nM dexamethasone. The plating medium was then replaced by culture medium [DMEM supplemented with 0.1% (*v/v*) BSA, 1% (*v/v*) antibiotics and antimycotics, 100 nM dexamethasone].

Cell viability in primary rat hepatocytes

Primary rat hepatocytes were pre-treated with SP for 24 h which was then incubation in H_2O_2 for 24 h. After treatment, the medium was replenished with a medium (100

μL/well) containing 2% FBS. 20 μL of the CellTiter 96® AQueous One Solution Cell Proliferation Assay reagent (Promega Corporation, Madison, USA) was added into well containing 100 μL culture medium at 37°C for incubation for another 2 h. Absorbance at 490 nm was measured using a microplate reader (Bio-Rad, model 550, USA).

Determination of intracellular ROS level

The level of intracellular ROS was measured with a fluorescent dye, 2',7'-dichlorodihydrofluorescein diacetate, (DCFH-DA; Molecular Probes, Eugene, OR), which would be oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of intracellular ROS. Cells were pretreated with different concentrations of SP for 24 h, then treated with H_2O_2 (750 μM) for 6 h. They were then washed once with PBS and incubated with 10 μM DCFH-DA at 37°C for 30 min. Next, they were washed again with PBS to remove the dye, and the florescent signals were scanned with a plate reader (Wallac; PerkinElmer) at excitation at 485 nm and emission at 535 nm. Results were expressed as percentage increase in fluorescence intensity (Zhang *et al.* 2011).

PCR analysis for HO-1 expression

Primary rat hepatocytes were treated with 750 μM H_2O_2 in the presence or absence of SP for 24 h. After incubation for 24 h, total RNA was extracted from cells by using TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA) according to the standard protocol. RNA was precipitated by mixing with isopropyl alcohol. The RNA pellet was washed twice with cold 75% alcohol and dissolved in diethylpyrocarbonate-treated water. 2 μg of RNA was reverse transcribed to first strand DNA (cDNA) by Reverse transcriptase (Applied Biosystem) with oligo$(dT)_{18}$, dNTP Mix and RNase inhibitors, according to the manufacturer's instruction. PCR was then performed using 0.2 μM of primers, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP Mix (Invitrogen), 1U of *Taq* polymerase and 1 μL of cDNA to a final reaction volume of 20 μL. The reaction was carried out at a one-step denaturation of 94°C for 3 min, followed by 35 cycles of 30 s denaturation at 94°C, 1 min annealing at 60°C, and 1 min extension at 72°C. 10 μL of PCR products were run on 1.5% agarose gel and visualised by ethidium bromide staining. The sequences of the PCR primers for HO-1 and GAPDH were: HO-1 forward: 5′- AAGGCGGTCTTAGCCTCTTC-3′, HO-1 reverse: 5′- AGCATGTCCCAGGATTTGTC-3′; GAPDH forward: TGCACCACCAACTGCTTAG-3′, GAPDH reverse: 5′-AGTGGATGCAGGGATGATGT-3′. Expression levels of cDNA were compared with internal standard, GAPDH gene, to correct for the RNA quantity differences between

samples. Each reaction was performed in triplicate.

Hoechst staining assay

Chromatin condensation was detected by nucleus staining with Hoechst 33342. Primary rat hepatocytes (2×10^6 cells/well) grown in a 6-well plate were washed with ice-cold PBS. Primary rat hepatocytes were pre-treated with 0.5 mg/mL SP for 24 h which was then removed before incubation in 750 μM H_2O_2 for 4 h. Cells were then stained with Hoechst 33342 (5 μg/mL) for 5 min at 4°C. Nuclei were visualized using a fluorescence microscope at 400 × magnification.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was quantified using JC-1 dye (Life Technologies, Molecular Probes, USA). In brief, cells were harvested by trypsinization and centrifuged at 1200 rpm and 4°C for 5 min. Cell pellets were washed twice with PBS, and then re-suspended in 500 μL PBS. JC-1 dye (4 mM in stock) was added to samples at a final concentration of 4 μM and incubated at 37°C for 20 min. The green fluorescence represents JC-1 monomers, whereas red fluorescence represents JC-1 aggregates. The proportion of aggregated vs. monomeric JC-1 probe was quantified using the ratio of fluorescence emissions at 590 nm (red) over 530 nm (green) with a

FACSCalibur flow cytometer (Zhang, Mak, Cui, Li, Han, Hu, Ye, Pi and Han 2011).

Statistical analysis

Data were expressed as means ± standard error of mean (SEM). Analysis of variance (ANOVA) followed by Bonferroni's post-test was used for statistical comparisons, and $p < 0.05$ or less was considered to be of statistical significance.

RESULTS

HPLC Fingerprint

A representative HPLC-UV chromatogram is shown in Figure 1A. A total of three major peaks were identified from the sample by the mass spectrometer. Their identities (Figure 1B) were confirmed by comparing the MS fragmentation patterns from previous study (Wang *et al.* 2011) and relative content (in terms of peak area under the 210 nm detection wavelength) in the SP sample were hydroxy-ε-sanshool (1.03%), hydroxy-α-sanshool (6.93%), and hydroxy-β- sanshool (2.58%).

Total phenolic content and free radical scavenging capacity of SP

Flavonoid and phenolic compounds are major antioxidants found in medicinal herbs. In this study, the Folin-Ciocalteu method was employed to estimate the phenolic

components in SP. The result indicated that there was a significant amount of phenolic compounds in the SP. Total phenolic content was 56.53 ± 1.12 mg GAE/g dry extract. The overall free radical scavenging capacity of SP was determined using the DPPH method. The SC_{50} of SP was 0.01 mg/mL.

Inhibition of AAPH-induced RBC haemolysis

As shown in Figure 2, the inhibitory effect of SP on AAPH-induced haemolysis was evaluated. Without AAPH, a negligible haemolysis response (close to 0%) was observed in the experimental period (180 min). However, addition of AAPH to RBC suspension resulted in about 20% haemolysis after incubation for 60 min and a 100% haemolysis was achieved at 180 min (Figure 2A). Our results clearly showed that SP (0.5 – 5 mg/mL) significantly suppressed AAPH-induced haemolysis in a concentration-dependent manner in RBC (Figure 2B).

SP reduces H_2O_2-induced cell death in primary rat hepatocytes

The effect of H_2O_2 (250 - 1,500 μM) on the viabilities of primary rat hepatocytes was evaluated before applying SP. As shown in Figure 3A, cell viability was significantly reduced in a concentration-dependent manner. Based on these results, 750 μM H_2O_2 for 24 h was selected as an optimal treatment for the subsequent experiments. To

investigate the effects of SP on H_2O_2-induced toxicity, primary rat hepatocytes were pre-treated with SP (0.1 – 1 mg/mL) for 24 h and changed to drug free medium before being incubated in 750 μM H_2O_2 for another 24 h. Compared with 750 μM H_2O_2-treated cells, SP (0.3 – 1 mg/mL) showed hepatoprotective effect against H_2O_2-induced cell death in a concentration-dependent manner (63.50 ± 3.01%; $p < 0.01$; 79.75 ± 2.72%, $p < 0.001$ and 73.00 ± 1.47%, $p < 0.001$, respectively) (Figure 3B). Treatment with SP alone showed no effects (data not shown).

SP reduces H_2O_2-induced intracellular ROS generation in primary rat hepatocytes

The intracellular ROS in primary rat hepatocytes was measured by using the fluorescent probe DCFH-DA. It was found that incubating cells with H_2O_2 (750 μM) for 2 h significantly increased intracellular ROS by 8.4-fold compared with normal control. Pre-treatment of cells with SP (0.5 – 1 mg/mL) for 24 h significantly reduced this increase of ROS levels induced by H_2O_2 (Figure 4).

SP attenuates H_2O_2-induced apoptosis in primary rat hepatocytes

Hoechst 33342 staining assay showed that 0.5 mg/mL SP significantly decreased the number of cells with nuclear condensation and apoptotic bodies (Figure 5A). Treatment of SP alone had no effect on the cell nuclei as compared with that of the

control group. To further confirm that apoptosis is caused by H_2O_2, mitochondrial membrane potential assay was conducted on the above-mentioned primary rat hepatocyte cultures. Pre-treatment of cells with 0.5 mg/mL SP significantly prevented the changes of mitochondrial membrane permeability induced by H_2O_2 (Figure 5B and C).

SP up-regulates the gene expression of HO-1 in H_2O_2-stimulated primary rat hepatocytes

H_2O_2-induced down-regulation of HO-1 mRNA expression was observed (Fig. 6). SP (0.5 mg/mL) markedly reversed the H_2O_2-mediated decrease in HO-1 mRNA expression (0.79 ± 0.06%, $p < 0.001$) as compared with the H_2O_2 alone group (0.29 ± 0.04%).

DISCUSSION

Oxidative stress is a common pathogenetic mechanism contributing to the initiation and progression of hepatic damage in a variety of liver disorders. Excessive ROS can induce liver cell death by either apoptosis, necrosis or autophagy (Bhattacharya *et al.* 2011, Lai *et al.* 2011). Cell death is mediated by ROS-induced intracellular signal molecules such as mitogen-activated protein kinases (MAPKs), NF-κB, JNKs, tumor

necrosis factor-alpha (TNF-α), Akt and activated caspases (Cohen *et al.* 2009, Conde de la Rosa *et al.* 2006, Liu *et al.* 2011, Robinson and Mann 2010, Schlatter *et al.* 2011). Thus, antioxidants are believed to be effective on facilitating the treatment of liver diseases through antagonizing ROS's effects (Arteel 2003, Singal *et al.* 2011). Phenolic compounds, which are widely found in fruit, natural plants and Chinese medicinal herbs, possess ideal chemical structures for free radical scavenging activity (Kwok *et al.* 2013). Several studies have indicated that the antioxidant activities of some fruits and traditional Chinese medicine are highly correlated to their total phenolic contents (Guo, Cheng, Chan and Yu 2008, Negi *et al.* 2011).

SP contains complicated components which makes it impossible to estimate its antioxidant property by simply a single assay. Different methods have been adopted to measure the antioxidant activity of SP. In addition to the aforementioned cell-free methods, biological tests have been used to evaluate the protective effects of SP against AAPH-induced haemolysis in RBC. These indicate the possibility of employing SP as an antioxidant substance to ameliorate oxidative damage.

In this study, SP showed hepatoprotective effect against H_2O_2-induced cell death. The mechanism was further confirmed by Hoechst staining and mitochondrial membrane potential assays, showing that SP prevented primary rat hepatocytes against H_2O_2-induced apoptosis. By Hoechst 33342 staining assay, the number of

stained apoptotic nuclei caused by H_2O_2 decreased after the pretreatment of SP. For its anti-apoptotic mechanisms, it is further demonstrated that SP can suppress the generation of intracellular ROS induced by H_2O_2 in primary rat hepatocytes. Mitochondrial membrane is one of the important targets attacked by ROS (Tripathi *et al.* 2009, Weng *et al.* 2007). A decrease in mitochondrial membrane potential was observed in hepatocytes under H_2O_2 treatment (the ratio was 70.55 %), and SP pretreatment (the ratio was 86.00%) significantly attenuated H_2O_2-induced decrease of mitochondrial membrane potential (Figure 5B-C). HO-1, the rate-limiting enzyme of heme degradation, has powerful antioxidant properties. PCR assay demonstrated that SP upregulated HO-1 expression to protect primary rat hepatocyte against oxidative stress (Figure 6). Activation of HO-1 has a potential role in reducing H_2O_2-induced oxidative stress (Aggeli *et al.* 2006, Jian *et al.* 2011, Wu *et al.* 2006). Collectively, these results prove that SP could lead to the reduction of H_2O_2-induced oxidative stress and the prevention of H_2O_2-induced apoptosis in primary rat hepatocytes.

Sichuan pepper contains many phytochemicals. It was found that essential oils (e.g. linalool and limonene) (Yang 2008) and alkylamides (e.g. α-hydroxysanshool) (Zhao, Zhu, Zhong, He, Luo and Gao 2013) are major components in Sichuan pepper. Flavonoids and phenols are other key compounds found in it. Essential oils,

flavonoids and phenols contribute mainly to several biological activities including anti-oxidative and antibacterial properties of Sichuan pepper (Di *et al.* 2012, Dziri *et al.* 2012). SP's liver protective effect may contribute to its anti-oxidative effect. Alkylamides from the fruit of Sichuan pepper produce a strong tingling sensation in the mouth, making Sichuan pepper a well-known spice in Chinese and Japanese cuisines. Alkylamides such as hydroxy-α-sanshool are one of the active ingredients in Sichuan pepper and have been demonstrated to have stimulatory effects on cultured sensory neurons (Bautista *et al.* 2008). In this study, 3 major alkylamides, hydroxy-ε-sanshool (1.03%), hydroxy-α-sanshool (6.93%), and hydroxy-β-sanshool (2.58%), were determined. There is little research on their antioxidant and liver protection effects; further studies on investigating these activities are warranted.

CONCLUSIONS

The present study demonstrated the anti-apoptotic potential of SP *in vitro* as illustrated in Figure 7. This effect may be mediated, in part, by the antioxidative function of SP. Mechanistic studies demonstrated that SP suppressed oxidative stress to protect primary rat hepatocytes by decreasing ROS generation, maintaining mitochondrial membrane potential and upregulating HO-1 expression. Therefore, Sichuan pepper has potential therapeutic effects on treating/preventing oxidative

stress-related hepatocyte injuries and liver dysfunctions.

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CONFLICT OF INTEREST

There is no conflict of interest associated with the authors of this paper.

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Figure Legend

Figure 1. (A) Representative HPLC-UV (at 210 nm) chromatogram of the SP sample used in the current study. (1) hydroxy-ε-sanshool, (2) hydroxy-α-sanshool and (3) hydroxy-β-sanshool. (B) Mass spectrum of hydroxy-ε-sanshool, hydroxy-α-sanshool and hydroxy-β-sanshool.

Figure 2. Percentage hemolysis of AAPH-induced RBC hemolysis in control (A) and SP (B). Data are expressed as means ± SEM (n = 5). $^{###}p < 0.001$ versus control group (without AAPH) at the same time point. $^{*}p < 0.05$, $^{**}p < 0.01$ versus control group (AAPH only).

Figure 3. SP inhibited H_2O_2-induced cell death in a concentration-dependent manner. (A) H_2O_2 induced cytotoxicity in a concentration-dependent manner. Primary hepatocytes were exposed to 250, 500, 750, 1,000 or 1,500 μM H_2O_2 for 24 h. (B) SP concentration-dependently prevented H_2O_2-induced cell death. Hepatocytes were treated with SP at concentrations of 0.1, 0.3, 0.5 or 1 mg/mL for 24 h which was then removed before incubation in 750 μM H_2O_2 for 24 h. Cell viability was measured by MTS assay at 24 h after H_2O_2 treatment. Expressed as percentage of control, data were the mean ± SEM of three individual experiments. $^{#}p < 0.05$, $^{###}p < 0.001$ versus

control, and $^{**}p < 0.01$, $^{***}p < 0.001$ versus H_2O_2 alone group. White arrows indicate cells with nuclear condensation.

Figure 4. SP reduced H_2O_2-induced intracellular ROS generation in primary rat hepatocytes using DCFH-DA staining. Data were the mean ± SEM of six individual experiments. $^{###}p < 0.001$ versus control, $^{*}p < 0.05$ and $^{**}p < 0.01$ versus H_2O_2 alone.

Figure 5. SP attenuated H_2O_2-induced apoptosis in primary rat hepatocytes. (A) Hepatocytes were treated with 0.5 mg/mL SP for 24 h which was removed before incubation in 750 μM H_2O_2 for 4 h. The cells were then analyzed by Hoechst 33342 staining assay. (B) Effects of SP on the alteration of mitochondrial membrane potential induced by H_2O_2. The mitochondrial membrane potential was evaluated with the potential sensor JC-1 and analyzed by flow cytometry. (C) Relative ratio of geometric red/green fluorescence. Data were the mean ± SEM of three separate experiments. $^{#}p < 0.05$ versus control and $^{*}p < 0.05$ versus H_2O_2 alone.

Figure 6. SP increased the gene expression of HO-1 in primary rat hepatocytes treated with H_2O_2. Hepatocytes were treated with 0.5 mg/mL SP for 24 h which was then removed before incubation in 750 μM H_2O_2 for another 24 h. After 24 h of incubation, HO-1 mRNA expression was examined by real time RT-PCR. Data (the ratio of HO-1

mRNA level to GAPDH mRNA level) were expressed as the mean ± SEM of three independent experiments. $^{###}p < 0.001$ versus control and $***p < 0.001$ versus H_2O_2 alone.

Figure 7. Schematic illustration of the mechanisms of SP's hepatoprotective effects.