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Resistance investigation of wheat bran polyphenols extracts on HEK293 cells against oxidative damage

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Abstract:

Oxidative stress has been considered as a major cause of cellular injury in a variety of clinical abnormalities. One of popular methods to inhibit the reactive-oxygen-species (ROS)-induced cellular injury is dietary or pharmaceutical augmentation of endogenous antioxidant defense capacity. In this study, the resistance effects of wheat bran polyphenols extracts (WBPE) against H_2O_2-induced cytotoxicity in HEK293 cells were investigated. The phenolic components of WBPE were analysis using UPLC/TQD and the presence of ferulic acid, p-coumaric acid, o-coumaric acid and gallic acid components were confirmed. Cytotoxicity of such contents and their effects on cell morphology were also evaluated. The results demonstrated that incubation of WBPE-N9-4 with cell prior to H_2O_2 exposure could significantly improve cell viability, corresponding with increased catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) levels. On the other hand, reduction in the levels of lactate dehydrogenase (LDH), malondialdehyde (MDA), and ROS generation were obviously observed. The presence of WBPE-N9-4 inhibited H_2O_2-induced apoptosis in HEK293 cells, which was confirmed by flow cytometry of sub-G1 DNA content and Annexin V assay. It is reasonable to assume that WBPE-N9-4 have an excellent ability to prevent HEK293 cells from oxidization-damage. Our studies firstly reveal that WBPE-N9-4 have resistance effects against H_2O_2-induced cytotoxicity in HEK293 cells.

Keywords: *wheat bran polyphenols extracts, hydrogen peroxide, oxidative stress, HEK293 cells, reactive oxygen species*

1. Introduction

Cereal bran as outer grain layers which are separated from the milling process during the production of refined flours [1, 2]. Wheat bran, a byproduct generated abundantly during wheat processing, is regarded as good dietary source of wheat antioxidants [3]. The antioxidant components in wheat are mainly phenolics [4, 5], including ferulic acid [6] and as protocatechuic, sinapic, vanillic, *p*-hydroxybenzoic and *p*-coumaric acids [7-9], which distributed in the bran fractions [10, 11]. Wheat bran extracts (WBEs) have potential antiproliferative activity and the phenolics derived from WBEs possess various physiological activities include antioxidative activity and anti-radiation activity, [12] but the exact mechanism is not yet fully understood. The relationships between dietary antioxidant potential and tumor multiplicity have been revealed after ingesting whole wheat as well as wheat bran diets and after ingesting a wheat bran diet with tumor load [13]. The results indicated that antioxidant ability of wheat in the diets is associated with their antitumor activity. These studies demonstrated that WBEs may serve as an excellent dietary source for disease prevention and health promotion.

Although the antioxidant activities of WBEs have been demonstrated in previous reports [3, 14, 15], deep and comprehensive understanding about their possible resistance effects against radical-initiated oxidative damage is still infant. Thus, it's necessary to develop cell model exposure to specific components to investigate how these components affect biological system physiological processes [16-20]. In cellular system, generation and elimination of reactive oxygen species (ROS) is regulated by the antioxidant system. In normal physiological process, the mitochondria converts 1-2% of the consumed oxygen to ROS, while ROS levels increased dramatically under environmental stress (e.g. ultraviolet or heat exposure), [21]. The excessive ROS can

damage vital cellular structures such as lipids, DNA, RNA, and proteins [22, 23] via oxidation of relevant small molecular components, leading to severe biological response such as mutation and cell death [24, 25]. These ROS generation induced damages attribute to the pathogenesis of inflammatory disease, cardiovascular disease, cancer, diabetes, Alzheimer's disease, cataracts, autism and aging [24, 26]. Human embryonic kidney (HEK) 293 cells are a specific cell line originated from HEK cells grown in tissue culture, such cell lines have been widely used in cell biology as well as toxicology studies [27, 28]. In particular, hydrogen peroxide (H_2O_2) is one of major contributors to oxidative stress. Exogenous treatment with H_2O_2 in HEK293 cells could serve as an *in vitro* mimic models for investigating oxidative stress-induced injury [29]. It has been illustrated that natural antioxidants may increase cells resistance against H_2O_2-induced oxidative stress [30]. Some studies reported that ferulic acid ester of oligosaccharides (FQs) released either from microorganisms in the colon or from enzymatic hydrolysis of arabinoxylans present in wheat bran has strong ability against oxidative DNA damage in normal human peripheral blood lymphocytes induced by H_2O_2 [31]. To our best knowledge, very few studies involved evaluation of wheat bran polyphenols extracts (WBPE) effects on cell resistant ability toward radical-initiated oxidative damage. In addition, preparative enrichment and separation of polyphenol acid from WBEs with NKA-9 macroporous resin was performed before subsequent experiments. The products of WBEs in NKA-9 with 40% ethanol elution were named WBPE-N9-4. In order to have a deep and comprehensive understanding of WBPE effects on cell antioxidation ability, HEK293 cells were chosen as an oxidative stress model for effects of WBPE-N9-4 against H_2O_2-induced apoptosis, as well as the related mechanisms.

2. Materials and Methods

2.1 Chemicals, reagents and equipment

Raw wheat bran was provided by Yihai Kerry Food Industry Co., Ltd. (Kunshan, China). HEK293 cells were purchased from American Type Culture Collection, chemical reagents included ferulic acid, o-coumaric acid, p-coumaric acid, gallic acid, and olivetol (Sigma-Aldrich, St. Louis, USA), high-glucose Dulbecco's Modified Eagle's Medium (HG-DMEM) (Gibco BRL, Life Technologies, USA), trypsin-ethylene diamine tetraacetic acid (EDTA) (Beyotime, Jiangsu, China), fetal bovine serum (FBS) (Sijiqing, Zhejiang, China), and methyl thiazolyl tetrazolium (MTT) (Sigma, St. Louis, USA) were also purchased commercially. The lactate dehydrogenase (LDH) and glutathione peroxidase (GSH-Px) assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), Annexin V/FITC kit, Hochest33258 kit, Cell cycle and ROS assay kit, and mitochondrial membrane potential assay kit with JC-1 were all purchased from Beyotime Institute of Biotechnology (Haimen, China).

2.2 Preparation of WBPE.

Initially, wheat bran (1 kg) was washed with 5–6 volumes (w/v) of water 3 times to remove starch and heated and maintained at 50 °C for about 12 h. Then, the dried wheat bran was ground into powders by hammer mill and passed through 100 mesh. WBEs were prepared according to previously report [32]. The bran (30 g) was then extracted twice with 80% ethanol at a ratio of 8:1 (v/w) at room temperature for 15 h. The mixture was centrifuged at 9000×g for 20 min and drying at 40 °C using a rotary evaporator. The ethanol extract (4 g) was further freeze-dried and stored in a sealed container at 4 °C in a dark environment for further used and analysis. The freeze-dried dispersed product was WBEs.

In addition, preparative enrichment and separation of polyphenol acid from WBEs with 4 types of macroporous resins were performed before subsequent experiments. The WBEs then were subjected to NKA-9 macroporous resin column chromatography eluting with a gradient of ethanol–water (40:60). The fractions were collected, concentrated, and lyophilized for the subsequent experiments. The freeze-dried dispersed products were defined as WBPE-N9-4.

2.3 Chromatographic system and conditions

The products were analyzed using the Acquity UPLC/TQD system (Waters, Milford, MA), including an autosampler, photodiode array detector and an MS pump equipped with an electrospray ionization (ESI) probe as the interface. The samples were separated by ultra-high performance liquid chromatography on an Acquity UPLC BEH C18 column (2.1×50 mm, 1.7 μm) with a mobile phase consisting of acetonitrile solution (A) and 0.1% (v/v) formic acid water solution (B) at a flow rate of 0.3 mL/min (Table 1). Detection of phenolics was conducted in the multiple reaction monitoring (MRM) mode. Individual compounds were first identified using MRM, using specific precursor-production transition: *m/z* 163.08>119.64 for o-coumaric acid; *m/z* 163.08>119.15 for p-coumaric acid; *m/z* 193.00>134.10 for ferulic acid; *m/z* 169.05>125.00 for gallic acid and *m/z* 181.08>43.03 for olivetol. Detected phenolic compounds were quantified against standard curves generated with phenolic standards. Results were expressed as milligrams compound per gram of extract (mg compound/g extract).

2.4 Cell culture and treatment.

HEK293 cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (HG-DMEM) supplemented with 10% fetal bovine serum (FBS), maintained at 37 ℃ in an incubator with 5% CO_2. The medium was changed every two days. All

experiments were performed for 12 h after the cells were seeded in microplates. N9-4 were freshly prepared as a stock solution in ethanol and diluted with HG-DMEM. The control group was performed in the presence of 1% (v/v) ethanol [33] under the same culture conditions while ferulic acid was used as a positive control.

2.5 Cell viability and LDH release assay

Cell viability was determined using the MTT assay [34]. The MTT cytotoxicity assay was performed as previously described [35, 36] with minor modifications. Briefly, HEK293 cells were seeded at a density of 3×10^4 cells/well in 96-well plates (Costar 3599; Corning, NY) for 12 h attachment. WBPE-N9-4 and ferulic acid were dissolved separately in ethanol and diluted with HG-DMEM. The cells were incubated first with WBPE-N9-4 (0.1 -1 mg/mL) or ferulic acid (1 mM) for 1 h, then with 1 mM H_2O_2 for 2 h [37]. After being replaced with fresh medium, 20 μL of MTT (5 mg/mL in phosphate buffer solution, PBS) was added and incubated for 4 h at 37 °C in humidified incubator with presence of 5% CO_2. The cells were incubated in a dark incubator to avoid unexpected phototoxicity [34]. The medium was then carefully removed, and colored formazan were dissolved in 150 μL of dimethyl sulfoxide (DMSO). The plate was shaken for 10 min, and the absorbance of sample was measured at 570 nm using a SH-1000 microplate reader. The results were expressed as the percentage of viability (%) = [(optical density of treated cells/optical density of control cells)]. All assays were repeated as three independent experiments, each experiment containing at least six replicates.

When the cells were damaged by H_2O_2, the cell viability will decrease, and there will be a change in the value of LDH. The release of intracellular LDH into the culture medium is an indicator of irreversible cell death due to membrane damage [38]. LDH activity can be achieved on cytotoxicity quantitative analysis through the

detection of the release from the plasma membrane to rupture the cell culture medium. The LDH leakage was detected using an assay kit. First, HEK293 cells were seeded at a density of 9×10^5 cells/well in 6-well plates. The cells were incubated first with WBPE-N9-4 and ferulic acid for 1 h, then exposure to 1 mM H_2O_2 for 2 h. Then, the cells were washed twice with PBS and then lysed by the cell lysis buffer to release the LDH inside the living cells into the new supernatant. After the reaction, the samples absorptance was recorded at 450 nm. The results were presented as ratio of the control value.

2.6 Observation of morphological changes.

HEK293 cells were seeded in 96-well plates at a density of 3×10^4 cells/well. After the treatment with WBPE-N9-4 (1 mg/mL) or ferulic acid (1 mM) for 1 h and H_2O_2 for 2 h, the cells were examined under inverted phase contrast microscopy (BX41 Olympus Optical Co. Ltd., Japan). The cells were observed with a fluorescence microscope [39] (BX41 Olympus Optical Co. Ltd., Japan) after being stained with Hochest33258 dye, and were seeded into 6-well plates at a density of of 9×10^5 cells/well. Then, the cells were observed and cell images were recorded [40, 41].

2.7 ROS assay.

HEK293 cells were seeded at 3×10^4 cells/well in clear-bottom, black-walled 96-well plates (Costar 3606; Corning, NY) and for 12 h attachment. Then, the cells were incubated with carboxy-2',7'-dichloro-dihydro-fluorescein diacetate probe for 20 min and washed twice with PBS. The cells were followed exposed to WBPE-N9-4 (1 mg/mL) or ferulic acid (1 mM) for 1 h and H_2O_2 for 2 h. Fluorescence of samples was measured at 488 nm (excitation) and 525 nm (emission) wavelengths using SpectraMax M5 microplate reader. All assays were repeated for three independent experiments, each experiment containing at least six replicates.

2.8 Measurements of CAT, SOD, GSH-Px and MDA.

The activities of catalase (CAT), superoxide dismutase (SOD), and the content of malondialdehyde (MDA) were measured by assay kits (Beyotime Institute of Biotechnology, Haimen, China). GSH-Px activity was determined using an assay kit (Jiancheng Bioengineering). The assay for GSH-Px activity analysis was performed by quantifying the rate of oxidation of the reduced glutathione to the oxidized glutathione with H_2O_2 catalyzed by GSH-Px. Initially, the HEK293 cells were washed with PBS after treatment (1 mg/mL WBPE-N9-4, 1 mM ferulic acid and 1 mM H_2O_2), then scraped into ice-cold PBS and homogenized with sonication. The homogenate was centrifuged for 10 min at 12,000 g at 4 ℃ and supernatant was used to measure CAT, SOD, GSH-Px activities. Protein content was measured by Coomassie blue protein-binding method with bovine serum albumin as the standard [42]. The concentration of MDA can be determined at a wavelength of 532 nm by transfer it to form a stable chromophoric production via reacting with thiobarbituric acid (TBA). The level of MDA was expressed as nmol/mg protein.

2.9 Cell cycle analysis and sub-G1 DNA content assay.

The growing cells were seeded at 9×10^5 cells per well in six-well plates for 12 h attachment. After incubated with WBPE-N9-4 (1 mg/mL), ferulic acid (1 mM) and H_2O_2 (1 mM), the cells were washed with PBS, then trypsinized, fixed in 70% (v/v) ice-cold ethanol and stored at 4 ℃ for 24 h. Then, cell pellets were washed with ice-cold PBS, centrifuged, resuspended in 1 mL of PBS containing 1 mg/mL RNase and 50 μg/mL propidium iodide (PI), and incubated in the dark at 37 ℃ for 30 min and then on ice. Samples were filtered through a nylon mesh and the cell cycle was analyzed using the flow cytometer with the software package Cell Quest Pro (Becton Dickinson, CA, USA).

2.10 Cell apoptosis test.

HEK293 cells were seeded at 9×10^5 cells/well in 6-well plates for 12 h attachment. Cell apoptosis was detected by Annexin V-FITC kit after 3h treatment with WBPE-N9-4 (1 mg/mL), ferulic acid (1 mM) and H_2O_2 (1 mM). Briefly, the cells were gently trypsinized, washed with PBS, suspended in binding buffer, and incubated with Annexin V-FITC and PI at room temperature in the dark for 10 min. The cells were analyzed immediately by the flow cytometer. Specially, cells were delivered at a high flow rate of 200-300 cells/s. Digital signals of forward scatter (FS), sideward scatter (SS), and green and red fluorescence (FL1, FL2) were recorded for further analysis. The cells stained positively by Annexin V-FITC but remaining impermeable to PI (AV+/PI−) were regarded as early apoptotic cells[43].

2.11 Measurement of mitochondrial membrane potential ($\Delta\psi_m$).

Mitochondrial membrane potential ($\Delta\psi_m$) was measured using a dual-emission potential-sensitive probe JC-1 in the monomeric form, with excitation at 490 nm and emission at 527 nm[44]. The treated HEK293 cells (1 mg/mL WBPE-N9-4 and 1 mM ferulic acid for 1 h, and then 1 mM H_2O_2 for 2 h) were harvested and incubated with 10 μg/mL JC-1 at 37 °C in the dark for 10 min. The cells were then washed twice with PBS, resuspended into 0.5 mL of PBS and analyzed using flow cytometry for green and orange fluorescence with a 525 and 575 nm filter respectively for changes in $\Delta\psi_m$. The values of fluorescence intensity were averaged.

2.12 Statistical analysis.

All the experiments were performed in triplicate and data were expressed as mean ± standard deviation (SD). Statistical analysis was performed according to Student's t-test and one-way analysis [45] on SPSS 18.0 (SPSS Inc. US Chicago), p values <0.05 were considered to be significant.

3. Results and Discussion

3.1 UPLC/TDQ analysis.

UPLC-MS/MS analysis of WBPE-N9-4 was conducted in order to quantify contained phenolic compounds, which may be responsible for their antioxidant activity. Total ion chromatograms of the standard solution for five phenolic compounds were shown in Fig. 1. The presence of standard phenolic compounds by UPLC/TQD was confirmed in Table 2. These analysis results confirmed the existence of ferulic acid (35.49 ± 2.7 mg/g), p-coumaric acid (16.79 ± 0.68 mg/g), o-coumaric acid (0.92 ± 0.05 mg/g) and gallic acid (2.08 ± 0.09 mg/g); however olivetol was not detected. Ferulic acid was the most abundant phenolic compound in WBEs. In previous study, similar results were obtained from phenolic acid concentrations in spring and winter wheat [9]. The results showed that the content of ferulic acid was higher than other phenolic acids in WBPE-N9-4, which may be associated with WBPE-N9-4's antioxidative activity. And in ROS assay, the antioxidant activity of WBPE-N9-4 was very close that of the positive control-ferulic acid (Fig. 3).

3.2 Effects of WBPE-N9-4 on H_2O_2-injured growth and viability in HEK293 cells.

Cell viability was determined by MTT assay; cell count after exposure to H_2O_2 decreased in dose- and time- dependent manner. Specially, Cell viability was decreased significantly after exposure to H_2O_2 for 2 h, suggesting that HEK293 cells were injured at IC_{50} value of 0.98±0.02 mM. Therefore, H_2O_2 with concentration 1 mM was used for further experiments. Moreover, no cytotoxic and proliferative effects of WBEs or WBPE-N9-4 were observed when normal HEK293 cells were treated for 12 or 24 h. As shown in Table 3, cell viability dropped to 50.90±2.20% after exposure to only 1 mM H_2O_2. However, cell viability significantly increased to 66.65±2.39% and 72.78±2.34%, respectively, when treated with WBPE-N9-4 (0.50 &

1.00 mg/mL) prior to H_2O_2 exposure for 1 h. LDH, which is a stable cytoplasmic enzyme in cells, is an important indicator of cytotoxicity[46]. LDH will be released into the culture supernatant when the cell membrane is damaged[47] Thus, the percentage of LDH leakage could also be employed to investigate the resistance effect of WBPE-N9-4 (Table 3). The results indicated that when treated with WBPE-N9-4, LDH decrease from 13.09 to 7.64, which agreed with cell viability results.

3.3 Effects of WBPE-N9-4 on the morphology of H_2O_2-treated HEK293 cells.

The morphological alteration was observed to cells exposure to WBPE-N9-4 using a phase-contrast microscope. The HEK293 cells in the control group grew well and maintained normal round morphology (Fig. 2A1). However, cell exposure to H_2O_2 for 2 h could result in obvious reduction in viable cells counts as well as cell body shrinkage (Fig. 2A2). In contrast, after treatment with WBPE-N9-4 (1 mg/mL) and ferulic acid (1 mM) for 1 h before exposure to H_2O_2, the majority of the cells maintained in a good status without remarkable cell number reduction and cells congregating (Fig. 2A3, and 2A4). These results revealed that WBPE-N9-4 protected against H_2O_2-induced oxidative damage in HEK293 cells.

To further investigate the effects of different treatments on DNA and nuclear structure in HEK293 cells, the DNA fluorescent dye Hoechst 33258 was used. Upon H_2O_2 treatment, shrinkage of cell nuclei, delineation of condensed chromatin material adjacent to plasma membrane was obviously observed. Nuclei broke into small fragments and cellular debris was extruded out via budding from the plasma membrane to form apoptotic bodies (Fig. 2B2). Compared to the control group, cells incubated with H_2O_2 alone contained many small white dots, representing chromatin condensation and/or nuclear fragmentation. Similar morphological characteristics were detected in PC12 cells upon treatment with 500 μM H_2O_2 for 24h [48]. However,

these morphological changes were inhibited by prior addition of WBPE-N9-4 and ferulic acid (Fig. 2B3 and Fig. 2B4). The majority of cells nuclei maintained normal shape and size.

3.4 Effect of WBPE-N9-4 on H_2O_2 intracellular ROS accumulation.

It is well known that treatment with H_2O_2 could cause nuclear damage, loss of $\Delta\psi m$, and higher ROS levels. In normal physiological process, mitochondria convert 1-2% of the consumed oxygen to ROS, while ROS levels increased dramatically under environmental stress [21]. The curve of control cells demonstrated ROS levels under the normal state of the cells (Fig. 3). After adding WBPE-N9-4 and ferulic acid were added to cells and standing for 20 min, the plates were placed in microplate reader and the value of (dichlorofluorescin) DCF relative fluorescence was measured. Then after 30 min, the H_2O_2 was added to the cell samples. The results showed that the curves of control cells and H_2O_2 were coincident in 30 min. However, the curves of WBPE-N9-4 and ferulic acid were obey similar tendency in 30 min, and the ROS levels were significantly lower compared with the control cells. These results clearly illustrated that WBEs could reduce the level of ROS. The decrease of ROS level was the same as ferulic acid when the H_2O_2 was absent. When H_2O_2 was added, the WBPE-N9-4 curve had an increase and also maintained ROS levels the same as control cells, which was significantly lower than the curve of H_2O_2. These results suggested that WBPE-N9-4 had ability to reduce intracellular ROS levels in HEK293 cells and prevent cell death induced in the presence of H_2O_2. It is reasonable to conclude that WBPE-N9-4 has strong ability to decrease ROS levels (Fig. 3).

3.5 Effects of WBPE-N9-4 on the activities of antioxidant enzymes and lipid peroxide level in H_2O_2-treated HEK293 cells.

The accumulation of highly-reactive oxygen radicals causes damage to biomolecules in cells as well as disrupts the membrane, alters enzyme activities, and eventually leads to DNA damage. However, several enzymes, forming part of the cellular antioxidant system, could play crucial roles in scavenging ROS and reducing oxidative damage, including CAT, SOD and GSH-Px [49]. Treatment of HEK293 cells with 1 mM H_2O_2 caused an increase in intracellular MDA levels by 139.08%, while pre-incubation of cells with WBPE-N9-4 or ferulic acid significantly attenuated the increase (Table 4). In addition, HEK293 cells treated with H_2O_2 exhibited decrease in activities of SOD, CAT, and GSH-Px by 41.96%, 66.44%, 77.33%, respectively. However, pretreatment with WBPE-N9-4 significantly inhibited the changes of SOD, CAT, and GSH-Px activities in a dose-dependent manner (Table 4).

3.6 Effect of WBPE-N9-4 on the apoptosis of H_2O_2-treated HEK293 cells.

Flow cytometry was used to monitor the changes of DNA contents in PI-stained and H_2O_2-treated HEK293 cells in the presence of WBPE-N9-4. Compared to the control group, the percentage of sub-G1 peak, indicative of necrosis and reduced DNA content [50], increased after exposure to H_2O_2. However, it decreased from 15.2% to 12.8% and 11.7% after pretreatment with WBPE-N9-4 and ferulic acid, respectively. Annexin V-FITC/PI assay based on flow cytometry was used to confirm cell apoptosis in presence of WBPE-N9-4. As shown in Fig. 4B, control cells were AV-/PI- and appeared in the lower left quadrant. The cells in the lower right quadrant were AV+/PI-, indicating early apoptosis. The majority of normal cells were AV-/PI-, and H_2O_2-injured cells increased the AV+ population from 0.1% to 1.69%, the PI+ population increase from 0.00% to 40.04%, AV-/PI- in H_2O_2-injured cells were only 50.11%. However, pretreatment with WBPE-N9-4 and ferulic acid could increase the AV-/PI- cell population to 86.78% and 89.86%, respectively. Similarly, decrease in

apoptotic cell numbers were observed in PC12 cells pretreated with WGPIH5 [51]. In conclusion, our results demonstrated that H_2O_2 suppressed the proliferation of HEK293 cells and induced cells death. Furthermore, WBPE-N9-4 could protect H_2O_2-injured HEK293 cells against the induction of necrosis.

The $\Delta\psi m$ in HEK293 cells was also examined by flow cytometry using JC-1. The effects of various treatments on changes of $\Delta\psi m$ are shown in Fig. 4C. The ratio of red to green arithmetic mean (FL2-H, FL1-H) was used to demonstrate cell damage. The ratio was 1.578 in control cells, and 0.203 in H_2O_2-injured cells. However, the ratio was increased after pretreatment with WBPE-N9-4 (0.979) or ferulic acid (1.218). A decreased ratio (from 1.587 to 0.203) indicated cells were damaged by H_2O_2. The ratio increased after pretreatment with WBPE-N9-4 (from 0.203 to 0.979), suggesting that WBPE-N9-4 can prevent the HEK293 cells from oxidation damage.

4. Conclusions

Incubation of HEK293 cells with WBPE-N9-4 prior to H_2O_2 induced stress was successful to enhance cell resistance ability to oxidation damage. The WBPE-N9-4 exhibited anti oxidation activities through restoring cell viability and diminishing ROS content and LDH release, which also displayed DNA protective effects in HEK293 cells exposed to H_2O_2-induced oxidative stress. The improved resistance impacts of WBPE-N9-4 on cell might be due to phenolic acids which could neutralize radicals and ROS. Further investigations to determine presence of phenolics using UPLC/TQD analysis revealed that WBPE-N9-4 with high antioxidant activity containing large amounts of ferulic acid. Further work is being carried out to isolate and purify the bioactive phenolic acid from WBPE-N9-4, and to clarify their structure-function relationship for elucidating the specific antioxidant pathway for cell protection.

Acknowledgments

This work was sponsored by Qing Lan Project, China Postdoctoral Science Foundation (Grant No. 2014M560396), Jiangsu Planned Projects for Postdoctoral Research Funds (Grant No.1402072C), and the National Key Technology R&D Program (Grant No. 2013AA102201).

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464 **Figure Captions**

465 **Fig. 1.** TIC chromatogram from standard solution of gallic acid (A), p-coumaric acid
466 (B), ferulic acid (C), o-coumaric acid (D), olivetol (E) in methanol, TIC tatol ion
467 chromatogram. Only olivetol (E) is channels ES+.

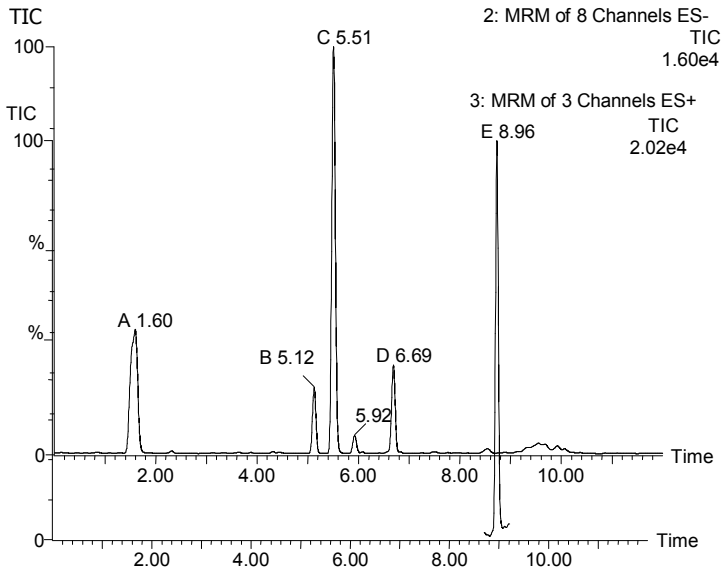
468 **Fig. 2.** Effects of WBPE-N9-4 and ferulic acid on the morphology of H_2O_2-treated
469 HEK293 cells. Morphological changes of HEK293 cells observed by (A) phase-
470 contrasted microscopy (200×) and (B) fluorescence microscope after staining
471 with Hochest33258 (200×). (A1/B1) control cells; (A2/B2) cells treated with 1
472 mM H_2O_2 for 2 h, cells displayed the extent of programmed cell death.
473 Condensed chromatin and apoptotic nuclei were considered as apoptotic cells.
474 (A3/B3) cells pretreated with WBPE-N9-4 prior to H_2O_2 treatment and after 2 h
475 incubation did not exhibit such nuclear condensation. (A4/B4) Cells pretreated
476 with 1 mM ferulic acid prior to H_2O_2 treatment and after 2 h incubation did not
477 exhibit such nuclear condensation.

478 **Fig. 3.** Inhibition of WBPE-N9-4 on H_2O_2-induced ROS in HEK293 cells. Cells were
479 incubated with carboxy-2',7'-dichloro-dihydro-fluorescein diacetate probe for 20
480 min and washed twice with PBS. Cells pretreated with 1 mg/mL WBPE-N9-4
481 and 1 mM ferulic acid for 1 h then added 1 mM H_2O_2 for 2 h. Fluorescence of
482 samples was measured at 488 nm (excitation) and 525 nm (emission)
483 wavelengths using SpectraMax M5 microplate reader.

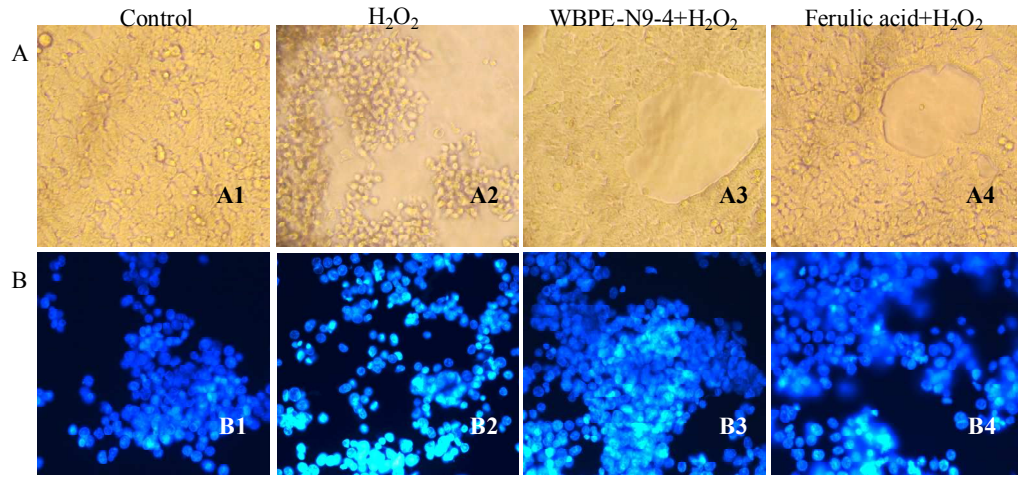
484 **Fig. 4.** Protective effects of WBPE-N9-4 against H_2O_2-induced apoptosis in HEK293
485 cells. (A) Changes in the DNA content monitored by flow cytometry with PI-
486 staining. (B) Apoptosis detected by flow cytometry with Annexin V-FITC assay.
487 (C) Alterations of mitochondrial membrane potential ($\Delta\psi_m$) examined by flow
488 cytometry using JC-1. FL2-H: red arithmetic mean; FL1-H: green arithmetic

mean. The ratio of red to green arithmetic mean (FL2-H, FL1-H) was used to demonstrate cell damage.

491

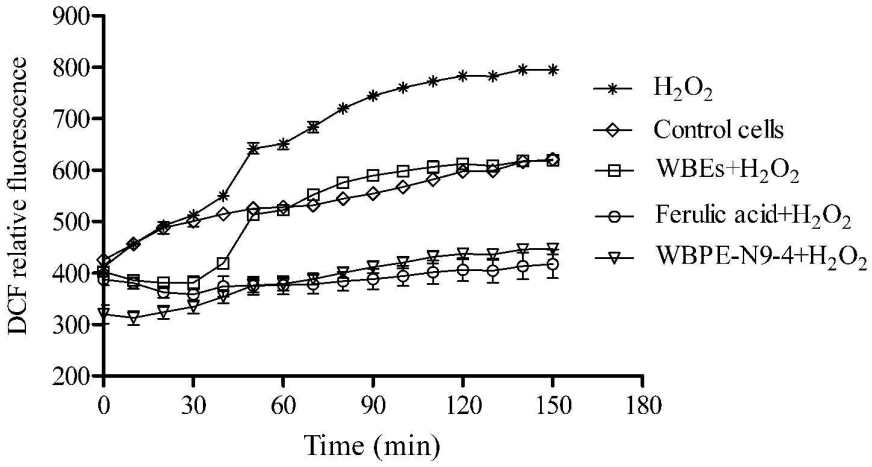


492 Fig. 1



493

494 Fig. 2



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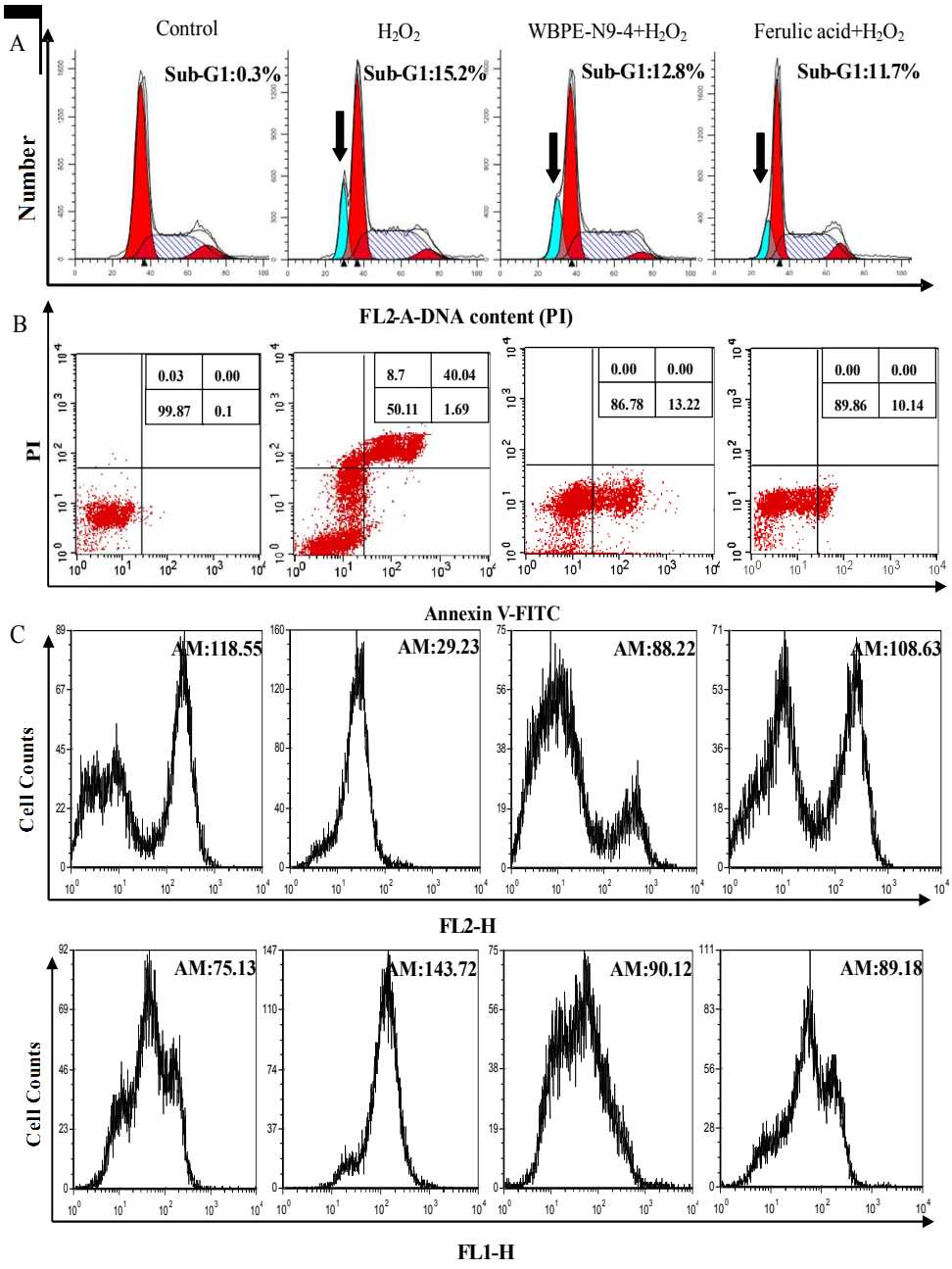
496 Fig. 3



497 Fig. 4

498 Table 1 Gradients: UPLC-TQD (A: acetonitrile solution and B: 0.1% (v/v) formic acid water
499 solution)

Time (min)	Flow (mL/min)	Eluent A (%)	Eluent B (%)
0.00	0.30	5.0	95.0
0.80	0.30	5.0	95.0
1.20	0.30	10.0	90.0
1.90	0.30	10.0	90.0
2.40	0.30	15.0	85.0
3.70	0.30	15.0	85.0
4.00	0.30	21.0	79.0
5.20	0.30	21.0	79.0
5.50	0.30	27.0	73.0
7.80	0.30	50.0	50.0
8.80	0.30	100.0	0.0
9.30	0.30	5.0	95.0

500 Table 2 Identification of standard phenolic compounds by UPLC-TQD.

Compound	Retention time (min)	Molecular weight (g/mol)	MS (*m/z*)	MS/MS MRM (*m/z*)	Cone voltage (V)	Collision energy (V)
o-coumaric acid	6.68	164.16	163.08	163.08>93.00	22	24
				163.08>119.64	22	26
p-coumaric acid	5.12	164.16	163.08	163.08>93.03	24	26
				163.08>119.15	24	30
Ferulic acid	5.51	194.1	193.00	193.00>134.10	28	13
				193.00>149.20	28	10
				193.00>178.20	28	12
Gallic acid	1.57	170.0	169.05	169.05>79.01	28	22
				169.05>97.02	28	16
				169.05>125.00	28	13
Olivetol	8.96	180.24	181.08	181.08>43.03	26	18
				181.08>71.03	26	12
				181.08>110.97	26	12

Table 3 Effects of WBPE-N9-4 on the cell viability and LDH leakage of H_2O_2-induced HEK293 cells.

	Cell viability (%)	LDH leakage (%)
Control	100.52±3.04^{f}	2.91±0.52^{a}
H_2O_2 (1 mM)	50.90±2.20^{a}	13.09±1.14^{g}
WBPE-N9-4(0.10 mg/mL) + H_2O_2 (1 mM)	53.59±3.47^{a}	11.86±1.05^{f}
WBPE-N9-4(0.25 mg/mL) + H_2O_2 (1 mM)	61.64±2.75^{b}	10.14±0.09^{e}
WBPE-N9-4(0.50 mg/mL) + H_2O_2 (1 mM)	66.65±2.39^{c}	9.15±0.71^{d}
WBPE-N9-4(1.00 mg/mL) + H_2O_2 (1 mM)	72.78±2.34^{d}	7.64±0.40^{c}
Ferulic acid (1mM) + H_2O_2 (1 mM)	78.58±1.81^{e}	5.75±0.48^{b}

Cells were incubated with 1 mM H_2O_2 for 2 h for MTT assay and LDH assay. WBPE-N9-4 were added to the culture 1 h prior to H_2O_2 addition. Ferulic acid (1mM) served as a positive control. Results are mean±SD (n=6). Numbers followed by different letters are significantly different at the level of $p<0.05$ according to Duncan test.

507 Table 4 Effects of WBPE-N9-4 on lipid peroxidation and antioxidant enzyme activities in H_2O_2-
508 treated HEK293 cells.

Treatment	MDA (nmol/mgprot)	SOD (U/mgprot)	CAT (U/mgprot)	GSH-Px (U/mgprot)
Control	1.74 ± 0.06^{a}	110.64 ± 1.85^{g}	12.01 ± 1.05^{e}	22.36 ± 1.16^{f}
H_2O_2 (1 mM)	4.16 ± 0.13^{g}	64.22 ± 0.94^{a}	4.03 ± 0.64^{a}	5.07 ± 1.06^{a}
WBPE-N9-4 (0.10 mg/mL) + H_2O_2 (1 mM)	3.91 ± 0.03^{f}	71.15 ± 1.06^{b}	5.18 ± 0.06^{ab}	7.01 ± 0.63^{b}
WBPE-N9-4 (0.25 mg/mL) + H_2O_2 (1 mM)	3.75 ± 0.05^{e}	75.29 ± 1.10^{c}	5.85 ± 0.08^{b}	8.21 ± 0.45^{bc}
WBPE-N9-4 (0.50 mg/mL) + H_2O_2 (1 mM)	3.11 ± 0.12^{d}	81.71 ± 0.53^{d}	7.66 ± 0.61^{c}	9.28 ± 0.57^{c}
WBPE-N9-4 (1.00 mg/mL) + H_2O_2 (1 mM)	2.79 ± 0.09^{c}	91.34 ± 1.51^{e}	8.73 ± 0.96^{cd}	11.27 ± 0.87^{d}
Ferulic acid (1 mM) + H_2O_2 (1 mM)	2.09 ± 0.06^{b}	102.43 ± 1.24^{f}	9.43 ± 1.04^{d}	13.52 ± 1.13^{e}

509 Cells were incubated with 1 mM H_2O_2 for 2 h for assay of MDA, SOD, CAT and GSH-Px.
510 WBPE-N9-4 were added to the culture 1 h prior to H_2O_2 addition. Ferulic acid (1mM) served as a
511 positive control. Results are mean±SD (n=6). Numbers followed by different letters are
512 significantly different at the level of $p<0.05$ according to Duncan test.