

## Hydrophobicity of whey protein hydrolysates enhances the protective effect against oxidative damage on PC 12 cells

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The relationship between hydrophobicity and the protective effect of whey protein hydrolysates (WPHs) against oxidative stress was studied. Whey protein was first hydrolysed by pepsin and trypsin to obtain WPHs. After absorbed by macroporous adsorption resin DA201-C, three fractions named as M20, M40, and M60 were eluted by various concentrations of ethanol. The hydrophobicity showed a trend of increase from M20 to M60. Antioxidant ability test *in vitro* indicated that all the three components of WPHs displayed reasonably good antioxidant ability. Moreover, with the increase of hydrophobicity, antioxidant ability of WPHs improved significantly. Then rat pheochromocytoma line 12 (PC12) cells oxidative model was built to evaluate the suppression of oxidative stress of three components on PC12 cells induced by H<sub>2</sub>O<sub>2</sub>. Morphological alterations, cell viability, apoptosis rate, and intracellular antioxidant system tests all indicated that WPHs exert significant protection on PC cells against H<sub>2</sub>O<sub>2</sub>-induced damage. Among them, M60 had the highest protective effect by increasing 19.3% cell survival and reducing 28.6% cell apoptosis. These results suggested hydrophobicity of WPHs was contributing to the antioxidant ability and the protective effect against oxidative damage.

**Keywords:** Antioxidant peptides, hydrophobicity, whey protein hydrolysates, oxidative damage.

Oxidative stress reflects an imbalance between reactive oxygen species and a biological antioxidant system. It can cause toxic effects against all the human tissues and important biomacromolecules such as proteins, lipids and DNA. It is believed that various diseases are connected tightly to oxidative stress (Lau et al. 2005). Thus, food-derived antioxidant peptides that can be consumed in diet have attracted increasing attention due to the growing consumer preference for naturally derived products and the increased concern over the quality and safety in food industry.

Many researches and our previous study revealed that peptides obtained from natural food such as soybean (Beermann et al. 2009), eggs (Sakanaka & Tachibana, 2006), rice bran (Parrado et al. 2006), and whey protein (Zhang et al. 2012) have antioxidant activities. However, the

antioxidant activities of these peptides, even the peptides from the same source, were significantly different from each other using the same evaluation system. For example, the antioxidant activities of enzymatic whey protein peptides on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals differed from 19.68 to 84.78 (our unpublished data). It is said that hydrophobicity is generally considered as advantageous regarding antioxidant properties, because hydrophobic antioxidants are more active in emulsions than their hydrophilic homologues. Laguerre's et al. (2011) study implied that there was a dependency, though nonlinear, between hydrophobicity and antioxidant capacity of phenolipids. Amarowicz et al. (1997) also found that hydrophobic components from flax extract showed stronger antioxidant effect than hydrophilic fractions. Thus, this study tried to explore the connection between hydrophobicity and antioxidant abilities of peptides derived from whey protein hydrolysates (WPHs).

Whey is a by-product of the cheese-making process. It is a rich and varied mixture of globular proteins possessing

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a wide range of nutritional and functional properties. In this study, WPHs were fractionized according to their hydrophobicity. And H<sub>2</sub>O<sub>2</sub>-induced rat pheochromocytoma line 12 (PC12) oxidative model was established to assess the antioxidative stress activity.

## Materials and methods

### Materials

Heat-stable ALACEN392 whey protein concentrate was a product of Fonterra (Auckland, New Zealand). Pepsin (EC. 3.4.23.1, 1:10 000 U) and trypsin enzymes (EC. 3.4.21.4, 1:2500 U) were purchased from Sangon Biotech (shanghai) Co., Ltd. PC12 cells were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum were obtained from Gibco (Grand Island, NY, USA). Total antioxidant capacity (T-AOC), superoxide dismutase (SOD) activity, catalase (CAT) activity, malondialdehyde (MDA), and lactate dehydrogenase (LHD) Assay Kits were sourced from Institute of Biological Engineering of Nanjing Jian-cheng (Nanjing, China). Propidium iodide (PI) and Hoechst 33342 were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and provided by commercial suppliers.

### Preparation and fractionation of whey protein hydrolysates

The preparation of WPHs involved the method described by Zhang et al. (2012) with pepsin and trypsin. After preparation, WPHs were adsorbed by macroporous adsorption resin DA201-C and eluted by three various concentrations of ethanol (20, 40 and 60%), the graded elution fractions obtained were named M20, M40 and M60, respectively.

### HPLC molecular weight profile and amino acid composition

Molecular weight distribution (MWD) of WPHs was determined using an HPLC system (Agilent 1100<sup>®</sup>, CA, USA). A total of 17 amino acids were determined using oxidative acid hydrolysis for cysteine/cystine and methionine and conventional acid hydrolysis for the others.

### The degree of hydrophobicity

The degree of hydrophobicity is calculated according to the Q rule established by Ney (1971). Ney's Q value is the average free energy for the transfer of the amino acid side chains from ethanol to water. Ney proposed the Q value as a means to theoretically measure hydrophobicity by the equation:  $Q = \Sigma \Delta f / n$ , where Q = average hydrophobicity of a peptide,  $\Sigma \Delta f$  = the sum of free energy for the transfer of amino acid side chains from ethanol to water in cal/mol for each residue, and n = the number of amino acid residues.

**Table 1.** Amino acid content of WPHs (g/100 g protein)

Amino acid	Hydrophobicity value (kJ/mol)	Amino acid content g/100 g		
		M20	M40	M60
Asp	2.26	4.53	3.07	3.08
Glu	2.30	10.10	6.78	7.04
Ser	0.17	2.24	2.73	2.29
His	2.09	0.25	0.26	0.44
Gly	0.00	1.56	1.76	1.88
Thr <sup>b</sup>	1.84	2.20	1.17	1.49
Arg	3.06	0.65	1.80	1.57
Ala <sup>a</sup>	3.06	2.47	3.34	2.90
Tyr <sup>a</sup>	12.01	1.04	1.63	2.29
Cys-s	4.19	0.19	0.25	0.31
Val <sup>ab</sup>	7.07	2.02	2.27	3.10
Met <sup>ab</sup>	5.44	0.31	1.24	1.21
Phe <sup>ab</sup>	11.09	1.02	1.74	2.56
Ile <sup>ab</sup>	12.43	2.86	2.32	2.85
Leu <sup>ab</sup>	10.13	5.20	7.39	7.70
Lys <sup>b</sup>	6.28	4.25	3.77	3.87
Pro <sup>a</sup>	10.88	6.64	5.08	6.16
Total amount of hydrophobic amino acid (g/100 g)		21.56	25.01	28.77
Grand average of hydrophobicity Value Q (kJ/mol)		5.61	5.73	6.58

Note: <sup>a</sup>Hydrophobic amino acid

<sup>b</sup>Essential amino acid

### Determination of the antioxidative activity

The reducing ability of WPHs is determined by potassium ferricyanide method, using the method described by Peng et al. (2009) and Zhu et al. (2006). The free radical DPPH scavenging activity was determined by the method of by Wu et al. (2003). The suppression rate of lipid peroxidation is determined by thiobarbituric acid (TBA) assay (Osawa & Namiki, 1985).

### Cell morphological assay

PC12 cells were plated in cell culture plates at  $3 \times 10^5$  cells per well according to the method described by Lu et al. (2010). To produce oxidative stress, H<sub>2</sub>O<sub>2</sub> was freshly prepared from a 30% stock solution prior to each experiment. Cells in WPHs-group were pretreated with 100 or 200 µg/ml WPHs for 2 h, following co-incubated with 100 µmol/l H<sub>2</sub>O<sub>2</sub> for another 24 h. The H<sub>2</sub>O<sub>2</sub>-group cells were only treated with 100 µmol/l H<sub>2</sub>O<sub>2</sub> for 24 h, while the control-group cells were cultured with the same medium without H<sub>2</sub>O<sub>2</sub> or WPHs. At the end of the experiment, all the cells were photographed by phase-contrast microscopy (Nikon).

### Cells viability assay

The cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method

**Table 2.** Antioxidant ability of WPHs

	DPPH radical scavenging capacity (IC <sub>50</sub> value, mg/ml)	Suppression rate of lipid peroxidation (IC <sub>50</sub> value, mg/ml)	Reducing power (A <sub>700 nm</sub> , 10 mg/ml)
M20	11.48±0.52 <sup>A</sup>	8.44±0.28 <sup>A</sup>	0.573±0.059 <sup>A</sup>
M40	7.25±0.13 <sup>B</sup>	6.44±0.13 <sup>B</sup>	1.273±0.024 <sup>B</sup>
M60	4.39±0.08 <sup>C</sup>	5.68±0.15 <sup>C</sup>	1.416±0.025 <sup>C</sup>

Note: A, B and C represent the significant difference among three samples,  $P < 0.05$

**Table 3.** The effect of WPHs on T-AOC, SOD, CAT, MDA and LDH on H<sub>2</sub>O<sub>2</sub>-induced PC12 cells ( $X \pm SD$ )

	T-AOC (U/mg)	SOD (U/mg)	CAT (U/mg)	MDA (nmol/mg)	LDH (U/l)
Control	9.39±0.29 <sup>d</sup>	201.22±3.99 <sup>e</sup>	22.66±1.11 <sup>e</sup>	2.32±0.20 <sup>d</sup>	557.42±19.34 <sup>e</sup>
H <sub>2</sub> O <sub>2</sub>	2.10±0.14 <sup>a</sup>	96.90±2.98 <sup>a</sup>	5.69±0.99 <sup>a</sup>	7.19±0.52 <sup>a</sup>	1026.32±21.22 <sup>a</sup>
M20	5.79±0.10 <sup>b</sup>	111.77±4.77 <sup>b</sup>	7.52±0.79 <sup>b</sup>	5.03±0.52 <sup>b</sup>	953.76±17.66 <sup>b</sup>
M40	7.80±0.24 <sup>c</sup>	151.63±6.12 <sup>c</sup>	14.79±0.55 <sup>c</sup>	4.25±0.12 <sup>c</sup>	901.01±11.32 <sup>c</sup>
M60	7.83±0.18 <sup>c</sup>	179.81±2.25 <sup>d</sup>	18.77±1.00 <sup>d</sup>	2.28±0.28 <sup>d</sup>	843.43±12.90 <sup>d</sup>

Note: a, b and c represent the significant difference among samples,  $P < 0.05$

as described by Zhao et al. (2007). Briefly, MTT was dissolved in phosphate buffer saline (PBS) solution at a concentration of 5 mg/ml, and cells ( $5 \times 10^3$  cells/well) seeded in 96-well plates. After exposure to different concentration of esculin and 50 mM Dopamine for 24 h, 10 ml MTT solution was added to each well and incubated for additional 4 h. Then, the medium was aspirated off. To achieve solubilization of the formazan crystal formed in viable cells, 200 ml dimethylsulphoxide was added to each well. The absorbance was read at 570 nm.

#### Nuclear staining for assessment of apoptosis

PC12 cells were stained with two fluorescent dyes, PI and Hoechst 33342 (Lieberthal et al. 1998). Each group of cells was photographed twice (Olympus Optical., Japan magnification,  $\times 400$ ). Apoptotic cells were counted for five independent microscopic fields for each group (Giovannini et al. 2000).

#### T-AOC, SOD, CAT, MDA and LDH assays

To analyse T-AOC, SOD, CAT, and MDA activities, the cells were washed with PBS twice and lysed on ice for 30 min and then centrifuged at 12000 g for 5 min at 4 °C. Protein content was measured using a bicinchoninic acid (BCA) kit (Beyotime Institute of Biotechnology, Nantong, Jiangsu, China). In order to analyse the level of LDH activity, the medium was collected. The activities of T-AOC, SOD, CAT, MDA and LDH were tested by Assay Kits according to the manufacturers' instructions.

#### Statistical analysis

Results were expressed as mean  $\pm$  SD of at least three independent experiments. Multiple groups' comparisons

were evaluated by Duncan's multiple range test, and performed in SPSS (SPSS 18.0, USA). Differences between the means were determined and were considered statistically significant when  $P < 0.05$ .

## Results and discussion

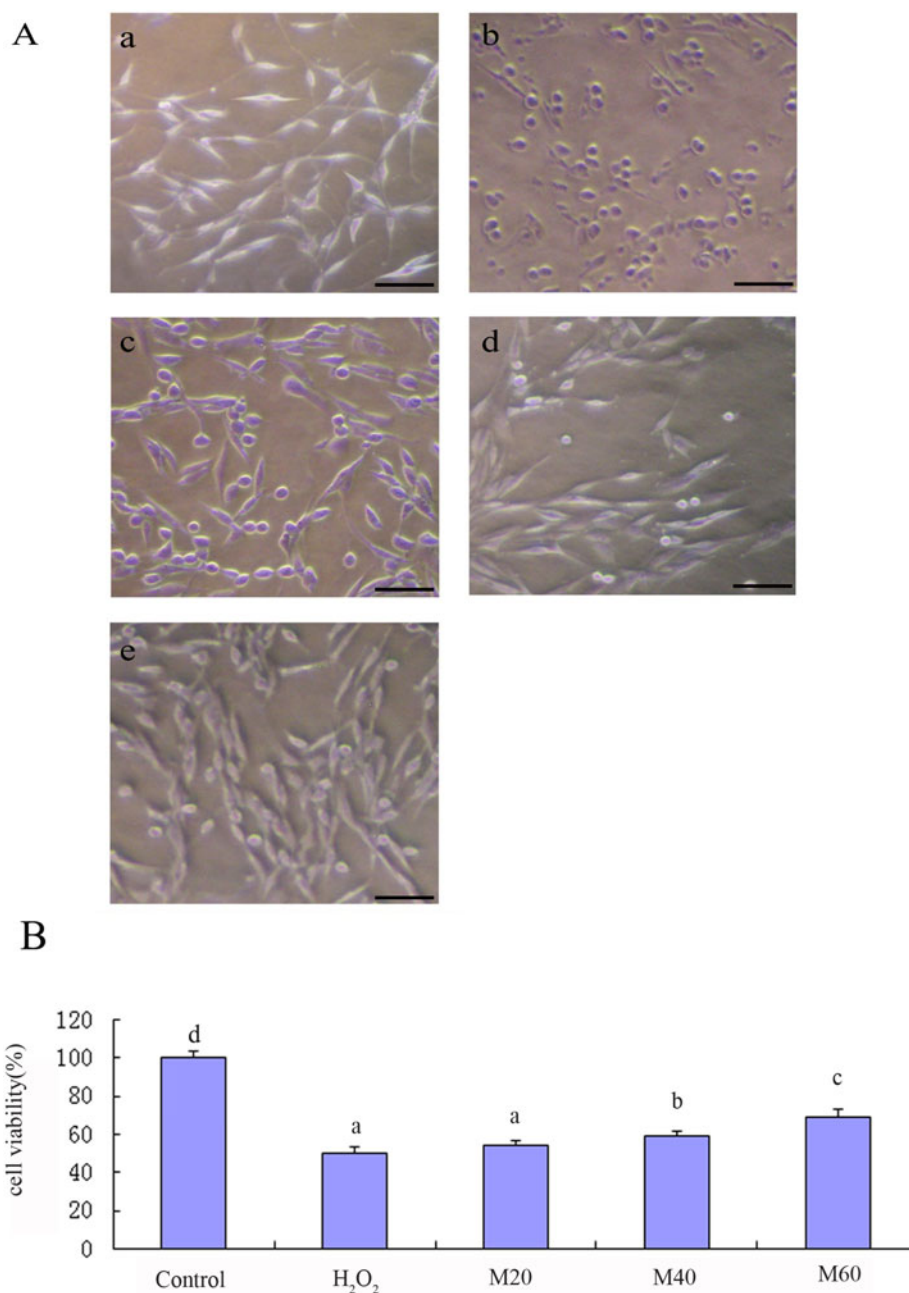
### Fractionation of WPHs and Amino acid composition of each component

The amino acid composition and the degree of hydrophobicity of M20, M40 and M60 were analysed. As shown in Table 1, component of hydrophobic amino acids Tyr, Val, Phe, Leu and hydrophobicity Q value showed a trend of increase from M20 to M60. Many researchers have confirmed that some kinds of amino acid residues, such as Pro, Tyr, Val, Ile, and Leu were possibly responsible for the high antioxidant effects (Chen et al. 1996; Zhu et al. 2008; Alemán et al. 2011). As an antioxidant needs to be stable enough to donate electron to stabilize and neutralize free radicals (Patil et al. 2010), these hydrophobic amino acids could act as qualified electron donors to stabilize free radicals and break free radical chain reaction.

### Antioxidant ability of WPHs in vitro

DPPH radical scavenging capacity, suppression rate of lipid peroxidation and reducing power of WPHs were tested to evaluate antioxidant abilities in vitro from different perspectives. Based on the data in Table 2, three components of WPHs displayed reasonably good antioxidant ability. Moreover, with the increase of hydrophobicity, antioxidant ability of WPHs improved significantly ( $P < 0.05$ ).

Porter et al. (1989) first formulated a polar paradox hypothesis that regarding oil-in-water emulsion, hydrophobic antioxidants are more active than their hydrophilic



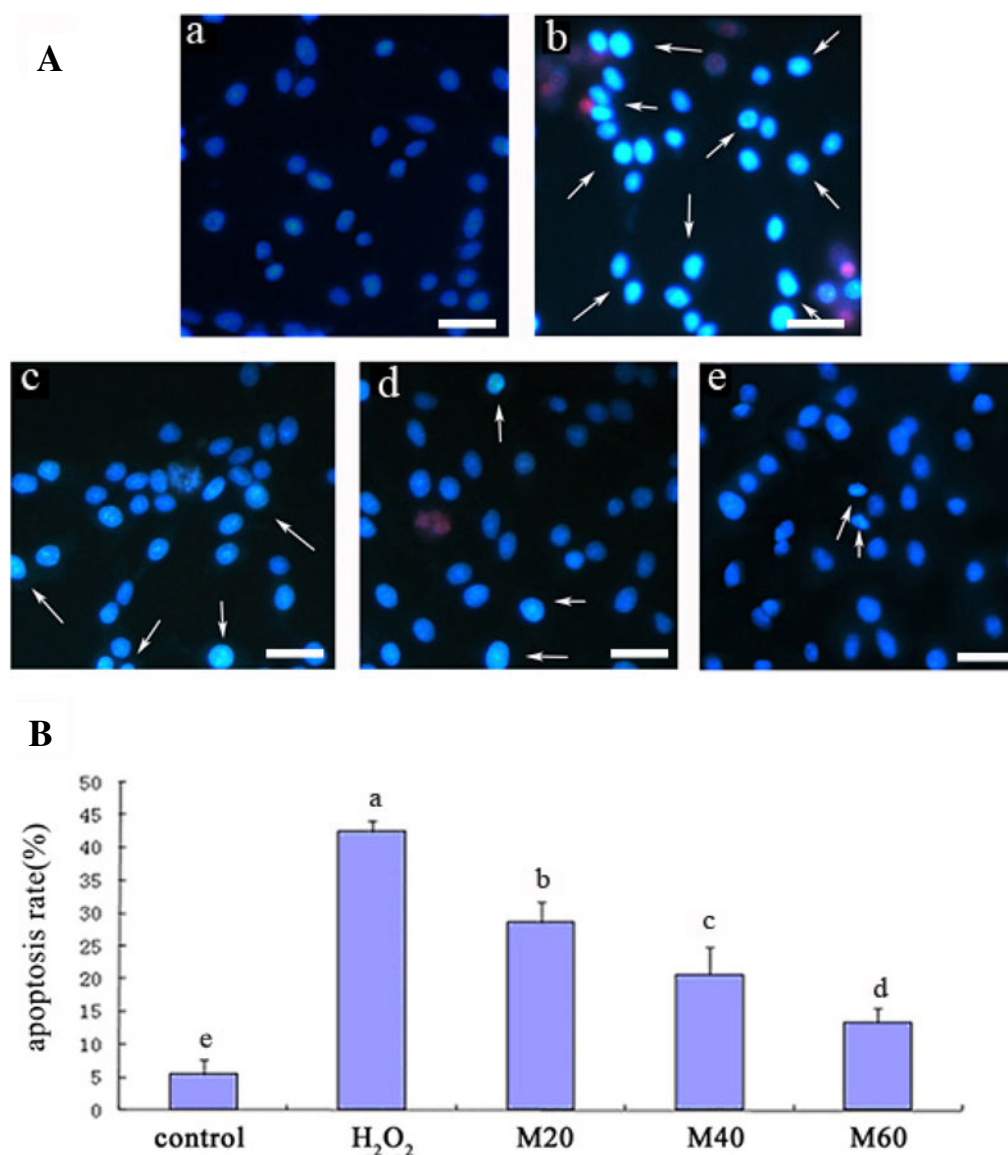
**Fig. 1.** The effect against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress of PC12 cells morphology and viability. (A) Effect of Graded elution fractions of WPHs on H<sub>2</sub>O<sub>2</sub>-induced morphological alterations in PC12 cells (a Control, b Model (200 μmol/l H<sub>2</sub>O<sub>2</sub>), c M20+200 μmol/l H<sub>2</sub>O<sub>2</sub>, d M40+200 μmol/l H<sub>2</sub>O<sub>2</sub>, e M60+200 μmol/l H<sub>2</sub>O<sub>2</sub>). bar=20 μm. (B) Graded elution samples of WPHs inhibit the reduction of cell viability induced by H<sub>2</sub>O<sub>2</sub> in PC12 cells (a, b, c, and d represent the significant difference among samples,  $P<0.05$ ).

homologues and there was a linear dependency between the antioxidant capacity and the hydrophobicity. Frankel et al. (1994) explained that this phenomenon might be because hydrophobic antioxidants tend to concentrate at the interfacial membrane where the oxidation is supposed to occur. Laguerre et al. (2010) confirmed that there was indeed a dependency between the antioxidant capacity and the hydrophobicity though in a nonlinear trend. Our

results showed that the more hydrophobic amino acids WPHs contained, the better antioxidant ability they presented.

#### *WPHs increased intracellular antioxidant system load*

Monitoring the change of antioxidant system could help us to understand the antioxidative activities in living cells.



**Fig. 2.** Graded elution samples of WPHs protected PC12 cells against H<sub>2</sub>O<sub>2</sub>-induced apoptosis. (A) Morphological apoptosis was determined by staining with Hoechst 33342 and PI. Arrowheads indicate apoptosis cells. (a Control, b Model (200  $\mu$ mol/l H<sub>2</sub>O<sub>2</sub>), c M20 + 200  $\mu$ mol/l H<sub>2</sub>O<sub>2</sub>, d M40 + 200  $\mu$ mol/l H<sub>2</sub>O<sub>2</sub>, e M60 + 200  $\mu$ mol/l H<sub>2</sub>O<sub>2</sub>). bar = 50  $\mu$ m. (B) Apoptosis ratio was determined by counting under fluorescence microscope after PC12 cells were stained with Hoechst 33342 and PI. (a, b, c, d, and e represent the significant difference among samples,  $P < 0.05$ ).

The T-AOC reflects the ability of the body to remove free radicals, which may be one of the most effective defences of a living body against various diseases. The CAT level reflects catalase activity, which decompose H<sub>2</sub>O<sub>2</sub> into water and oxygen. SOD catalyses the neutralization of superoxide anion to H<sub>2</sub>O<sub>2</sub>.

Compared with control-group, T-AOC, CAT, SOD levels of H<sub>2</sub>O<sub>2</sub>-group declined significantly ( $P < 0.05$ ) (Table 3) owing to H<sub>2</sub>O<sub>2</sub>-induced oxidative damage of living cells. While pretreatment with (M20, M40 and M60), all the three indexes increased significantly ( $P < 0.05$ ) compared with the H<sub>2</sub>O<sub>2</sub>-group. These results suggested that WPHs could

increase the clearance rate of H<sub>2</sub>O<sub>2</sub> and protect the cells against oxidative injury. Among the three WPHs, M60 exhibited the highest protective effect.

While MDA reflects the degree of lipid peroxidation in cells and LDH suggests the level of cell injury. The MDA and LDH levels of H<sub>2</sub>O<sub>2</sub>-group increased ( $P < 0.05$ ) compared with that of the control group (Table 3), indicating that the lipid system had been damaged. However, the MDA and LDH levels of the WPHs group decreased significantly compared with the H<sub>2</sub>O<sub>2</sub>-group. The results indicated that WPHs could reduce the invasion of reactive oxygen and inhibit lipid peroxidation, thus protect the cell membrane to

a certain extent. And the ability of inhibiting oxidative damage was enhanced from M20 to M60 in turn.

#### *Morphological alterations and cell viability of PC12 Cells induced by H<sub>2</sub>O<sub>2</sub>*

It is believed that H<sub>2</sub>O<sub>2</sub> could cause apparent damage and result in cell morphological alterations (Li et al. 2003). Different concentrations of H<sub>2</sub>O<sub>2</sub> were used to induce dose-dependent oxidative damage in PC12 cells and 200 µmol/l H<sub>2</sub>O<sub>2</sub> was median lethal dose (data not show). Normal cells are multipolar, have elongated shapes, and grow attached to a substrate (Fig. 1Aa). Morphological change was observed under inverted microscope after 200 µmol/l H<sub>2</sub>O<sub>2</sub> affect cells. PC12 cells are spherical in shape and in suspension without attaching to a surface (Fig. 1Ab). WPHs exerted a protective effect against H<sub>2</sub>O<sub>2</sub>-induced morphological change (Fig. 1Ac–e). The data were consistent with quantitative data on cell viability (Fig. 1B). These results indicated that WPHs can protect PC12 cells against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity and significantly improve the morphology of H<sub>2</sub>O<sub>2</sub>-injured cells.

Attention should be concentrated on the increasing protective effect of graded elution samples of WPHs. A better cell morphology and a significantly 19.3% increase in cell viability were presented ( $P < 0.05$ ) while PC12 cells were cultured with M60. It is interesting to find that highly hydrophobic samples of WPHs achieved a more effective protecting ability against oxidative stress.

#### *Nuclear staining for assessment of apoptosis*

The effects of WPHs on H<sub>2</sub>O<sub>2</sub>-induced apoptosis in PC12 cells are shown in Fig. 2. The PC12 cells nuclei of control group exhibited diffuse homogeneous blue fluorescence (Fig. 2Aa). When exposed to 200 µmol/l H<sub>2</sub>O<sub>2</sub>, PC12 cells showed the condensation of chromatin, and the appearance of brilliant condense blue fluorescence in nuclei, compared with the control group (Fig. 2Ab). However, when the cells were pre-incubated with 200 µg/ml WPHs (M20, M40 and M60), H<sub>2</sub>O<sub>2</sub>-induced cell apoptosis was significantly attenuated. The nuclear staining count assay indicated that compared with control group, up to 42.6 ± 1.6% of cells incubated with 200 µmol/l H<sub>2</sub>O<sub>2</sub> for 24 h apoptosis and that pretreatment with WPHs (M20, M40 and M60) reduced 13.4, 20.7 and 28.6% of apoptotic cells respectively ( $P < 0.05$ ) (Fig. 2B).

Excessive reactive oxygen species (ROS) could promote oxidative reaction and ultimately leads to apoptotic or necrotic cell death. Many studies have shown that nuclear condensation and DNA fragmentation are closely related to H<sub>2</sub>O<sub>2</sub>-induced neuronal cell death (Zhou et al. 2008; Zhao et al. 2011). Our results indicated that WPHs quenched excess free radicals and prevented DNA from being attacked by ROS. There was a positive correlation between the ability of inhibiting apoptosis and hydrophobicity of WPHs.

These results illustrated that WPHs exerted significant protection on PC12 cells against H<sub>2</sub>O<sub>2</sub>-induced cell apoptosis. This might be one of the important antioxidant abilities WPHs represented. What's more, highly hydrophobic samples of WPHs showed a more effective anti-apoptosis ability.

#### **Conclusion**

Oxidative stress damaged PC12 cells were protected by WPHs. In terms of chemical antioxidant ability and cultured cell model systems, WPHs improved antioxidant ability, maintained cell viability, and inhibited apoptosis by increasing clearance rate of H<sub>2</sub>O<sub>2</sub> and inhibiting cell membrane's lipid peroxidation. These results indicate the potential benefits of WPHs as valuable food antioxidative additives. And more notably, hydrophobicity enhanced the protective effect of WPHs against oxidative stress.

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