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In vitro and *in vivo* studies on the antioxidant activities of the aqueous extracts of Douchi (a traditional Chinese salt-fermented soybean food)

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Abstract

The aqueous extracts of Douchi were obtained and evaluated for their antioxidant properties. The isoflavones and peptides contents of extracts were determined. Antioxidant activities *in vitro* of extracts were conducted by determining the α,α-diphenyl-β-pricrylhydrazyl (DPPH) and 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging activities, and the chelating ability of ferrous ions, of which IC_{50} values were found to be 0.658, 0.204 and 206 mg/mL, respectively. Antioxidant enzymatic activities of extracts in cholesterol-fed rats and an index of lipid peroxidation (thiobarbituric acid reactive substances (TBARS)) were determined, and hepatic tissue ultramicrostructure was also observed under transmission electron microscope (TEM). These results showed that, in Douchi extracts groups, superoxide dismutase (SOD) activities in liver and kidney, catalase (CAT) activity in liver, and glutathione peroxidase (GSH-Px) activity in kidney increased significantly compared with the negative control group ($p < 0.05$). TBARS in liver and kidney of extracts groups decreased significantly ($p < 0.05$). Less fatty degeneration in hepatocytes of extracts groups was found on TEM photos. The percentage of total isoflavones and peptides contents in aqueous extracts were 0.087% and 40.7%, respectively. These results showed that Douchi extracts had excellent antioxidant activities, might affect the activities of antioxidant enzymes and lipid peroxidation, and mitigate the lipidosis of hepatocytes.
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Keywords: Douchi; Antioxidant activity; Isoflavones; Peptides

1. Introduction

Oxidative-free radicals are byproducts of the normal reactions within our body. These reactions include the generation of calories, the degradation of lipids, the catecholamine response under stress, and the inflammatory processes (Ikeda & Long, 1990). If the balance between oxidative-free radical production and eradication is maintained, the harmful effects of free radicals would be minimized in the body. However, if the unwanted free

Abbreviations: ABTS, 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid); CAT, catalase; DPPH, α,α-diphenyl-β-pricrylhydrazyl; EDTA, ethylene diaminetetraacetic acid; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; OPA, *o*-phthalaldehyde; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TEM, transmission electron microscope; VE, vitamin E.

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radicals are not eradicated efficiently, oxidative stress would occur. Oxidative stress, caused by reactive oxygen or free radicals, has been shown to be associated with the progression of many diseases including cancer, heart disease, and depression, among others (Kovacic & Jacintho, 2001; McCord, 2000; Parola & Robino, 2001). In order to protect tissues and organs from oxidative damage, the body possesses both enzymatic and non-enzymatic systems. The main enzymes include superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT). These enzymes are our frontline defenders against oxidative damage. In addition to these enzymatic systems, the non-enzymatic mechanisms can also protect the body from the damages caused by oxidative stress.

One of the most popular natural antioxidants is vitamin E (VE). It acts as a peroxyl radical scavenger. Recently, investigators and consumers are interested in seeking natural antioxidant components in the diet, which may help to reduce oxidative damage. Epidemiologic studies have shown that the consumption of soybean products as foods can reduce the occurrence of cancer, osteoporosis, and cardiovascular diseases in humans (Carrol, 1991; Messina, Persky, Setchell, & Barnes, 1994). Many antioxidant components have been found in soybean foods, such as isoflavones, tocopherols, phospholipids, chlorogenic acid isomers, caffeic acid, ferulic acid, peptides, amino acids, and melanoidin (Hayes, Bookwalter, & Bagley, 1977; Pratt & Birac, 1979).

The fermented soybean food considered in this study is "Douchi", which is one kind of many traditional Chinese foods consisting of fermented soybeans. It has played very important roles in Chinese diets for centuries. Douchi has been used as seasoning in foods and for medicinal purpose even before the *Han Dynasty* (206 BC). Today it is still added to some traditional Chinese medicines. As the population who consume foods with Douchi added is increasing, there is an increasing interest in its physiological properties. These include antioxidant activity (Chen et al., 2005; Wang et al., 2007a), anti-hypertensive activity (Zhang, Tatsumi, Ding, & Li, 2006a), and α-glucosidase inhibitory activity (Fujita, Yamagami, & Ohshima, 2001a, 2001b; Fujita & Yamagami, 2001c).

Natto and tempeh are fermented soybean foods similar to Douchi (Li, Zhang, Li, & Tatsumi, 2003). They were found to have remarkable antioxidant activities (Esaki, Nohara, Onozaki, & Osawa, 1990; György, Murata, & Ikehata, 1964; Iwai, Nakaya, Kawasaki, & Matsue, 2002a, 2002b; Rilantono, Yuwono, & Nugrahadi, 2000; Sheih, Wu, Lai, & Lin, 2000; Yokota, Hattori, Ohishi, Hasegawa, & Watanabe, 1996). As such, the *Aspergillus*-type Douchi, the most common type, which grows wildly in China, is expected to have certain antioxidant activity. In a previous study, we found that some antioxidant compounds were synthesized during Douchi fermentation (Wang et al., 2007a). So far, there are no reported studies on the antioxidative activities of Douchi extracts *in vivo*, and their roles in liver lipid metabolism.

In this study, the antioxidative properties of Douchi extracts *in vitro* were investigated by determining the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and the 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging activities, and the chelating ability of ferrous ions. In the *in vivo* study subsequently, rats were fed with cholesterol-rich diets containing different amounts of Douchi extracts and vitamin E (VE). Enzyme activities of SOD, GSH-Px, CAT and the values of thiobarbituric acid reactive substances (TBARS) in liver and kidney were detected. The ultramicrostructure of liver was observed under transmission electron microscope (TEM).

2. Materials and methods

2.1. Preparation of Douchi extracts

Douchi was prepared as previously described (Wang et al., 2007a). (1) Pre-treatment. Soybeans were washed, and soaked in tap water for 8 h at room temperature (24 ± 2 °C). After draining, the soybeans were steamed for 30 min at 121 °C in a retort (YMQ.L31.400, Beijing Jiangtai Medical Instrument Co. Ltd., Beijing, China). (2) Pre-fermentation. The cooked soybeans were cooled to 30–35 °C, and then inoculated quickly with *Aspergillus oryzae* 3.951 incubated at 30 °C for 60 h, around 80% relative humidity in an incubator (LTI-601SD; Tokyo Rikakikai CO., Ltd, Tokyo, Japan). Semi-finished products were called the Douchi qu (koji). (3) Post-fermentation. The Douchi qu were salted until the content of NaCl reached about 5% (w/w). The sample was ripened for four weeks at 35 °C in the same incubator.

Douchi extracts were isolated from the fresh Douchi. Firstly, fresh Douchi sample (1000 g) was milled to paste and suspended in 9000 mL water before boiling for 60 min and was left at room temperature. After 16 h, the mixture was centrifuged at 2000*g* for 15 min at room temperature, then the supernatant was filtered by filter paper and the filtrates were concentrated by a vacuum rotary evaporator. Finally, the concentrated extract was lyophilized to make into powder using a mortar and a pestle.

2.2. Proximate compositions analysis of Douchi extracts

Freeze-dried powder (Douchi extracts) was measured. Official methods (AOAC., 2000) were used to analyze chemical compositions, including moisture (Vacuum Oven Method 925.09), protein (Method 955.04 using 6.25 as conversion factor), lipids (Method 945.39), ashes (Method 924.05), and salts (Method 937.09).

2.3. Measuring isoflavones contents of Douchi extracts

The contents of isoflavones in the Douchi extracts were determined as described previously (Wang et al., 2007b). Freeze-dried powder (2 g) was extracted with 50 ml of 80% methanol at 80 °C for 4 h by a Soxhlet extractor

and the extractants filtered through a 0.45 μm filter unit. Isoflavones were analyzed quantitatively by high pressure liquid chromatography (HPLC). The HPLC system used was a Shimadzu HPLC (Kyoto, Japan), consisting of an LC-10AT pump, a UV detector (SPD-10AVVP), and a Dikma Diamonsil C_{18} column (4.6 × 250 mm) (Dima Co., Ltd., Orlando, FL). The mobile phases for HPLC consisted of solvent (A) 0.1% (v/v) acetic acid in filtered MilliQ water, and (B) 0.1% (v/v) acetic acid in acetonitrile. The solvent gradient was as follows: Solvent B was increased from 15 to 25% over 35 min, then increased to 26.5% within the next 12 min, and finally increased to 50% within 30 s prior to being held for 14.5 min. The flow rate was 1.0 ml/min. The column temperature was 40 °C and the absorption was measured at 254 nm. Quantitative data for each isoflavone was obtained by comparison to known standards. In order to estimate total isoflavone amounts, individual isoflavone glucodides and aglycones were normalized for their molecular weight differences and summed and reported as total isoflavones.

2.4. Measuring peptides contents of Douchi extracts

The contents of peptides were determined according to the OPA (*o*-phthalaldehyde) method (Church, Swaisgood, Porter, & Catignani, 1983) with some modifications. Samples (50 μL) were added into 2 mL of OPA mixture (50 mL of a mixture containing 25 mL of 100 mM sodium tetraborate, 2.5 mL of 20% (w/w) sodium dodecyl sulphate, 40 mg of OPA dissolved in 1 mL methanol, 100 μL of β-mercaptoethanol, and 21.4 mL distilled water). After 4 min incubation at room temperature, the absorbance at 340 nm of the resulting solution was measured. The peptide content was calculated on the basis of standard curve with glutathione as the standard.

2.5. Measuring the antioxidative activities of Douchi extracts in vitro

2.5.1. Measuring DPPH and ABTS radical scavenging activities

The DPPH radical scavenging activity of Douchi extracts were determined according to the methods described by Suda (2000) with some modifications. Diluted Douchi extracts (100 μL) were mixed with 100 μL of 200 μM DPPH solution in 0.1 M MES buffer (pH 6.0), containing 80% ethanol was then added. The mixture was kept at room temperature for 20 min, then the absorbance of the resulting solution was measured at 520 nm using a 96-well plate reader (Model 550, Bio-Rad Laboratories, Tokyo, Japan).

ABTS radical scavenging assay was based on the method developed by Robert et al. (1999) with some modifications. ABTS·+ was generated by reacting ABTS (7.4 mM) with potassium persulphate (2.6 mM). The solution was diluted to obtain an absorbance of 1.4 units at 414 nm with ethanol. The ABTS·+ antioxidant reaction mixture contained 200 μL of ABTS·+ solution, and 50 μL of Douchi extracts or water for the control. The absorbance at 420 nm of the resulting solution was measured at 6 min of the reaction by a 96-well plate reader (Model 550, Bio-Rad Laboratories, Tokyo, Japan).

The scavenging activities were expressed as IC_{50}, which is the inhibitory concentration of the test samples that inhibits 50% substrate. Trolox was used to compare the DPPH and ABTS radical scavenging activities.

2.5.2. Measuring chelating ability for ferrous ions

Chelating abilities of the Douchi extracts were determined by the method of Dinis, Madeira, and Almeida (1994). Sample solution (0.1 mL) and 0.1 mL, 0.2 mM $FeCl_2 \cdot 4H_2O$ were added into 3 mL deionized water; the mixture was stored at room temperature (28 ± 2 °C) for 30 s. The reaction mixture thus obtained was added with 0.1 mL 5 mM ferrozine (Sigma, St. Louis, MO, USA) later and changes in the absorbance of the Fe^{2+}–ferrozine complex were monitored at 562 nm against a blank with a spectrophotometer (UV mini-1240, Shimadzu, Kyoto, Japan) after 10 min resting time at room temperature. The chelating effects were expressed as IC_{50}, which is the inhibitory concentration of the test samples that inhibits 50% substrate. Ethylenediaminetetraacetic acid (EDTA) was used to benchmark the chelating abilities.

2.6. Measuring the antioxidant activities of Douchi extracts in vivo

2.6.1. Grouping of animals and preparation of diets

The experimental animals, three-week-old male Wistar rats (80–100 g), were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences of China, Beijing, China. The rats were housed individually in stainless wire netting cages in an air-conditioned room (temperature: 21–23 °C; relative humidity: 55–60%). These animals were randomized into four groups and seven rats each (named as negative control, positive control, Douchi extracts 1 and Douchi extracts 2 groups, respectively). The negative control rats were fed with basic diet without any Douchi extracts or VE. The diet of positive control rats was added with 0.0025% VE. The diet of Douchi extracts 1 and Douchi extracts 2 groups were added with 2% and 4% freeze-dried Douchi extracts flour, respectively.

The basic diet of rat was purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences of China, Beijing, China. The diet compositions are given in Table 1. VE of analytical grade was obtained from Sigma Chemical (St. Louis, MO, USA). The dietary cholesterol was analyzed using the high performance liquid chromatography (HPLC) (Ansari, Smith, & Smart, 1979). No cholesterol oxides were found in the basic diet. The 0.5% (w/w) cholesterol batches were mixed carefully into the basic diet just before the rats were fed. After four weeks of the experimental period, all rats were fasted 12 h before operation.

Table 1
Compositions of experimental diets for four groups of rats (%, w/w)

	Negative	Positive	Douchi extracts 1	Douchi extracts 2
Corn starch[a]	50	50	48.9	47.9
Casein[a]	23.2	23.2	22.3	21.3
Soybean oil	5.6	5.6	5.6	5.6
Cellulose	3.2	3.2	3.2	3.2
Mineral mixture	6.7	6.7	6.7	6.7
Vitamin mixture[b]	1	1	1	1
Cholesterol	0.5	0.5	0.5	0.5
Water	9.8	9.8	9.8	9.8
VE	0	0.0025	0	0
Douchi extracts	0	0	2	0
Douchi extracts	0	0	0	4

[a] Starch and casein were adjusted by the contents of proteins in Douchi extracts.
[b] There was no VE in the vitamin mixture.

2.6.2. Collection of in vivo samples

The animals were cared for according to the Guiding Principles in the Care and Use of Animals. The experiments were approved by Peking University Council on Animal Care Committee. The anesthetized rats (by inhaling diethyl ether) were fixed on an experimental desk. Blood was collected from a fine catheter inserted into the celiac artery. When the collection of blood sample finished, 20 mL normal saline were perfused through the catheter to wash the blood out. The liver and kidney were then harvested.

The liver and kidney were cut into pieces and milled to paste, added with normal saline to prepare 10% solution of tissue homogenate, and then the tissue homogenate was centrifuged at 2000*g* for 10 min and the supernatant was kept. All the samples of liver and kidney were used to measure enzyme activities of SOD, GSH-Px, and the values of TBARS.

The 10% tissue homogenate was then diluted to a 1% solution with normal saline and homogenized by centrifugation at 2000*g* for 10 min and the supernatant was kept. These samples were used to measure the CAT activity.

2.6.3. Measuring protein content

The protein content in each sample was measured using a bovine serum albumin (BSA) protein Assay Kit (Institute of Biological Engineering of Nanjing Jianchen, Nanjing, China) with BSA as the standard (Smith et al., 1985).

2.6.4. Measuring SOD activity

SOD activity was determined with SOD Assay Kit A001 (Institute of Biological Engineering of Nanjing Jianchen, Nanjing, China). Superoxide was generated in xanthing oxidase and hypoxanthine, and the superoxide scavenging effect of serum and tissue was determined according to Oyanagui's method (Oyanagui, 1984). Fifty percent inhibition was defined as one unit of SOD activity. SOD activities of the liver and kidney were expressed in nmol per mg protein of the sample.

2.6.5. Measuring CAT activity

Catalase activity was determined with CAT Assay Kit A007 (Institute of Biological Engineering of Nanjing Jianchen, Nanjing, China) by colorimetric method. It is based upon alteration of H_2O_2 optical density, depending on enzymatic decomposition of H_2O_2 (by the effect of CAT in the sample) (Summner & Dounce, 1937). CAT of hemoglobin was changed to k/g Hb after the '*k*' value was determined, taken into account suitable absorbance for each analysis according to calculated regression. The CAT activities of liver and kidney were expressed in units per gram of protein.

2.6.6. Measuring GSH-Px activity

GSH-Px activity was determined with a GSH-Px Assay Kit A005 (Institute of Biological Engineering of Nanjing Jianchen, Nanjing, China). The GSH-Px had the ability to decompose hydrogen peroxide (H_2O_2) and other organic hydroperoxides (ROOH). The reaction uses glutathione to complete the reaction. Hydrogen peroxide, or H_2O_2, was used as substrate of glutathione. Consumption of nicotinamide adenine dinucleotide phosphate (NADPH) was used to determine the GSH-Px activity.

2.6.7. Measuring the values of TBARS

The value of TBARS, an index of lipid peroxidation, was determined with a malondialdehyde (MDA) Assay Kit A003 (Institute of Biological Engineering of Nanjing Jianchen, Nanjing, China). The value of TBARS was estimated according to the thiobarbituric acid (TBA) method (Asakawa & Matsushita, 1980). The samples added with TBA were heated in an acidic environment. The absorbance of resulting solution was measured at 532 nm. The value of TBARS was expressed in nmol per mg protein of the sample.

2.6.8. Histological study (TEM of liver)

Samples of fresh liver were cut into small pieces (about $1 \times 1 \times 1$ mm), fixed in 3% (v/v) glutaraldehyde for 1 h and rinsed 3 times with phosphate buffer (0.1 mol/L, pH 7.4). The samples were then fixed in 1% (w/v) OsO_4 for 2 h, rinsed 2 times with phosphate buffer, and dehydrated in a graded acetone series [(50–70–90–100–100–100)% (v/v); 10 min/step]. The samples were infused in epoxypropane/Epon 812 (50%/50%) solution for 2 h and then infused in Epon 812 again for 2 h in vacuum condition. The samples were embedded in Epon 812 and reacted at 80 °C overnight. Dried samples were cut into ultra-thin sections by Ultramicrotome (Leica UCT, Wetzlar, German) and stained with uranium for 15 min and lead for 10 min. Micrographs were captured with a JEOL JEM-100CXII electron microscope (JEOL, Tokyo, Japan) at an acceleration voltage of 80 kV.

2.7. Statistical analysis

The mean values of each experiment were calculated. The SAS system (SAS for Windows 6.12, SAS Institute

Table 2
Antioxidative activities of Douchi extracts as expressed by 50% inhibition concentrations (IC_{50}) *in vitro*

Method	Samples		
	Douchi extracts (mg/mL)	Trolox (μg/mL)[a]	EDTA (μg/mL)[a]
Scavenging effect on DPPH radical	0.658	14.7	–[b]
Scavenging effect on ABTS radical	0.204	4.50	–[b]
Chelating ability for ferrous ions	206	–[b]	540

[a] The activities of Trolox and EDTA were used as controls.
[b] Not detected.

Inc., Cary, NC, USA) was used for statistical analysis. Duncan's multiple range tests were used to estimate significant differences among the mean values at the 5% probability level.

3. Results

3.1. Chemical composition, contents of isoflavones and peptides in Douchi extracts

Douchi extracts contain 46.7% protein, 1.1% fat, 9.8% water, 15.9% salt and 26.5% other components. Total isoflavones content was 0.087% in Douchi extracts. The peptides content was 40.7% in Douchi extracts, and the percentage of total protein in Douchi extracts was 87.1%.

3.2. Antioxidant activities of Douchi extracts in vitro

Three different methods were used to evaluate the antioxidant potential of Douchi extracts *in vitro*; Trolox as control sample was also detected to compare the DPPH and ABTS radical scavenging activities. Trolox was found to have no chelating ability for ferrous ions (Chung, Chang, Chao, Lin, & Chou, 2002), so EDTA was used for comparison purpose. According to results shown in Table 2, scavenging activity of Douchi extracts on DPPH and ABTS radical and chelating abilities of ferrous ions of Douchi extracts were evident; the average IC_{50} values were 0.658, 0.204 and 206 mg/mL, respectively, which are higher than those of Trolox and EDTA.

3.3. Antioxidant activities of Douchi extracts in vivo

Table 3 shows the SOD, CAT, GSH-Px activities and the values of TBARS in liver and kidney. Compared with the negative control, the SOD activity and the CAT activity of the positive control increased significantly in liver ($p < 0.05$), and Douchi extracts 1 and Douchi extracts 2 groups also produced significant increase ($p < 0.05$). Moreover, the SOD activity of Douchi extracts 1 group in liver was significantly higher than the positive control and Douchi extracts 2 group ($p < 0.05$). But, the GSH-Px activities (in positive, Douchi extracts 1 and Douchi extracts 2 groups) of liver increased compared with negative control group, but there was no significant difference ($p > 0.05$).

The significant increase of the SOD activities and the GSH-Px activities in kidney was observed in the positive, Douchi extracts 1 and Douchi extracts 2 groups ($p < 0.05$), compared with the negative control. The effects in the SOD activities and the GSH-Px activities of Douchi extracts 1 and Douchi extracts 2 groups were almost equivalent to the positive control group in kidney. The CAT activities (in positive and Douchi extracts 2 groups) of kidney increased compared with the negative group, but there was no significant difference ($p > 0.05$).

The values of TBARS in the positive group, Douchi extracts 1 and Douchi extracts 2 groups all significantly decreased in liver and kidney compared with the negative control ($p < 0.05$).

3.4. Histological study

TEM changes of hepatocytes of experimental animals (Fig. 1) were compared. In negative control group (Fig. 1a), fatty degeneration of hepatocytes was remarkable; many lipid droplets (1) were seen in the plasma of hepatocytes; the mitochondria (2) swelled and cell nuclear

Table 3
The SOD, CAT, GSH-Px activities and the values of TBARS in liver and kidney of cholesterol-fed rats supplemented with vitamin E or Douchi extracts[a,b]

Diet	SOD activities (nmol/mg protein)	CAT activities (U/g protein)	GSH-Px activities (U/g protein)	TBARS (nmol/mg protein)
Liver				
Negative	81.26 ± 4.66^{C}	320.03 ± 18.46^{B}	168.18 ± 41.15^{A}	6.93 ± 0.41^{A}
Positive	109.33 ± 15.86^{B}	341.30 ± 6.53^{A}	200.71 ± 21.79^{A}	5.63 ± 0.64^{B}
Douchi extracts 1	139.87 ± 6.40^{A}	355.16 ± 9.32^{A}	204.45 ± 9.85^{A}	5.92 ± 0.33^{B}
Douchi extracts 2	114.07 ± 8.03^{B}	341.62 ± 11.18^{A}	198.33 ± 11.35^{A}	6.17 ± 1.19^{B}
Kidney				
Negative	68.68 ± 7.73^{B}	284.23 ± 42.18^{A}	142.32 ± 37.03^{B}	7.57 ± 1.38^{A}
Positive	109.28 ± 23.28^{A}	318.32 ± 25.60^{A}	184.42 ± 29.94^{A}	6.18 ± 0.85^{B}
Douchi extracts 1	100.72 ± 23.02^{A}	285.04 ± 51.58^{A}	199.21 ± 24.71^{A}	6.13 ± 1.09^{B}
Douchi extracts 2	99.51 ± 20.61^{A}	316.28 ± 44.28^{A}	185.73 ± 40.70^{A}	5.97 ± 1.37^{B}

[a] Values represent the mean ± standard deviation of duplicate assays in seven animals in each group.
[b] Values in a column with different superscripts were significantly different ($p < 0.05$).

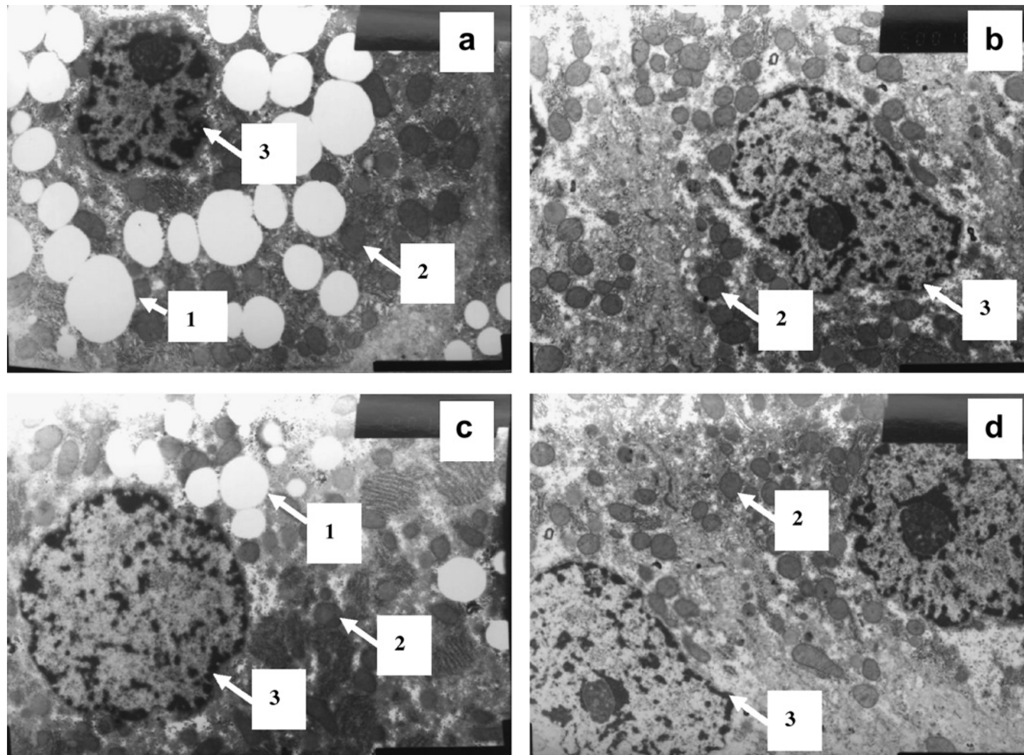

Fig. 1. TEM photos of liver sections. a: Negative; b: positive; c: Douchi extracts 1; d: Douchi extracts 2. 1: Lipid droplets; 2: mitochondria; 3: cell nuclear membrane. Magnification: × 5000.

membrane (3) was incomplete. On the contrary, in positive control group (Fig. 1b), fatty degeneration of hepatocytes was less obvious; there was almost no lipid droplets found in the plasma of hepatocytes, and the architecture of mitochondria stayed intact. In Douchi extracts 1 and Douchi extracts 2 groups (Fig. 1c and d), the degree of fatty degeneration of hepatocytes and the changes of mitochondria architecture were milder when compared with the negative control group but were more severe when compared with the positive control group, only a few lipid droplets in hepatocytes were observed. Hence, the microscope images suggested that the Douchi extracts might play a role in preventing the progression of the lipidosis of hepatocytes.

4. Discussion

As compared with the commercial antioxidants, the Douchi extracts showed less scavenging activity on DPPH and ABTS radicals than Trolox, and chelating ability for ferrous ions than EDTA. It was, however, evident that the Douchi extracts did show the antioxidant activity *in vitro*. Firstly, the antioxidant activity of the Douchi extracts might be attributed to its proton-donating ability as evidenced through DPPH and ABTS radical scavenging results. Secondly, Douchi extracts exhibited the chelating ability for ferrous ions and might afford protection against oxidative damage.

Our study demonstrated that after being fed with Douchi extracts, the body weight gain of rats was unchanged (data not shown). This result suggests that Douchi extracts have no influence on rats body weight gain. However, the activities of antioxidant enzymes and TBARS changed in liver and kidney to a certain degree (Table 3).

Antioxidant enzymes are capable of eliminating reactive oxygen species and lipid peroxidation products, thereby protect cells and tissues from oxidative damage. Antioxidant enzymes include SOD, CAT and GSH-Px, of which the first one can mutate the superoxide radicals to form molecular oxygen and H_2O_2, and the last two ones can decompose H_2O_2 to molecular oxygen and water.

In our test, we found that in Douchi extracts groups, SOD and CAT activities increased significantly in liver tissue; nonetheless, GSH-Px activities had no change in liver tissue. SOD and GSH-Px activities increased significantly in kidney tissue; however, CAT activities exhibited no change in kidney tissue. These results show that Douchi extracts might have different effects on different antioxidant enzymes in liver and kidney, respectively, or the rat organs might absorb Douchi extracts differently and the metabolic enzymes in organs were different as well. Therefore, the activities of antioxidant enzymes may vary in different organs. We hypothesize that the change of enzyme activities is related to the components or metabolites of Douchi extracts, which could affect enzymatic activities or enzyme contents. Further studies are needed to confirm this hypothesis.

The cholesterol-rich diets of rats could cause the increase of lipid peroxidation and expose the animals to

oxidative stress (Tasi, 1975). TBARS is a good indicator of lipid peroxidation. In liver and kidney, the values of TBARS of positive control and two Douchi extracts groups all decreased significantly (Table 3). This result indicated that the protective role of Douchi extracts against oxidative damage *in vivo* might be due to the decrease of lipid oxidation.

It is indicated that Douchi extracts had effect on hepatic lipid metabolism, and influenced the lipidosis of hepatocytes. Yokota et al. (1996) reported that an antioxidant-containing crude fraction from natto depressed the incidence of atherosclerotic lesions in cholesterol-fed rabbits by the histological studies. Their results agree with ours.

It is known that tocopherols and isoflavones can participate in the antioxidant activity of soybean. Esaki et al. (1990) reported that the antioxidant activity of natto depended on the increase of water-soluble fractions of natto with fermentation time, rather than on the increase of isoflavone aglycons, such as daizen and genistein. Liu, Chang, and Wiesenborn (2005) also reported that a laboratory-prepared tofu containing approximately 50 ppm isoflavones had greater effects than soybean extract with 250 ppm isoflavones, which indicated that molecules other than isoflavones might have synergistic effects on *in vivo* antioxidant enzyme induction of tofu. Peptides and amino acids were liberated during the Douchi fermentation process (Zhang, Tatsumi, Fan, & Li, 2006b). Our results showed the peptide percentage was 87.1% of protein of Douchi extracts. Antioxidant peptides are found in the hydrolysates of soy proteins, such as histidine-containing peptides or tyrosine-containing peptides (Chen, Muramoto, & Yamauchi, 1995; Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998). Markus, Hem, and Heinz (1997) suggested the antioxidant activity of tempeh had a synergistic effect with tocopherols (presented in the soybeans) and amino acids (liberated during the fermentation). Meanwhile, Fan (2006) isolated a strong antioxidant peptide from Douchi. It consisted of about 5–8 amino acid residues, which could scavenge ABTS and DPPH radicals, and exert a strong inhibition effect on linoleic acid oxidation in the linoleic acid model system. Moreover, the aqueous extracts of natto and tempeh also have strong antioxidant potential *in vivo* (Iwai et al., 2002b; Sheih et al., 2000). Soybean protein could inhibit lipid peroxidation in plasma (Carrol, 1991). The aqueous extracts of Douchi contained very little isoflavone, only 0.087 mg/g, however, the peptide content was 40.7% of Douchi extracts, and the percentage of Douchi extracts total protein was 87.1%. Therefore, the peptides and free amino acids might play an important role as antioxidant components in Douchi extracts.

Attention has focused on oxidative stress caused by a high cholesterol diet and many dietary supplements have been developed as the result of the finding of potential antioxidant such as VE. Douchi extracts showed many characteristics of an ideal antioxidant. Douchi is the popular traditional fermented soybean food and is very cheap. The antioxidant property of Douchi extracts implied that Douchi extracts might be used as an economical material to help fight against aging or some chronic diseases. In the absence of comparative investigation and based on this limited experiments, according to the ratio of the experimental rice weight and people weight, thus, people might consider eating about 75 g Douchi extracts/day/60 kg body weight for the preventive purpose as a dietary supplement. However, Douchi extracts still have a high level NaCl content (15.9%), so a desalting process should be considered in subsequent works.

5. Conclusions

The results of antioxidant activities *in vitro* of Douchi extracts show that the average IC_{50} values for DPPH and ABTS radical scavenging activities, and the chelating ability of ferrous ions, were 0.658, 0.204 and 206 mg/mL, respectively. The activity of SOD increased in liver and kidney significantly; CAT activity increased significantly in liver but there was no significant difference in kidney; GSH-Px activity increased significantly in kidney but there was no significant difference in liver; the values of TBARS decreased significantly in liver and kidney. Fewer lipid droplets were found in hepatocytes of Douchi extracts groups compared with negative control group. This work demonstrated that Douchi extracts might have some ideal antioxidant characteristics and inhibitory effect on lipid peroxidation and may mitigate the degree of the lipidosis of hepatocytes to improve lipid metabolism in liver.

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