Wageningen Academic Publishers

# Antioxidant capacity and antitumor activity of *Fructus Kochiae* extracts

**J. Wang, L. Yuan, B. Cheng, W. Li, C. Xiao, Y. Wang and X. Liu***

*Northwest A&F University, College of Food Science and Engineering, Taicheng Road 3, Yangling, 0086-712100 Shaanxi, China P.R.; xueboliu@yahoo.com.cn*

Received: 29 October 2012 / Accepted: 3 May 2013
© 2014 Wageningen Academic Publishers

**RESEARCH ARTICLE**

## Abstract

*Fructus Kochiae* (dried fruit of *Kochia scoparia* (L.) Schrad., broom cypress fruit), widely distributed in the northwest of China, has been used as medicinal and edible plant for thousands of years. The antioxidant activities and antitumor effect of aqueous and 50% ethanol *Fructus Kochiae* extracts were investigated. Furthermore, the content of momordin Ic by HPLC and the antioxidant activity by protein oxidation model were evaluated. *Fructus Kochiae* extracts could effectively scavenge different free radicals and suppress growth of cancer cells. The activity of ethanol extracts was more remarkable than aqueous ones especially for hydroxyl-induced oxidation. Moreover, ethanol extract was found to have higher amount of momordin Ic than aqueous ones. Momordin Ic was effective in inhibiting protein oxidation and carbonylation. *Fructus Kochiae* extracts could be developed as appreciable food additives with antioxidant activity and momordin Ic was an active component which partly contributed to the antioxidant activity.

**Keywords:** carbonylation, free radical, HepG2 cell, momordin Ic, oxidative damage

## 1. Introduction

Exposure to metals, air pollution, pesticides and diet are potential risks for the induction of oxidative stress. Extensive research has revealed that continued oxidative stress could mediate most chronic diseases including cancer, diabetes and cardiovascular, neurological and pulmonary diseases (Glade, 2010; Reuter *et al*., 2010). Natural extracts of plants, fruits and vegetables are complex mixtures of substances or single compounds with good resistance capacity and better security, which could be beneficial to reduce the risk of human cancers, arteriosclerosis and inflammation (Matkowski, 2008). A great many extracts such as sea buckthorn (Xu *et al*., 2011), mushroom (Cheung *et al*., 2003), centellab (Hussin *et al*., 2007), cedrela sinensis (Yu *et al*., 2012), garlic (Dziria *et al*., 2012), tea leaves (Ho and Su, 2012) and spices (Hinneburg *et al*., 2006) are proved to be natural components ideal for food additives and for managing various incurable diseases. Thus, there is a growing trend in identifying natural antioxidants of plant origin which are effective in health improvement and disease prevention.

*Fructus Kochiae* (dried fruit of *Kochia scoparia* (L.) Schrad., broom cypress fruit), a highly popular fruit which belongs to the goosefoot family, is widely distributed in the northwest of China. It first appears in 'Shen Nong's Herbal Classic', described as cold, spicy and bitter. The major bioactive compounds identified in *Fructus Kochiae* are triterpenoid saponins, steroid compounds, fatty oil, alkaloids and flavonoids (Xia *et al*., 2003). As a traditional Chinese herbal medicine, *Fructus Kochiae* has attracted considerable attention for its pharmacological activities, including antiinflammation, antidiabetics, insecticide, antianaphylaxis, antipruritic and anti-rheumatoid arthritis (Han *et al*., 2006; Kubo *et al*., 1997; Lee *et al*., 2011; Lirussi *et al*.,2004; Lu *et al*., 2012; Matsuda *et al*., 1997a,b; Shin *et al*., 2004). However, investigations on biological activities of free radical-scavenging capacity, oxidative injury inhibition and antitumor effect with regard to *Fructus Kochiae* are scarce. Momordin Ic, an oleanolic acid oligoglycoside isolated from the fruit of *K. scoparia*, has already been identified. However, whether momordin Ic is responsible for the biological activity, especially for antioxidant capacity remains to be explored (Choi *et al*., 2002).

ISSN 1757-8361 print, ISSN 1757-837X online, DOI 10.3920/QAS2012.0218

The aim of the present work was to compare the antioxidant activities (iron reduction, 1,1-di-phenyl-2-picrylhydrazyl (DPPH), ABTS·+, superoxide anion radical ($O_2^-$), hydroxyl radical (·OH) scavenging capacity and inhibition of biomolecules oxidation) and anti-cancer effect of aqueous and 50% ethanol *Fructus Kochiae* extracts. Moreover, the content of momordin Ic and its antioxidant activity were also evaluated, attempting to uncover the reason for the different bioactivity between aqueous and ethanol extracts and to provide additional evidence for *Fructus Kochiae* research and development.

## 2. Materials and methods

### Chemicals and reagents

1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tris-2,4,6-tri-piridyl-2-triazine (TPTZ), iron (III) chloride (dry), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH), herring sperm DNA and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Momordin Ic (98% purity) was purchased from Sichuan Victory Biotechnology Co. Ltd (Chengdu, China P.R.) and soy lecithin was from Beijing Huamaike Biotechnology Co. Ltd (Beijing, China P.R.). Modified RPMI 1640 medium and foetal bovine serum (FBS) were purchased from Thermo Fisher Scientific Co. (Hanover Park, IL, USA). All other chemicals were of analytical grade or HPLC grade purity.

### *Fructus Kochiae* extracts preparation

*Fructus Kochiae* were purchased from Shaanxi Huike Botanical Development Co. Ltd (Xian, China P.R.). Dried fruits of *Fructus Kochiae* were ground to fine powder for extraction preparation. For aqueous samples, fine powder was mixed in a 1:8 (w/v) ratio with distilled water under infusion condition for 4 h at room temperature. Ethanol samples were obtained by 50% (v/v) ethanol under reflux condition for 4 h. The crude extracts were then centrifuged at 4,000 g (SC3610; AnHui ustc Zonkia Scientific instruments Co. Ltd, Anhui, China P.R.) for 5 min and the supernatant were collected. The residue was re-extracted under the same conditions, the supernatants combined and evaporated under reduced pressure using a rotary evaporator (RE-210D; Ji'nan Gaoke Instrument Co. Ltd, Jinan, China P.R.) followed by vacuum drying with a vacuum drier (DZF-6000; Beijing surplus AmMed Scientific Instrument Co. Ltd, Beijing, China P.R.). The dried powder was stored at -4 °C for later use.

### Ferric-reducing antioxidant power assay

The ferric-reducing antioxidant power(FRAP)assay was determined according to the procedure of Benzie and Strain (1996) with slight modifications. Briefly, the freshly prepared FRAP reagent (5 ml, containing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution (2,4,6-tripyridyl-s-triazine) in 40 mM HCl and 20 mM iron (III) chloride solution in proportions of 10:1:1 (v/v)) were mixed with different concentrations of extracts (1 ml) and incubated for 30 min in dark place. The absorbance of the reaction mixture was then recorded at 593 nm using a spectrophotometer (UV1101; Techcomp, Shanghai, China P.R.). FRAP value was calculated using the calibration curve of $FeSO_4 \cdot 7H_2O$. The values were expressed as micromole of Fe(II) equivalents per gram wet tissue weight.

### Free radical-scavenging activity

#### *DPPH radical assay*

DPPH radical scavenging capacity of the extract was evaluated using absolute ethanolic solution of DPPH following Mellors and Tappel (1996). DPPH solutions were mixed with different concentrations of extracts in a ratio of 5:1 and incubated for 30 min in the dark. The reduction capability of DPPH· was determined by the decrease in its absorbance at 517 nm. Radical scavenging activity was reported as the inhibition percentage of free radical versus sample concentrations (mg/ml) according to Equation 1. The antioxidant activities of the extracts were expressed as $IC_{50}$, defined as the concentration of the extract required to cause a 50% decrease in initial free radical concentration.

$$\text{Free radical scavenging activity (\%)} = (1 - A_{sample} / A_{control}) \times 100 \quad (1)$$

Where $A_{sample}$ refers to the absorbance in the presence of extracts, and $A_{control}$ to the absorbance without extracts.

#### *Radical cation ABTS·+ scavenging activity*

ABTS·+ scavenging activity was measured according to the standard method described by Miller *et al.* (1993) with minor modifications (Re *et al.*, 1999). The blue-green ABTS radical form was produced by reacting the ABTS (7 mM) stock solution with potassium persulfate (7.35 mM) in the dark at room temperature for 12-16 h. Different concentrations of the extract sample (1 ml) were added to ABTS·+ solution (5 ml) and the decrease in absorbance at 734 nm were determined after 30 min. Results were expressed as the inhibition percentage of free radical by the sample using Equation 1.

#### *Hydroxyl radical scavenging activity*

Hydroxyl radical scavenging activity was adopted according to a literature procedure with some modifications (Smirnoff and Cumbes, 1989). The reaction mixture included $FeSO_4$ and sodium salicylate (6 mM), $H_2O_2$ (2.5 mM) and varying concentrations of extracts (1:1:1:1). The absorbance of the hydroxylated salicylate complex was measured at 500 nm after a reaction for 0.5 h at 37 °C. Hydroxyl radical scavenging activity was calculated as the inhibition percentage of ·OH by the sample. All determinations were carried out in triplicate and calculated using Equation 1.

#### *Superoxide anion radical scavenging activity*

Scavenging of anion superoxide was determined by the pyrogallol auto-oxidative systems with some modifications (Marklund and Marklund, 1974). Briefly, phosphate buffer (50 mM, pH 8.34, 4.5 ml) and sample solution (0.5 ml) were mixed uniformly and the absorbance at 510 nm were recorded quickly at 1 min and 4 min as soon as pyrogallol (45 mM, 10 μl) was added to start the auto-oxidation. The results were computed according to Equations 2 and 3.

$$C_{control} = (A_4 - A_1) / 3 \quad (2)$$

$$\text{Superoxide anion radical scavenging activity (\%)} = (1 - C_{sample} / C_{control}) \times 100 \quad (3)$$

Where $C_{control}$ is the pyrogallol auto-oxidation rate, $A_4$ and $A_1$ is the absorbance in the absence of extracts at 4 min and 1 min, respectively. $C_{sample}$ is the auto-oxidation rate in the presence of extracts.

### Inhibition on biomolecules oxidative damage

#### *Lipid peroxidation*

Lecithin (20 mg) was sonicated in an ultrasonic cleaner (KQ-500DE; Kun Shan Ultrasonic Instruments Co. Ltd, Kunshan, China P.R.) in 5 ml of phosphate buffer (0.05 mM, pH 7.4) for 0.5 h. Different concentrations of extracts (500 μl) were mixed with sonicated solution (0.2 ml) and $FeSO_4$ (50 mM, 50 μl) successively, the solutions were incubated at 37 °C for 40 min accompanied by vortexing every 5 min. Then TBA (10% w/v, 1 ml) and TCA (0.8% w/v, 1 ml) were added and heated at 100 °C for 15 min followed by centrifugation (5,000 r/min) for 10 min, the final absorbance of the reaction supernatant were recorded at 532 nm. The lipid peroxidation inhibition activity of the extracts was calculated using the Equation 4 (Fagali and Catalá, 2009).

$$\text{Inhibition activity (\%)} = (1 - A_{sample} / A_{control}) \times 100 \quad (4)$$

#### *DNA oxidative damage*

Hydroxyl radical was generated by phen-$CuSO_4$-$V_C$ system (Stoewe and Prütz, 1987). Different concentrations of extracts (500 μl) were added to the mixture of $CuSO_4$ (1 mM, 50 μl), phen (1.75 mM, 114 μl) and DNA (3 mg/ml, 100 μl). Then the reaction was started by $V_C$ (17.5 mM, 100 μl) and incubated at 37 °C for 90 min followed by TBA assay. The absorbance was then recorded at 532 nm. The result was calculated using Equation 4.

#### *Protein oxidative damage*

The effects of *Fructus Kochiae* extracts on protein oxidation were carried out by the method of Miura *et al* (1992). The reaction mixture (100 μl), containing extracts sample (10 μl), phosphate buffer (10 mM, pH 7.4), bovine serum albumin (0.5 mg/ml), $CuSO_4$ (100 μM) and $H_2O_2$ (2.5 mM) were incubated under open condition at 37 °C for 1 h. The results were indicated by R250 dyeing after sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Similarly, AAPH induced BSA oxidative damage was assayed following the same procedure except that the AAPH (250 mM, 20 μl) instead of $CuSO_4/H_2O_2$ was mixed with the extracts and were incubated at 37 °C for 3 h under sealed condition (Volpi and Tarugi, 1999).The result was represented as the residue of intact proteins.

### Anti-cancer activity

#### *Cell culture*

HepG2 cell line, a human hepatocyte-carcinoma cell line derived from a well-differentiated human hepatoblastoma and was purchased from Collection of Cell Cultures of Fourth Military Medical University (Shaanxi, China P.R.). HepG2 cells were cultured in RPMI medium supplemented with 10% FBS, benzylpenicillin (100 kU/l) and streptomycin (100 mg/l) at 37 °C and in atmosphere of 5% $CO_2$.

#### *Cell viability assay*

HepG2 cells were seeded in 96-well plates in a density of $2\times10^4$/well and incubated overnight. Cells were treated with different concentrations of extracts for 4 and 24 h, respectively. At the end of time interval, MTT was added to a final concentration of 0.5 g/l. After 4 h incubation, cultures were removed and the formazan crystals formed by live cells were dissolved with DMSO and absorbance at 490 nm was recorded by use of an enzyme-linked immune sorbent assay reader (Bio-Rad model 680; Bio-Rad, Hercules, CA, USA). The results were expressed as a percentage of viable cells in comparison to the control (100%).

### The inhibitory effect of momordin Ic on protein oxidation and carbonylation

The inhibitory effect of momordin Ic on protein oxidation and carbonylation was analysed according to the procedure in 2.5.3. The results of BSA oxidative damage were indicated by R250 dyeing after SDS-PAGE and the bands were visualised under UV light. The results of protein carbonylation were analysed by derivatisation of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) followed by immunodetection with DNPH-specific antibodies in gels and the bands were photographed by enhanced chemiluminescence (ECL) using an ECL commercial kit (Bio-Rad ChemiDoc XRS) (Yan, 2009).

### HPLC analysis of momordin Ic in *Fructus Kochiae* extracts

HPLC were carried out on a Shimadzu chromatograph (LC-10A; Shimadzu Corporation, Kyoto, Japan) equipped with SEDEX55 detector and performed on a $C_{18}$ column (4.6 mm × 5 μm). The mobile phase constituted of methanol, water and ethyl acetate (83:17:0.2) and the flow rate was 1.0 ml/min. The analyses were performed at 40 °C with a nitrogen pressure of 0.21 MPa and the injection volume was 20 μl. The drift tube temperature of ELSD parameters was 70 °C (Xia *et al.*, 2002).

### Statistical analysis

All analyses were performed in triplicate and the data were expressed as means ± standard deviations. Analysis of variance was performed by the DPS 9.50 software (Data Processing System; Sinyosoft, Yunfu, China P.R.) and the significance of the difference between means was determined by Duncan's multiple range test. Values of $P<0.05$ were considered to indicate statistical significance.

## 3. Results

### Ferric reducing power and free radical-scavenging activity

FRAP assay measures the change in absorbance at 593 nm due to the formation of a blue coloured Fe(II)-tripyridyltriazine compound from colourless oxidised Fe(III) form by the action of electron donating antioxidants. The ferric-reducing antioxidant power of the aqueous and ethanol extracts expressed as μmol ferrous iron equivalents per g of sample were indicated in Figure 1A. The FRAP value were 142-170 μmol Fe(II)/g for aqueous extracts and 178-179 μmol Fe(II)/g for ethanol extracts. The FRAP value for ethanol extracts were higher than that for aqueous ones.

Generally, the antioxidant activity of extracts is directly associated with its scavenging capacity. Results of *Fructus Kochiae* extracts on DPPH radical-scavenging activity were represented in Figure 1B. *Fructus Kochiae* extracts showed increased scavenging capacity with increased concentration in the range of 0.1-1 mg/ml. Compared with ethanol extracts, the scavenging effect of aqueous ones was lower with the $IC_{50}$ values being 0.24 mg/ml for ethanol extracts and 0.31 mg/ml for aqueous extracts, respectively. The ability of *Fructus Kochiae* extracts to scavenge ABTS radical was indicated in Figure 1C, both of the *Fructus Kochiae* extracts could greatly eliminate ABTS radical in a dose-dependent manner and $IC_{50}$ value of ethanol extracts was found to be 0.36 mg/ml, whereas the $IC_{50}$ value of aqueous extracts was 0.43 mg/ml. The hydroxyl radicals are highly active species and capable of damaging almost every molecule in biological systems. The results suggested that hydroxyl radical was greatly cleared by both of the extracts in a dose-dependent manner (Figure 1D). However, the $IC_{50}$ value of aqueous extract on hydroxyl radical scavenging was 2.25 mg/ml, which was nearly ten times as much as that of ethanol extracts being 0.23 mg/ml. In the present study, superoxide radicals were generated by pyrogallol auto-oxidative systems. The reduction of absorbance at 325 nm demonstrated the consumption of superoxide anion by *Fructus Kochiae* extracts. Different concentrations of extracts (1-7 mg/ml) displayed good superoxide scavenging capacity and ethanol and aqueous extracts (7 mg/ml) exhibited 84% and 54% inhibition rate, respectively (Figure 1E). The $IC_{50}$ values were 5.42 mg/ml for ethanol extracts and 6.64 mg/ml for aqueous extracts, both of which were higher than the $IC_{50}$ values of DPPH, ABTS·+ and ·OH.

### Inhibitory effect of *Fructus Kochiae* extracts on DNA oxidation and lipid peroxidation

As shown in Figure 2A, *Fructus Kochiae* extracts could greatly inhibit liposome peroxidation as indicated by the amount of malondialdehyde produced. *Fructus Kochiae* extracts showed 3-80% peroxidation inhibition in the concentration of 0.1-7 mg/ml compared with the control group, suggesting that the observed inhibition was concentration-dependent. In addition, ethanol extracts could almost completely inhibit the lipid peroxidation with a higher concentration. However, the inhibition rate of aqueous extracts on lipid peroxidation did not increase even the concentration was up to 3 mg/ml.

The effect of *Fructus Kochiae* extracts on the inhibition of hydroxyl radical-mediated DNA damage was determined by means of the copper (II)-dependent damage assay. It was obvious that herring sperm DNA was quickly degraded by hydroxyl radicals in the MCO system using $Cu^{2+}$ salts as an important catalytic component (Figure 2B). *Fructus Kochiae* extracts could dramatically protect herring sperm DNA against oxidative damage in a concentration-dependent manner. The aqueous extracts at the range of 0.1-7 mg/ml showed 4-40% inhibition rate on DNA oxidation while ethanol extracts displayed 19-70% with the same concentration.

**Figure 1. Antioxidant activities of aqueous and ethanol *Fructus Kochiae* extracts. (A) Iron reducing power, (B) DPPH· scavenging activity, (C) ABTS·+ scavenging activity, (D) $O_2$-· scavenging activity, (E) ·OH scavenging activity. All the results are presented as mean ± standard deviation (n=3). *$P$<0.05 and **$P$<0.01 represent statistically differences between aqueous extract and ethanol extract.**

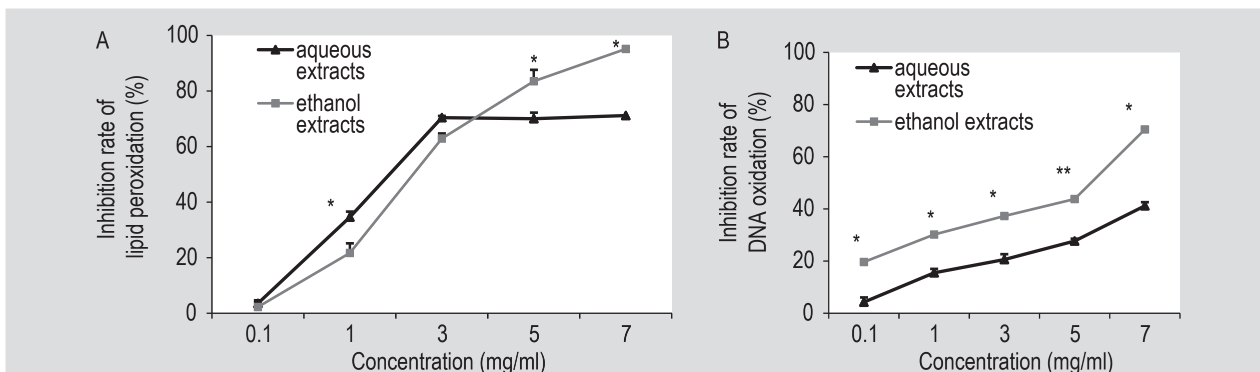


**Figure 2. Inhibitory activity of *Fructus Kochiae* extracts on DNA and lipid oxidation. (A) lipid peroxidation inhibition activity, (B) DNA oxidative damage inhibition activity. *$P$<0.05 and **$P$<0.01 represent significant differences between aqueous and ethanol extracts.**

### Inhibitory effect of *Fructus Kochiae* extracts on protein oxidation

In the present study, hydroxyl radical-induced damage of BSA was employed to determine the effect of *Fructus Kochiae* extracts on protein damage. As shown in Figure 3A and B, *Fructus Kochiae* extracts could wonderfully protect BSA from oxidation damage and the inhibition rate increased with increased concentration of the extracts. Especially, ethanol extracts of 0.1 mg/ml could almost completely suppress protein oxidation (73% intact protein remaining), while aqueous extracts could achieve the similar protection with a dose of 10 mg/ml.

AAPH is a water-soluble azo compound which decomposes at physiological temperature (37 °C) to generate peroxyl radicals (ROO·) in the presence of molecular oxygen in aqueous solutions. The activity of *Fructus Kochiae* extracts on AAPH-mediated protein damage was also revealed in Figure 3C and D. The results indicated that the residue of intact protein was increased with the increased concentration of extracts, suggesting an appreciable protection of *Fructus Kochiae* extracts on AAPH-mediated protein damage. However, there was no significant difference between ethanol and aqueous groups.

### The inhibition activity of *Fructus Kochiae* extract on HepG2 cells progression

Hepatocellular carcinoma (HCC), one of the most common malignant neoplasms around the world, is responsible for considerably high cases of death from cancer. The results showed that *Fructus Kochiae* extract exerted a strong growth inhibitory effect on human liver cancer HepG2 cells in an incubation time- and concentration-dependent manner, as depicted in Figure 4. Compared with untreated cells (100±6%), treatment of HepG2 cells with 0.01 mg/ml of extracts for 4 h had no effect on cell viability (Figure 4A). However, incubation with the extracts of 1 mg/ml for 4 h or 0.1 mg/ml for 24 h resulted in a significant decrease in viability (60-70%). Both of the extracts exerted a great antitumor activity at a dose of 5 mg/ml for 4 h (Figure 4A, B). There was no significant difference between the aqueous and ethanol extracts treatment.

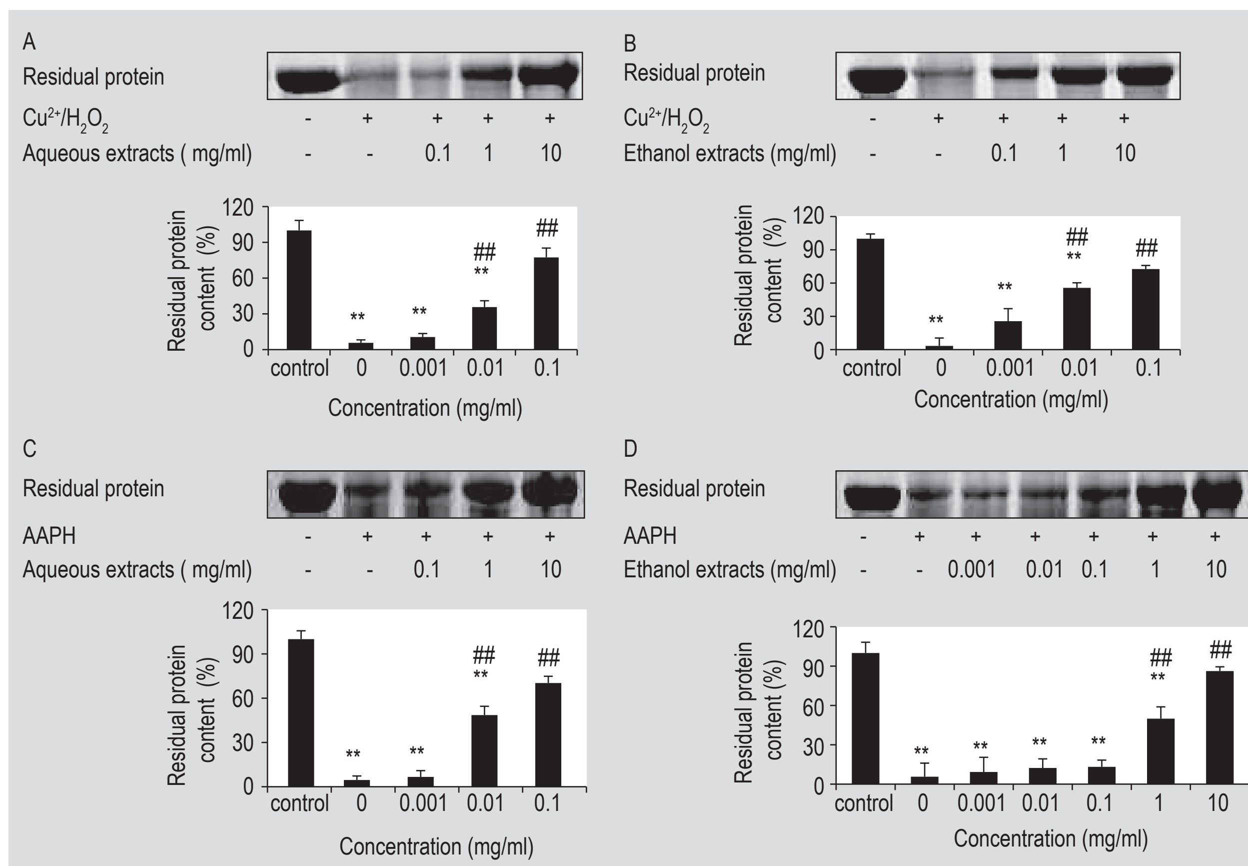


**Figure 3. Inhibitory activity of *Fructus Kochiae* extracts on BSA oxidation. (A, B) Inhibition effect of *Fructus Kochiae* extracts on BSA oxidation induced by $Cu^{2+}/H_2O_2$, (C, D) Inhibition effect on AAPH-induced BSA oxidation. ***P*<0.01 represents significant differences between the extract and control groups. ##*P*<0.01 represents significant differences between the extract and the group only treated with $Cu^{2+}/H_2O_2$ or AAPH.**

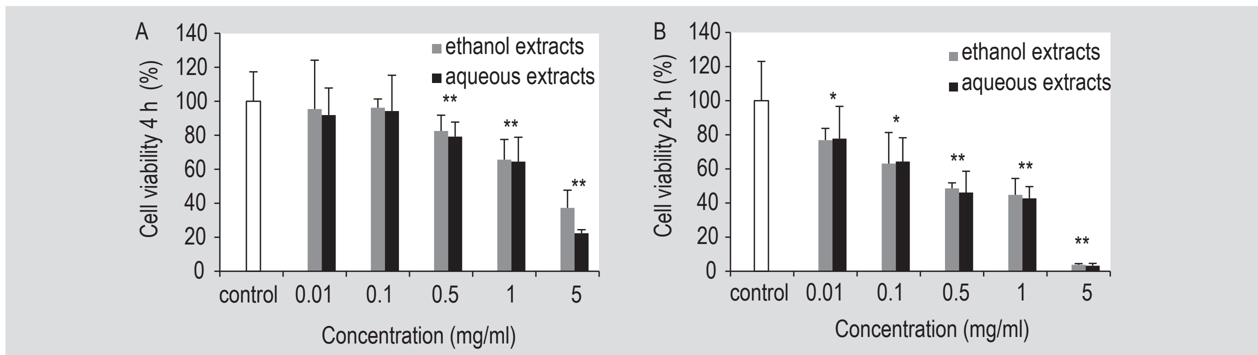


**Figure 4. The anti-tumor effect of *Fructus Kochiae* extracts on HepG2 cell viability. Cells were treated with different concentrations of extracts for 4 h (A) or 24 h (B). *$P$<0.05 and **$P$<0.01 represent significant differences between the extract and control groups.**

### The antioxidant activity of momordin Ic

As shown in Figure 5, momordin Ic could effectively inhibit protein oxidation induced by free radicals. Much of the protein (>80%) are protected when pretreated by 1 μM (0.76 μg/ml) of momordin Ic in the model of $Cu^{2+}/H_2O_2$-mediated oxidative damage. Meanwhile, the content of carbonyl protein was greatly decreased after addition of momordin Ic, compared with the group without momordin Ic (Figure 5A). Similarly, in the model of AAPH-mediated oxidative damage, there was a same trend indicating a better inhibition rate on protein oxidation and carbonylation with a gradually increased concentration of momordin Ic (Figure 5B).

### HPLC analysis of momordin Ic in *Fructus Kochiae* extracts

The content of momordin Ic in the aqueous and ethanol *Fructus Kochiae* extracts were assayed by HPLC analyses. The results showed that both of the extracts were found

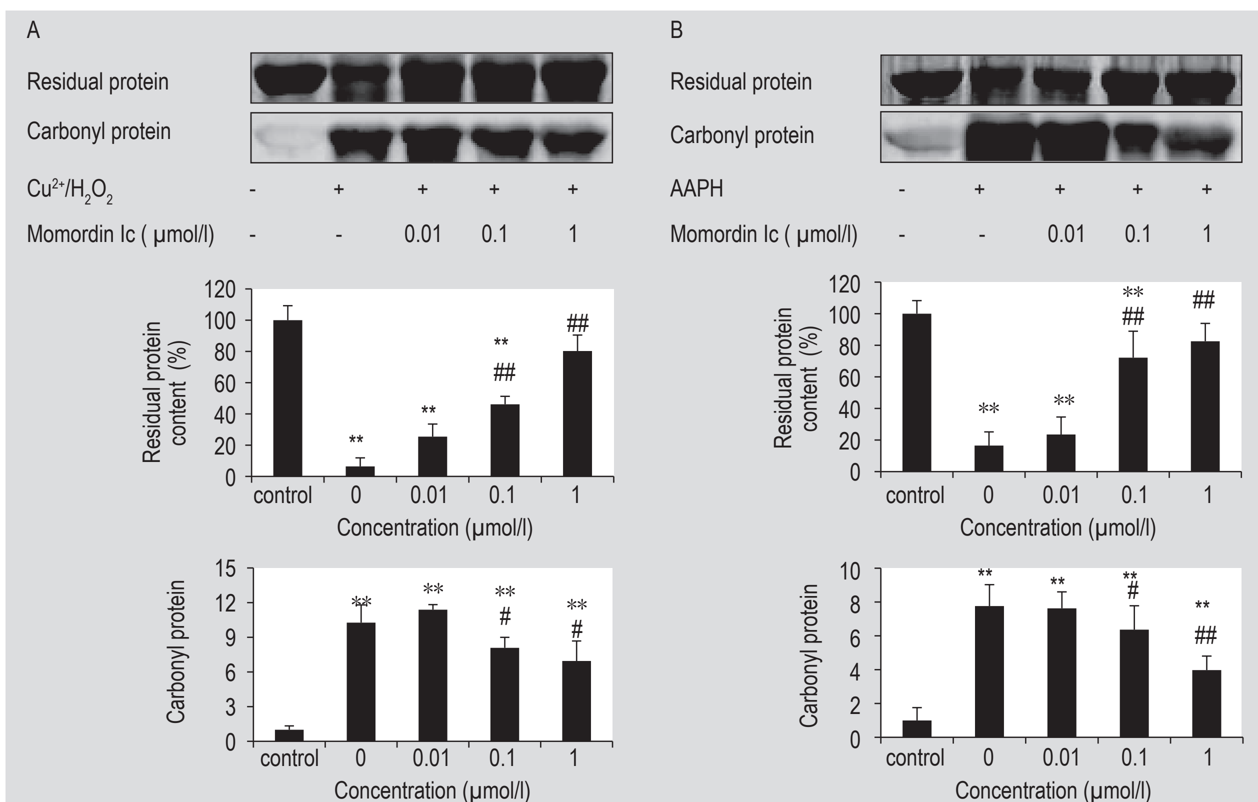


**Figure 5. Inhibitory activity of momordin Ic on protein oxidation or carbonylation. (A) Inhibitory activity of momordin Ic on $Cu^{2+}/H_2O_2$-induced BSA damage, (B) Inhibitory activity of momordin Ic on AAPH-induced BSA damage. **$P$<0.01 represents significant differences between momordin Ic and control groups. $^{\#\#}P$<0.01 represents significant differences between momordin Ic and the group only treated with $Cu^{2+}/H_2O_2$ or AAPH.**

to have an appreciable amount of momordin Ic (Figure 6). Ethanol extracts were detected to have a higher content of momordin Ic (5.75%) which was twice as much as that in aqueous extracts (2.88%).

## 4. Discussion

Antioxidants can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals and also by acting as oxygen scavengers. A number of methods for measuring antioxidant activity have been suggested recently, such as systems of oil oxidation, free radicals scavenging system, total reduction power, etc. Generally, total antioxidant activity of fruits, vegetables and other plants cannot be evaluated through only one antioxidant method, due to various characteristics of the reaction mechanism, the complexity of the composition, certain limitations of each method as well as different determination principles. Among them, the FRAP assay has gained popularity due to its fast, reproducible and versatile characteristics for both aqueous and ethanol extracts of different plants. According to the data reported, the traditional Chinese medicinal plants were classified into four groups on the basis of their antioxidant activities:

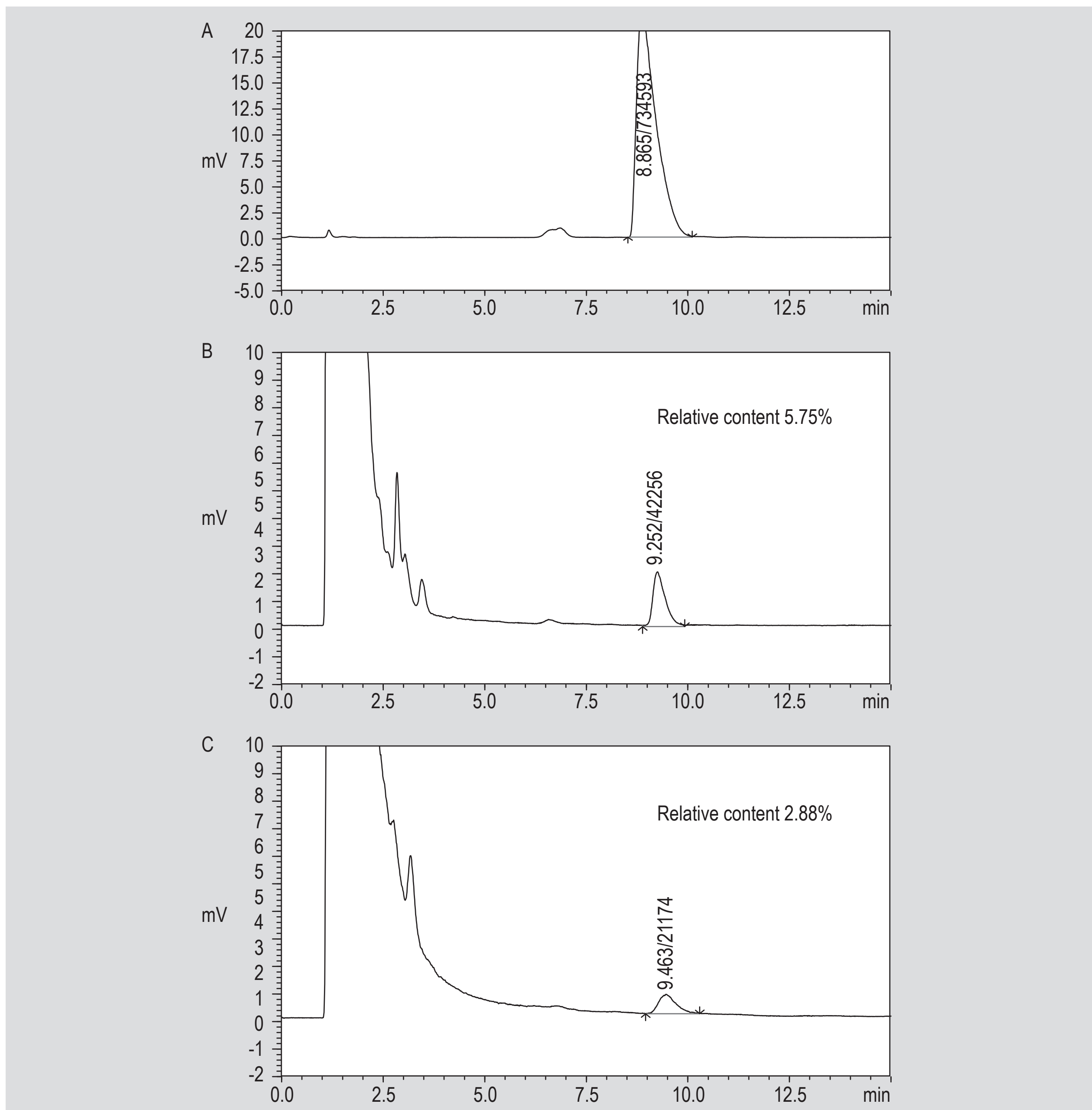


**Figure 6. The content of momordin Ic in different *Fructus Kochiae* extracts analyzed by HPLC. (A) The standard of monordin Ic, (B) Momordin Ic in ethanol extract, (C) Momordin Ic in aqueous extract.**

extremely high (>500 μmol Fe(II)/g), high (100-500 μmol Fe(II)/g), medium (10-100 μmol Fe(II)/g) and low (<10 μmol Fe(II)/g). According to this classification, both of the *Fructus Kochiae* extracts showed high antioxidant activity (142-170 μmol Fe(II)/g for aqueous extracts; 178-179 μmol Fe(II)/g for ethanol extracts). Ethanol extracts were found to have higher antioxidant activity than aqueous ones.

From a methodological point of view, the DPPH, ABTS and ORAC assays are recommended as simple and accurate methods for assessing the radical scavenging activity of extracts derived from fruit, juice and vegetables. The superoxide and hydroxyl radicals are highly active species and capable of damaging almost every molecule in biological systems. It was therefore significant to measure the interceptive capacity of the antioxidant to quench these radicals. In the present study, both of the *Fructus Kochiae* extracts could significantly eliminate the four free radicals (DPPH, ABTS·+, ·OH, $O^{2-\cdot}$). The higher free radical scavenging capacity is related to a lower $IC_{50}$ value. However, ethanol extracts showed stronger scavenging effect and both of the *Fructus Kochiae* extracts were much more effective in scavenging DPPH·, ABTS·+ and ·OH than $O^{2-}$·. Interestingly, the $IC_{50}$ of ethanol extracts for ·OH were relatively lower than that of aqueous extracts.

Vitamin C, iron and copper ions, which universally exist in biological systems, could readily initiate hydroxyl generation in aerobic metabolism. The extremely reactive hydroxyl radical has the capacity to damage DNA, lipids and proteins by oxidation. Particularly, the previous results showed that ethanol extracts were much more effective in quenching hydroxyl radical than aqueous extracts (the $IC_{50}$ of the two extracts were greatly different), models of biomolecule oxidative damage induced by hydroxyl radical were applied to verify the biological activity of *Fructus Kochiae* extracts further.

It is known that virtually every disease state involves free radicals. Oxidative species, such as superoxide anion, hydroxyl (·OH), peroxyl, alkoxyl radicals and their by-products (hydrogen peroxide and hypochlorite ion ($OCl^-$), if exceeded the normal level, may attack biological macromolecules, such as proteins, lipids and nucleic acids causing cellular damage and even diseases. Much of the damage resulting from oxidative stress has been attributed to the highly reactive hydroxyl radical. The metal-mediated production of hydroxyl radicals from $H_2O_2$ was one important mechanisms of biological deterioration. It has been shown that hydroxyl radicals are generated under physiological and pathological conditions and closely linked to oxidative stress-originated diseases, such as stress-induced gastric ulceration, neuronal damage, spinal cord injury, hepatitis, etc. (Das *et al.*, 1997; Gutteridge and Wilkins, 1983; Liu *et al.*, 2003; Rauhala *et al.*, 1996; Yamamoto *et al.*, 2001). Thus, hydroxyl radical should be well-regulated to help maintain homeostasis on the cellular level in normal, healthy tissues. From the results indicated above, it could be steadily summarised that *Fructus Kochiae* extracts could inhibit the oxidative damage of lipids, DNA and protein mediated by hydroxyl radical, as was expected. This paper provides the evidence that *Fructus Kochiae* extracts can be used potentially as accessible and valuable natural antioxidants for improving physical disease resistance, especially for managing active hydroxyl radical.

HCC is one of the most prevalent cancers around the world. HepG2 cell line is one of the most widely used experimental models for *in vitro* studies on HCC. In this work, the *Fructus Kochiae* extracts showed inhibitory effect on cell growth but there was no significant difference between the two extracts.

A wide range of compounds have been found to be well-established physiological antioxidant *in vivo* and in foods. Many bioactive compounds with possible antioxidant effects may be associated with the groups of polyphenols and saponins. Antioxidant compounds derived from plants, especially phenols such as quercetin, catechins, morin and saponins such as ginsenoside, gypenosides, platycodigenin are of considerable interest from the perspective of dietary antioxidants (Oliva *et al.*, 2011; Ryua *et al.*, 2012; Subash and Subramanian, 2012; Wang *et al.*, 2010; Zhao *et al.*, 2009). In the present study, triterpenoid saponin was evaluated because most research revealed that the active component for *Fructus Kochiae* activities may be partially attributed to momordin Ic. It is suggested that momordin Ic could potentially inhibit ethanol-induced gastric mucosal lesionsin and alleviate carbon tetrachloride-induced hepatotoxicity by enhancing the hepatic antioxidant defense system in rats (Kim *et al.*, 2005; Matsuda *et al.*, 1999). Proteins can be oxidised by ROS in reversible or irreversible ways, of which carbonylation modification is irreversible and associated with progression of several human diseases (Dalle-Donne *et al.*, 2003). In the present work, momordin Ic was established to be effective in inhibiting protein modifications by hydroxyl or AAPH. Moreover, the content of momordin Ic in ethanol extracts was twice as much as that in aqueous extracts. Thus, these findings provided basic information for the establishment of momordin Ic as an efficacious antioxidant. Moreover, this may provide evidence that momordin Ic was an important factor for antioxidant determination between the two extracts although there is still much more evidence needed considering the complexity of the extracts.

It has been widely reported that natural extracts of plant origin are promising for food ingredients with high antioxidant activity (Itagi *et al.*, 2012; Otles and Selek, 2012), such as sea buckthorn (Xu *et al.*, 2011), mushroom (Cheung *et al.*, 2003) and tea leaves (Ho and Su, 2012). These dietary plants, which were demonstrated to have various bioactivities, especially remarkable antioxidant

capacity, have been used for functional food production. *Fructus Kochiae*, a kind of edible and pharmaceutical product, has been traditionally used for its pharmacological activities, whereas, its biological activities of antioxidant capacity and antitumor effect are not well explored. In this study *Fructus Kochiae* extracts could effectively scavenge different free radicals, inhibit oxidative damage and suppress growth of cancer cells. The antioxidant capacity of *Fructus Kochiae* extracts was comparable to that of the previous plant extracts.

Generally, different extracts may display various activities due to their complex components and characteristics in the extracts. Some main active components in the natural extracts have already been elucidated. For example, polyphenols mainly contributed to good bioactivity for the tea leaves. Whereas, for *Fructus Kochiae*, momordin Ic, a triterpenoid saponin, exhibited well inhibitory effect on protein oxidation and the content was different in aqueous and ethanol extracts, which verified that momordin Ic was the main active component. These results and findings were valuable for *Fructus Kochiae* application as food antioxidants. These results and findings were valuable evidences for *Fructus Kochiae* application as food antioxidants and for further research.

In conclusion, although the antioxidant activities established *in vitro* experiment were only preliminary reference for the potential health enhancement, these results were considered significant for properly screening antioxidant activity and for thoroughly exploring the use of *Fructus Kochiae* extracts. Therefore, *Fructus Kochiae* may be promising for an available source of natural antioxidants with consequent health benefits.

## Acknowledgements

This work was supported by the Young Scientists Fund of the National Natural Science Foundation of China (grant no. 31000757).

## Conflict of interest

The authors declare that they have no conflict of interest.

## References

Benzie, I.F. and Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of ‘antioxidant power’ the FRAP assay. Analytical Biochemistry 239: 70-76.

Cheung, L.M., Cheung, P.C.K. and Ooi, V.E.C., 2003. Antioxidant activity and total phenolics of edible mushroom extracts. Food Chemistry 81: 249-255.

Choi, J., Lee, K.T., Jung, H., Park, H.S. and Park, H.J., 2002. Anti-rheumatoid arthritis effect of the *Kochia scoparia* fruits and activity comparison of momordin Ic, its prosapogenin and sapogenin. Archives of Pharmacal Research 25: 336-342.

Dalle-Donne, I., Giustarini, D., Colombo, R., Rossi, R. and Milzani, A., 2003. Protein carbonylation in human diseases. Trends in Molecular Medicine 9: 169-176.

Das, D., Bandyopadhyay, D., Bhattacharjee, M. and Banerjee, R.K., 1997. Hydroxyl radical is the major causative factor in stress-induced gastric ulceration. Free Radical Biology and Medicine 23: 8-18.

Dziria, S., Hassenb, I., Fatnassia, S., Mrabet, Y., Casabianca, H., Hanchi, B. and Hosni, K., 2012. Phenolic constituents, antioxidant and antimicrobial activities of rosy garlic (*Allium roseum* var. *odoratissimum*). Journal of Functional Foods 4: 423-432.

Fagali, N. and Catalá, A., 2009. $Fe^{2+}$ and $Fe^{3+}$ initiated peroxidation of sonicated and non-sonicated liposomes made of retinal lipids in different aqueous media. Chemistry and Physics of Lipids 159: 88-94.

Glade, M.J., 2010. Oxidative stress and cognitive longevity. Nutrition 26: 595-603.

Gutteridge, J.M. and Wilkins, S., 1983. Copper salt-dependent hydroxyl radical formation: damage to proteins acting as antioxidants. Biochimica et Biophysica Acta – General Subjects 759: 38-41.

Han, L.K., Nose, R., Li, W., Gong, X.J., Zheng, Y.N., Yoshikawa, M., Koike, K., Nikaido, T., Okuda, H. and Kimura, Y., 2006. Reduction of fat storage in mice fed a high-fat diet long term by treatment with saponins prepared from *Kochia scoparia* fruit. Phytotherapy Research 20: 877-882.

Hinneburg, I., Damien Dorman, H.J. and Hiltunen, R., 2006. Antioxidant activities of extracts from selected culinary herbs and spices. Food Chemistry 97: 122-129.

Ho, C.L. and Su, Y.C., 2012. Composition, antioxidant and antimicrobial activities of the leaf essential oil of *Machilus japonica* from Taiwan. Natural Product Communications 7: 109-112.

Hussin, M., Abdul-Hamid, A., Mohamad, S., Saari, N., Ismail, M. and Bejo, M.H., 2007. Protective effect of *Centella asiatica* extract and powder on oxidative stress in rats. Food Chemistry 100: 535-541.

Itagi, H.N., Rao, B.V.R.S., Jayadeep, P.A. and Singh, V., 2012. Functional and antioxidant properties of ready-to-eat flakes from various cereals including sorghum and millets. Quality Assurance and Safety of Crops & Foods 4: 126-133.

Kim, N.Y., Lee, M.K., Park, M.J., Kim, S.J., Park, H.J., Choi, J.W., Kim, S.H., Cho, S.Y. and Lee, J.S., 2005. Momordin Ic and oleanolic acid from *Kochiae Fructus* reduce carbon tetrachloride-induced hepatotoxicity in rats. Journal of Medicinal Food 8: 177-183.

Kubo, M., Matsuda, H., Dai, Y., Ido, Y. and Yoshikawa, M., 1997. Studies on *Kochiae Fructus*. I. antipruritogenic effect of 70% ethanol extract from *Kochiae Fructus* and its active component. Yakugaku Zasshi – Journal of the Pharmaceutical Society of Japan 117: 193-201.

Lee, M.Y., Shin, I.S., Lim, H.S., Seo, C.S., Ha, H. and Shin, H.K., 2011. *Kochia scoparia* fruit attenuates allergic airway inflammation in ovalbumin (OVA)-induced murine asthma model. Inhalation Toxicology 23: 938-946.

Lirussi, D., Li, J., Prieto, J.M., Gennari, M., Buschiazzo, H., Rios, J.L. and Zaidenberg, A., 2004. Inhibition of *Trypanosoma cruzi* by plant extracts used in Chinese medicine. Fitoterapia 75: 718-723.

Liu, D., Liu, J., Sun, D., Alcock, N.W. and Wen, J., 2003. Spinal cord injury increases iron levels: catalytic production of hydroxyl radicals. Free Radical Biology and Medicine 34: 64-71.

Lu, C., Zhang, H.Y., Ji, J. and Wang, G.X., 2012. *In vivo* anthelmintic activity of *Dryopteris crassirhizoma*, *Kochia scoparia*, and *Polygala tenuifolia* against *Dactylogyrus intermedius* (*Monogenea*) in goldfish (*Carassius auratus*). Parasitology Research 110: 1085-1090.

Marklund, S. and Marklund, N., 1974. Involvement of the superoxide anion radical in the autoxidation of pyorgallol and a convenient assay for superaxide dismutase. European Journal of Biochemistry 47: 469-474.

Matkowski, A., 2008. Plant *in vitro* culture for the production of antioxidants – a review. Biotechnology Advances 26: 548-560.

Matsuda, H., Dai, Y., Ido, Y., Ko, S., Yoshikawa, M. and Kubo, M., 1997a. Studies on *Kochiae Fructus*. III. Antinociceptive and antiinflammatory effects of 70% ethanol extract and its component, momordin Ic from dried fruits of *Kochia scoparia* L. Biological and Pharmaceutical Bulletin 20: 1086-1091.

Matsuda, H., Dai, Y., Ido, Y., Yoshikawa, M. and Kubo, M., 1997b. Studies on *Kochiae Fructus*. IV. Anti-allergic effects of 70% ethanol extract and its component, momordin Ic from dried fruits of *Kochia scoparia* L. Biological and Pharmaceutical Bulletin 20: 1165-1170.

Matsuda, H., Li, Y. and Yoshikawa, M., 1999. Roles of capsaicin-sensitive sensory nerves, endogenous nitric oxide, sulfhydryls, and prostaglandins in gastroprotection by momordin Ic, an oleanolic acid oligoglycoside, on ethanol-induced gastric mucosal lesions in rats. Life Sciences 65: 27-32.

Mellors, A. and Tappel, A.L., 1996. The inhibition of mitochondrial peroxidation by ubiquinon and ubiquinol. Journal of Biological Chemistry 241: 4353-4356.

Miller, N.J. Rice-Evans, C.A. Davies, M.J. Gopinathan, V. and Milner, A., 1993. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clinical Science 84: 407-412.

Miura, T., Muraoka, S. and Ogiso, T., 1992. Oxidative damage to bovine serum albumin induced by hydroxyl radical generating systems of xanthine oxidase + EDTA–$Fe^{3+}$ and ascorbate + EDTA–$Fe^{3+}$. Chemico-Biological Interactions 85: 243-254.

Oliva, J., Bardag-Gorce, F., Tillman, B. and French, S.W., 2011. Protective effect of quercetin, EGCG, catechin and betaine against oxidative stress induced by ethanol *in vitro*. Experimental and Molecular Pathology 90: 295-299.

Otles, S. and Selek, I., 2012. Phenolic compounds and antioxidant activities of chestnut (*Castanea sativa* Mill.) fruits. Quality Assurance and Safety of Crops & Foods 4: 199-205.

Rauhala, P., Sziraki, I. and Chiueh, C.C., 1996. Peroxidation of brain lipids *in vitro*: nitric oxide versus hydroxyl radicals. Free Radical Biology and Medicine 21: 391-394.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine 26: 1231-1237.

Reuter, S., Gupta, S.C., Chaturvedi, M.M. and Aggarwal, B.B., 2010. Oxidative stress, inflammation, and cancer: how are they linked? Free Radical Biology and Medicine 49: 1603-1616.

Ryua, C.S., Kima, C.H., Lee, S.Y., Lee, K.S., Choung, K.J., Song, G.Y., Kim, B.H., Ryu, S.Y., Lee, H.S. and Kim, S.K., 2012. Evaluation of the total oxidant scavenging capacity of saponins isolated from *Platycodon grandiflorum*. Food Chemistry 132: 333-337.

Shin, K.M., Kim, Y.H., Park, W.S., Kang, I., Ha, J., Choi, J.W., Park, H.J. and Lee, K.T., 2004. Inhibition of methanol extract from the fruits of *Kochia scoparia* on lipopolysaccharide-induced nitric oxide, prostaglandin [correction of prostagladin] E2, and tumor necrosis factor-alpha production from murine macrophage RAW 264.7 cells. Biological and Pharmaceutical Bulletin 27: 538-43.

Smirnoff, N. and Cumbes, Q.J., 1989. Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 28: 1057-1060.

Stoewe, R. and Prütz, W.A., 1987. Copper-catalyzed DNA damage by ascorbate and hydrogen peroxide: kinetics and yield. Free Radical Biology and Medicine 3: 97-105.

Subash, S. and Subramanian, P., 2012. Protective effect of morin on lipid peroxidation and lipid profile in ammonium chloride-induced hyperammonemic rats. Asian Pacific Journal of Tropical Medicine 2: 103-106.

Volpi, N. and Tarugi, P., 1999. The protective effect on $Cu^{2+}$ and AAPH-mediated oxidation of human low-density lipoproteins depends on glycosaminoglycan structure. Biochimie 81: 955-963.

Wang, P., Niu, L., Guo, X.D., Gao, L., Li, W.X., Jia, D., Wang, X.L., Ma, L.T. and Gao, G.D., 2010. Gypenosides protects dopaminergic neurons in primary culture against MPP+-induced oxidative injury. Brain Research Bulletin 83: 266-271.

Xia, Y.F., Wang, Q., Dai, Y. and Pei, K., 2002. Determination of the content of saponin in *Kochia scoparia* fruits from different producing areas. Zhongguo Zhong Yao Za Zhi 27: 890-893.

Xia, Y., Wang, Q. and Pu, Z., 2003. Identification of *Kochia scoparia* and its substitutes by scanning electron microscope and UV spectrum. Zhong Yao Cai 26: 323-326.

Xu, Y.J., Kaur, M., Dhillon, R.S., Tappia, P.S. and Dhalla, N.S., 2011. Health benefits of sea buckthorn for the prevention of cardiovascular diseases. Journal of Functional Foods 3: 2-12.

Yamamoto, H., Watanabe, T., Mizuno, H., Endo, K., Hosokawa, T., Kazusaka, A., Gooneratne, R. and Fujita, S., 2001. *In vivo* evidence for accelerated generation of hydroxyl radicals in liver of Long-Evans Cinnamon (LEC) rats with acute hepatitis. Free Radical Biology and Medicine 30: 547-554.

Yan, L.J., 2009. Analysis of oxidative modification of proteins. Current Protocols in Protein Science, Chapter14, Unit14.4.

Yu, W.J., Chang, C.C., Kuo, T.F., Tsai, T.C. and Chang, S.J., 2012. *Toona sinensis Roem* leaf extracts improve antioxidant activity in the liver of rats under oxidative stress. Food and Chemical Toxicology 50: 1860-1865.

Zhao, H., Li, Q., Zhang, Z., Pei, X., Wang, J. and Li, Y., 2009. Long-term ginsenoside consumption prevents memory loss in aged SAMP8 mice by decreasing oxidative stress and up-regulating the plasticity-related proteins in hippocampus. Brain Research 1256: 111-122.