JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

# *In Vitro* Antioxidant and Antiproliferative Activities of 5-Hydroxymethylfurfural

Ling Zhao,[†,§] Jianping Chen,[†,§] Jianyu Su,*[,†] Lin Li,[†] Songqing Hu,[†] Bing Li,[†] Xia Zhang,[†] Zhenbo Xu,[†] and Tianfeng Chen*[,‡]

[†]College of Light Industry and Food Sciences, South China University of Technology, Wu Shan Road 381, Guangzhou 510640, China

[‡]Department of Chemistry, Jinan University, Guangzhou, 510632, China

**ABSTRACT:** 5-HMF is widely presented in foods and produced through the degradation of hexoses and Maillard reaction during heat treatment of foods containing reducing sugars and amino acids in an acid environment. However, controversial conclusions on the biological effects of 5-HMF have been drawn in previous studies. Therefore, the main aim of this study was to investigate the antioxidant and antiproliferative activities of 5-HMF. The 2,2′-azinobis-3-ethylbenzothiazolin-6-sulfonic acid (ABTS) assay, the 1,1-diphenyl-2-picryhydrazyl (DPPH) assay, and the hemolysis assay induced by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were performed to evaluate the antioxidant capacity of 5-HMF. The results showed that 5-HMF exhibited novel antioxidant activity by scavenging the ABTS and DPPH free radicals and inhibited the AAPH-induced hemolysis in a dose-dependent manner. In the hemolysis assay, the reduction of ROS and MDA contents and the increase in enzyme activities of SOD, CAT, and GPx were found in erythrocytes pretreated with 5-HMF, which demonstrated that 5-HMF could prevent the peroxidation from the source to protect the erythrocytes. The morphological changes of erythrocytes was also verified by observation using atomic force microscopy. The inhibitory effect of 5-HMF on human cancer cell proliferation was investigated by MTT assay, flow cytometric analysis, and the TUNEL and DAPI costaining assay. The results showed that 5-HMF displayed higher antiproliferative activity on human melanoma A375 cells than other cell lines. Further investigation on the action mechanisms revealed that 5-HMF could induce A375 cell apoptosis and G0/G1 cell cycle arrest. The A375 cell apoptosis that 5-HMF induced was characterized by a TUNEL and DAPI costaining assay. These findings suggest that 5-HMF could be developed as a novel natural antioxidant with potential applications in cancer chemoprevention.

**KEYWORDS:** *5-HMF, antioxidant, antiproliferative, apoptosis*

## INTRODUCTION

5-HMF (5-hydroxymethylfurfural, $C_3H_6O_3$), shown in Figure 1, is a five-carbon-ring aromatic aldehyde that exists naturally in coffee, honey, dried fruits, fruit juices, and flavoring agents.[1,2] It is also a common intermediate produced through the degradation of hexoses and the Maillard reaction during heat treatment of foods containing reducing sugars and amino acids in an acid environment.[3] The concentration of 5-HMF in food products varies widely. It has been reported that 5-HMF levels in wine, spirits, and fruit juices had been found to be as high as 200 mg/L and in prune juice might be up to 1000 mg/L.[4] The daily intake of 5-HMF is about 30−150 mg/per person, which may come from pharmaceutical preparations, cigarette smoking, and the consumption of a large number of commonly available beverages and foods.[5] In the last decades, it was considered hazardous to human health. There have been a lot of studies suggesting that 5-HMF irritates skin, upper respiratory tract, eyes, and mucous membranes, damages striated muscles and viscera, is genotoxic, and generates an accumulation of toxin within human proteins.[6−8] Therefore, the content of 5-HMF in honey, beer, and glucose injections has been limited strictly.

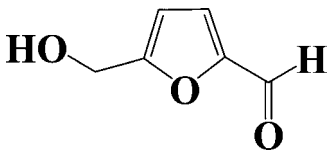


**Figure 1.** Structure of 5-hydroxymethylfurfural.

Despite the previous progress reviewed on the dangers of 5-HMF, there is an accepted theory concerning the biological activity of 5-HMF in recent years. There are more and more reports showing that 5-HMF has some pharmacological effects, such as antioxidant, antiischemic, and antityrosine enzyme effects, improving blood rheology, and affecting the role of glycyrrhizin metabolism.[9] For example, Abdulmalik et al. and Yamada et al. reported that 5-HMF had an inhibitory effect on the sickling of red blood cells *in vitro* and the treatment or prevention of type I allergic diseases.[10,11] Wang et al. suggested a morphological hepatocyte protective effect and the antiapoptosis mechanism by 5-HMF.[12] Ding et al. showed that 5-HMF protects L02 cells from damage induced by hydrogen peroxide through inhibiting the effect of cell apoptosis caused by promoting the S phase to the G2/M phase and the decreased caspase-3 activity and nitric oxide

**Received:** July 16, 2013
**Revised:** September 26, 2013
**Accepted:** October 9, 2013
**Published:** October 9, 2013

level.[13] The study by Gu et al. also showed that 5-HMF could restrain the apoptosis of cultured hippocampal neurons injured by $H_2O_2$.[14]

In pioneering studies, the authors attempted to demonstrate the antioxidant and antiproliferative effects of 5-HMF, but the experimental studies were limited and few were related to its mechanisms. Therefore, the aim of this study was to confirm the antioxidant and antiproliferative activities of 5-HMF and its mechanism. Interestingly, 5-HMF was found to be able to inhibit the hemolysis of human erythrocytes induced by AAPH and to induce apoptotic cell death in selected cancer cells. It was concluded that 5-HMF could decrease the oxidative damage of red blood cells induced by AAPH through reducing ROS generation and inhibit the proliferation of human melanoma A375 cells. Our findings demonstrate that 5-HMF could be developed as a novel natural antioxidant with potential applications in cancer chemoprevention.

## MATERIALS AND METHODS

**Chemicals.** 5-Hydroxymethylfurfural (chemical purity, >99.90%, molecular weight 126.11) was purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). ABTS (2,2′-azinobis-3-ethylbenzothiazolin-6-sulfonic acid), DPPH (1,1′-diphenyl-2-picryhydrazyl), MTT (thiazolyl blue tetrazolium bromide), and PI (propidium iodide) were obtained from Sigma. Ascorbic acid and AAPH [2,2′-azobis(2-amidinopropane) dihydrochloride] were obtained from Guangzhou Qiyun Biotechnology Co., Ltd. (Guangzhou, China). BCA protein kits and malondialdehyde (MDA) kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Assay kits for determination of ROS (reactive oxygen species), glutathione peroxidase (GPx) activity, catalase (CAT) activity, and superoxide dismutase (SOD) activity were purchased from Beyotime Institute of Biotechnology. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and the antibiotic mixture (penicillin−streptomycin) were purchased from Invitrogen (Carlsbad, CA, USA). The solvents used for the experiments were of high-performance liquid chromatography (HPLC) grade. Milli-Q water was used in our all experiments.

**$ABTS^+$ Free Radical Scavenging Assay.** Recently, the ABTS assay has been used in the antioxidant activity assay of water-soluble substances. The principle is that green $ABTS^+$ is generated under the appropriate oxidant, but is suppressed in the presence of antioxidants.[15] According to previous research methods,[16] a 5 mM ABTS stock solution in PBS (pH 7.4) and the right amount of $MnO_2$ were mixed until full reaction. Then the solution was filtrated and diluted appropriately to obtain an ABTS reaction solution. In the reaction system, 180 μL of ABTS reaction solution and 20 μL of samples were added to 96-well plates. The absorbance was measured once per minute at 734 nm for 30 min using a microplate reader (SpectraMax 250) at room temperature in the dark.

**$DPPH^+$ Free Radical Scavenging Activity.** In this assay, DPPH, a stable free radical, was dissolved in alcohol. The solution was dark purple, and an absorption peak at 515 nm was observed. In the presence of antioxidants, the color gradually faded and the absorbance at 515 nm was decreased due to the reduction of DPPH radicals in methanol. Therefore, the antioxidant capacity of 5-HMF could be measured based on the changes of absorbance and color.[17] According to methods from a previous study with some modifications,[18,19] a certain amount of DPPH was dissolved in methanol to obtain a stock solution of DPPH (6 mM), which was stored at −20 °C in the dark. The solution was diluted with methanol to a final concentration of 60 μM before using. In the reaction system, 180 μL of DPPH reagent and 20 μL of samples were added to 96-well plates, which were mixed and shaken. The absorbance was recorded once every minute at 515 nm for 30 min by microplate reader (SpectraMax 250) until the reading was stable. This experiment was carried out at room temperature in the dark.

**Preparation of Erythrocyte Suspension.** The fresh blood of a healthy volunteer containing heparin was centrifuged at 1500*g* for 10 min to obtain the erythrocytes; then the erythrocytes were washed three times with PBS buffer (pH 7.4). A 20% erythrocyte suspension was prepared with PBS (pH 7.4).

**Erythrocyte Hemolysis Assay Mediated by AAPH.** AAPH-induced erythrocyte hemolysis has been widely used to evaluate the *in vitro* antioxidant activity of tested samples. AAPH is a water-soluble free radical initiator that generates radicals such as ROO• and will attack the cell membrane including lipids and proteins and cause DNA peroxidation, thereby causing erythrocyte hemolysis.[20−22] In this study, the oxidative damage model was established in order to detect the antioxidant activity of 5-HMF.

In order to optimize the concentration, AAPH (at final concentrations 0, 50, 100, 200, 300, 400 mM) was mixed with 0.1 mL of 20% erythrocyte suspension and incubated at 37 °C for 2 h. The absorbance of the supernatant was recorded at 540 nm by a microplate reader (SpectraMax 250).

For time optimization, a final concentration of 100 mM AAPH was mixed with 0.1 mL of 20% erythrocyte suspension and incubated at 37 °C. The absorbance of the supernatant was recorded at 540 nm once per 30 min by the microplate reader.

An experiment was conducted by Cheung et al.[23] to investigate the inhibition activity of 5-HMF on hemolysis induced by AAPH. A 0.1 mL amount of a 20% erythrocyte suspension was pretreated with 0.1 mL samples at different concentrations at 37 °C for 20 min; then 0.2 mL of 200 mM AAPH was added, and the mixture was incubated at 37 °C for 2 h. A 0.1 mL amount of 20% erythrocyte suspension and 0.3 mL of PBS were mixed as a blank control. After incubation, 50 μL of the mixture was removed and diluted with 1 mL of PBS buffer (A) or distilled water (B), then was centrifuged at 1500*g* for 10 min. The absorbance of the supernatant was recorded at 540 nm by the microplate reader. Ascorbic acid (1.5 mM) was chosen as a positive control.

The percent hemolysis inhibition was calculated as follows:

$$\%\text{hemolysis inhibition} = (1 - A/B) \times 100\%$$

**Atomic Force Microscopy (AFM) Measurements.** The treated blood samples were centrifuged at 1500*g* for 10 min and washed with PBS; then the erythrocytes were resuspended appropriately. A 10 μL sample of the dilution was spread evenly on a clean silicon pellet, and the cells were fixed with 2.5% glutaraldehyde. After 5 min, the silicon pellet was washed with Milli-Q water three times. The morphology of erythrocytes was detected by atomic force microscopy (Asylum Research, CA, USA).

**Determination of ROS Generation, MDA Content, and Enzyme Activities of GPx, SOD, and CAT.** The treated blood samples were centrifuged, washed three times with PBS, then lysed with erythrocyte sedimentation with 500 μL of ultrapure water in an ice bath. The mixture was centrifuged at 10 000 r/min for 10 min at 4 °C, and the supernatant was collected and stored at −70 °C. Total protein concentration was measured by BCA kit.

The relative intracellular level of ROS was measured by DCFH-DA assay.[24] Specifically, the treated erythrocytes were collected by centrifugation at 1500*g* for 10 min, washed two times with PBS, then resuspended in DMEM without fetal bovine serum. A final concentration of 10 μM DCFH-DA was added in the cell suspension and co-incubated at 37 °C for 20 min. After the reaction, the loaded red blood cells were washed three times with PBS to remove the fluorescent agent adhered on their surfaces so as not to affect the results. A 100 μL amount of samples and 100 μL of loaded cell suspension were added to 96-well plates and co-incubated for 5−120 min. The generation of ROS was recorded by detecting the fluorescence intensity on a fluorescence reader (Thermo Fisher Variosban Flash, USA) (Ex = 488 nm, Em = 525 nm).

Determination of MDA in the cells can often reflect the degree of lipid peroxidation and indirectly reflects the degree of cell damage. MDA contents were measured by the thiobarbituric acid (TBA) method using a trace assay kit. Briefly, the samples and the appropriate reagents were added in some tubes and mixed by a vortex mixer. The

tubes with a hole were placed in a 95 °C water bath for 40 min. After a set time, the reactant was cooled with ice water. The absorbance was measured at 532 nm.

A large number of researchers have studied the activity detection of GPx,[25,26] SOD,[27] and CAT.[28] Experiments were performed according to the manufacturer's kit instructions. Activities of GPx and CAT were assayed by the decrease of the GSH and $H_2O_2$, respectively. The SOD activity level was determined with the nitroblue tetrazolium method, which utilized a tetrazolium salt to quantify the superoxide radicals generated by xanthine oxidase and hypoxanthine. Data were obtained by measuring the absorbance at 412 nm for GPx, 405 nm for CAT, and 550 nm for SOD.

**Cell Culture.** The human cell lines including normal kidney HK-2, lung carcinoma A549, melanoma A375, hepatocellular carcinoma HepG2, breast adenocarcinoma MCF-7, colon carcinoma SW480, and cervical carcinoma HeLa were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The normal liver L02 cell line was purchased from Nanjing KeyGEN Biotech. Co., Ltd. (Nanjing, China). The cell lines in the experiments were grown in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 50 units/mL streptomycin at 37 °C in a humidified (5% $CO_2$, 95% air) atmosphere.

**MTT Assay.** HK-2 and L02 cells with a density of $2 \times 10^3$ cells/well and A549, A375, HepG2, MCF-7, SW480, and HeLa cells with a density of $1.5 \times 10^3$ cells/well were seeded in 96-well plates and placed in a humidified atmosphere for 24 h. The cells were exposed to different concentrations of 5-HMF for 72 h. A 25 $\mu$L portion of MTT with 5 mg/mL in PBS was added into a well and incubated for another 4 h. The solution was deprived, while 150 $\mu$L/well of DMSO was added to dissolve the crystals with shaking for 10 min. The absorbance was measured at 570 nm by the microplate reader.

**Flow Cytometry.** The cell cycle distribution was shown by flow cytometric analysis according to a previous description.[29] A375 cells with a density of $1.5 \times 10^4$/mL were seeded in a culture dishes. After 24 h, different concentrations of 5-HMF were added and incubated for 72 h. The cells were collected by centrifugation at 1500 r/min for 5 min and washed with PBS or not based on the number of cells. The collected cells were fixed by adding 1 mL of 70% ethanol at −20 °C overnight, then stained with PI (1.21 mg/mL Tris, 700 U/mL RNase, 50.1 $\mu$g/mL PI, pH 8.0). The DNA content was analyzed by flow cytometry (Miami, FL, USA). MultiCycle software (Phoenix Flow Systems, San Diego, CA, USA) was used to analyze the cell cycle. G0/G1, S, and G2/M phase represents the histogram distribution of DNA content. The sub-G1 peak was used to calculate the hypodiploid DNA contents of apoptotic cells. A total of 10 000 events were recorded for every sample.

**TUNEL and DAPI Costaining Assay.** TUNEL and DAPI staining were used to detect cell apoptosis according to the literature by Chen et al.[29] The A375 cells were cultured in chamber slides for 24 h and were treated with different concentrations of 5-HMF. After 24 h, the cells were fixed with 3.7% formaldehyde and permeabilized with 0.1% Triton X-100 in PBS for 10 min. The fixed cells were dyed with 100 $\mu$L/well TUNEL reaction mixtures for 1 h and 1 $\mu$g/mL of DAPI for 15 min at 37 °C, respectively, then washed with PBS. DNA fragmentation was observed by a fluorescence microscope (Nikon Eclipse 80i).

**Statistical Analysis.** All experiments were performed at least three times or in triplicate. Data were given as mean ± SD and analyzed by SPSS statistical package (SPSS 13.0 for Windows; SPSS, Inc., Chicago, IL, USA). Two-tailed Student's $t$ test was used to analyze the difference between two groups. One-way analysis of variance multiple comparisons was used to analyze the difference between three or more groups. Differences were considered statistically significant with $p < 0.05$ (*) or $p < 0.01$ (**). Bars with different characters were statistically different at the $p < 0.05$ level.

## RESULTS AND DISSCUSION

***In Vitro* Antioxidant Activity of 5-HMF.** *ABTS Assay.* In the present study, the total antioxidant capacity of 5-HMF was assessed through the changes of the absorbance at 734 nm by a microplate spectrophotometer (SpectraMax 250). As shown in Figure 2A, at final concentrations ranging from 0.2 to 6.4 mM,

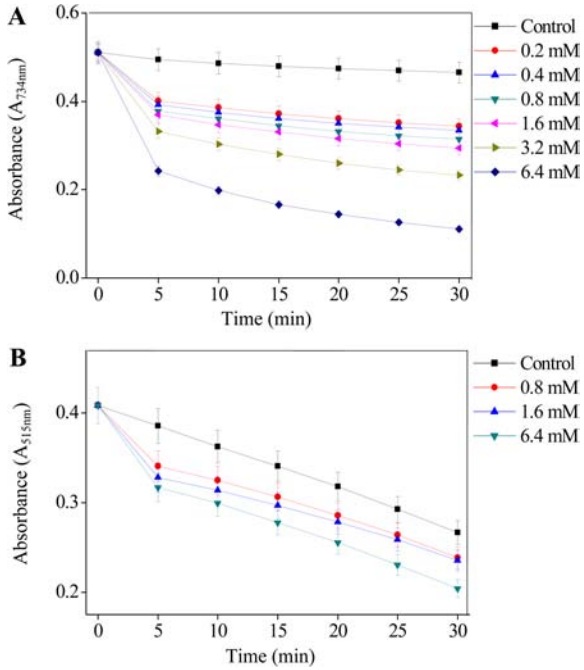


**Figure 2.** Antioxidant activities of 5-HMF as determined by the ABTS assay (A) and DPPH assay (B). Values expressed are means ± SD of triplicates.

the system characteristic absorption peak at 734 nm decreased significantly within 5 min, then stayed relatively more balanced until reaching a stable state. The greater the concentration of 5-HMF, the faster the absorbance value decreased, indicating that 5-HMF could effectively scavenge the ABTS free radical in a time- and dose-dependent manner.

*DPPH Assay.* The DPPH radical scavenging activities of 5-HMF are shown in Figure 2B. In the reaction system, the characteristic absorption peak at 515 nm decreased significantly in 5 min over a concentration range of 0.8−6.4 mM. The absorbance still declined as the time increased, but the system tended to be relatively stable. It showed that the scavenging activities of 5-HMF on DPPH radical were time- and dose-dependent. However, the scavenging activity of 5-HMF on DPPH radical is lower than the ABTS radical.

**Induction of Erythrocyte Hemolysis by AAPH.** As shown in Figure 3, AAPH induced a significant increase in erythrocyte hemolysis at a final concentration from 0 to 100 mM, while the hemolysis rate increased slowly with an increase in concentration. For the effect of time, a final concentration of 100 mM AAPH was chosen, and the hemolysis rate increased significantly before 2 h, then tended to level off. The oxidative damage induced by AAPH on erythrocytes was concluded to be dose and time dependent. The following experiments were carried out at a final concentration of 100 mM AAPH for 2 h.

In the paper, a 20% erythrocyte suspension was incubated with PBS (control) or preincubated with 5-HMF at the indicated concentration at 37 °C for 20 min and then co-incubated with or without 100 mM AAPH at 37 °C for 2 h. The hemolysis rate of erythrocytes by 5-HMF at 48.0 mM was 2.47 ± 0.006%, close to the control (1.95 ± 0.003%). Thus, it could be concluded that the oxidative damage was not caused

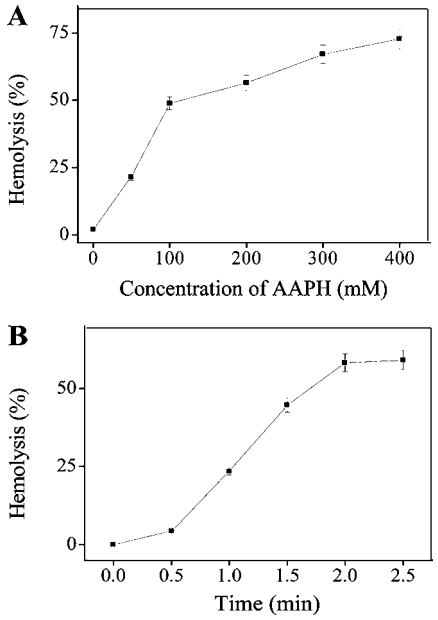


**Figure 3.** Optimization of concentration (A) and time (B) for APPH-induced hemolysis in erythrocytes. Values are expressed as means ± SD of triplicates.

by only 5-HMF. Moreover, erythrocytes were treated with 100 mM AAPH; the hemolysis rate was 46.33 ± 0.003%. The protection of 5-HMF on erythrocyte oxidative damage is shown in Table 1. When the concentration of 5-HMF increased from 1.5 mM to 48.0 mM, the hemolysis inhibition rate of 5-HMF was from 65.36 ± 0.041% up to 92.80 ± 0.002%, indicating the antioxidant activity of 5-HMF was raised with increased concentration. In addition, ascorbic acid at 1.5 mM was used as a positive control, whose inhibition rate of hemolysis reached 88.75 ± 0.006%.

**Table 1. 5-HMF Attenuated AAPH-Induced Erythrocyte Hemolysis[a]**

| group | final concentration (mM) | hemolysis inhibition (%) |
|---|---|---|
| VC (positive control) | 1.5 | 88.75 ± 0.006 |
| 5-HMF | 48.0 | 92.80 ± 0.002 |
| | 24.0 | 91.90 ± 0.003 |
| | 12.0 | 89.95 ± 0.001 |
| | 6.0 | 85.61 ± 0.003 |
| | 3.0 | 76.16 ± 0.007 |
| | 1.5 | 65.36 ± 0.041 |

[a]Values expressed are means ± SD of triplicates.

**Morphological Change As Examined by AFM.** AFM was used to observe the morphology changes of erythrocytes intuitively. Normal erythrocytes as control are shown in Figure 4A; we could observe the typical biconcave shape and smooth concave surface. When erythrocytes were treated with 100 mM AAPH, their morphology changed with a decrease in height due to cell collapse and a rough surface, as shown in Figure 4B. However, the damage to erythrocytes was improved by adding 5-HMF. As shown in Figure 4C, the typical biconcave shape and smooth concave surface were still found, an almost normal erythrocyte morphology. This intuitively confirmed that 5-HMF could protect the erythrocytes from oxidative damage by AAPH.

**5-HMF Inhibits AAPH-Induced ROS Generation and Changes in the Level of MDA, Activities of Antioxidant Enzymes.** In order to further determine the protective effects of 5-HMF on erythrocytes against oxidative stress by AAPH, ROS generation, accumulation of MDA, and changes of antioxidant enzymes (GPx, SOD, CAT) activities were investigated. ROS, such as superoxide anion, hydroxide, and hydroxyl radicals, can be constantly produced and eliminated in all biological systems and are an important index of oxidative stress. Excess ROS in the cells may attack cellular proteins, membrane lipids, and DNA, inhibit their normal functions, and finally cause oxidative damage.[30] Intracellular ROS generation was examined to reflect the level of oxidative damage. DCFH-DA fluorescent dye, the intensity of which increases with increasing intracellular ROS, was used to detect ROS level. The results are presented in Figure 5. It could be seen that when treated with only AAPH, the erythrocytes were damaged markedly ($p < 0.01$), as shown by the content of ROS, which was 627.98 ± 0.368% of the control. When 5-HMF was added, the ROS production significantly decreased ($p < 0.05$). However, the ROS level was stable when the concentration of 5-HMF changed from 6.0 mM to 24.0 mM. The study indicated that the dose-dependent protection of 5-HMF on erythrocytes was dependent on the inhibition of ROS generation.

MDA is an end product of lipid peroxidation caused by free radical attack on the cytomembrane. The reaction between MDA and proteins, nucleic acids (DNA, RNA) containing amino groups, can happen, which will lead to the damage of erythrocytes.[31−34] The content of MDA in cells was regarded as an indirect index of oxidative damage. As shown in Figure 6A, when the erythrocytes were treated with AAPH only, the content of MDA was up to 30.40 ± 0.005 nmol/mg protein more than the control (8.64 ± 0.005 nmol/mg protein). MDA content decreased with an increase of 5-HMF from 1.5 mM to 24.0 mM, indicating that 5-HMF could inhibit the oxidative damage of erythrocytes by AAPH. No obvious difference was found between 5-HMF and control.

When excess free radicals were generated in the cells that made the antioxidant system loaded, oxidative damage occurred. The intracellular antioxidant enzyme system plays a key role in resisting oxidative stress from SOD, GPX, and CAT, for example. These enzymes can individually or synergistically effect the removal of free radicals from the body, then protect the integrity of the cell structure and function. SOD can convert the superoxide anion into hydrogen peroxide by a disproportionation reaction; CAT can decompose hydrogen peroxide into water and oxygen; GPx can maintain the body's reduced state through catalyzing reduced glutathione GSH to reduction lipid peroxidation and hydrogen peroxide *in vivo*.[35] In this paper, changes in the level of activities of antioxidant enzymes were investigated. As shown in Figure 6B,C,D, 5-HMF could increase SOD, GPx, and CAT activity, decrease MDA content, and directly scavenge ROS. These results suggested 5-HMF could cut off the reaction of lipid peroxidation from the source, increase endogenous antioxidant enzyme activity, inhibit lipid peroxidation oxidation, protect the body from free radical attack, and maintain the integrity of the cell structure and function.

**Antiproliferative Activity of 5-HMF.** The MTT assay is a commonly used method to test cell survival. MTT is a color-developing agent that can accept a hydrogen atom from the dye. First, the HK-2, L02, A549, A375, HepG2, MCF-7,

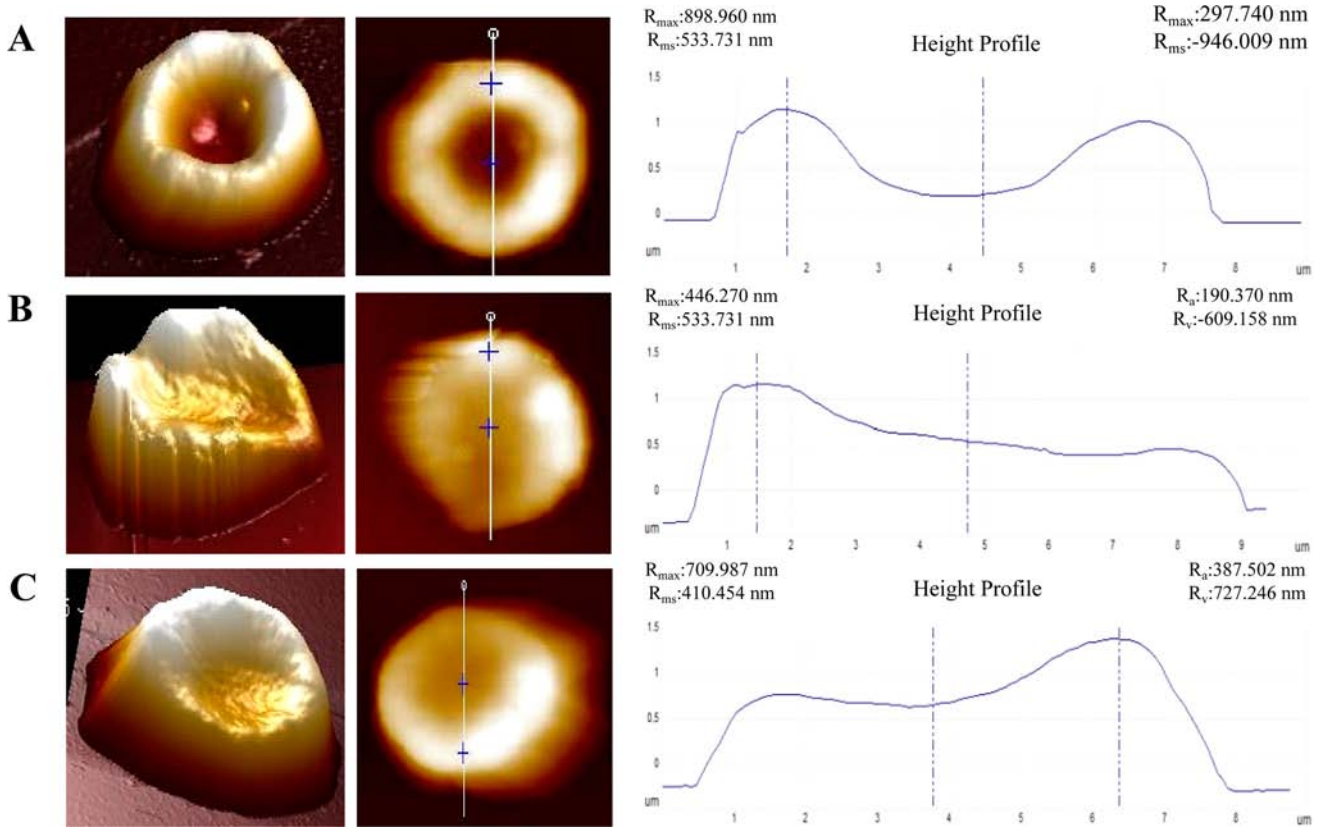


**Figure 4.** Characterization of morphological changes by AFM. (A) Normal erythrocytes. (B) Erythrocytes treated with 100 mM AAPH for only 2 h. (C) Erythrocytes pretreated with 48.0 mM 5-HMF for 20 min and further treated with 100 mM AAPH for 2 h.

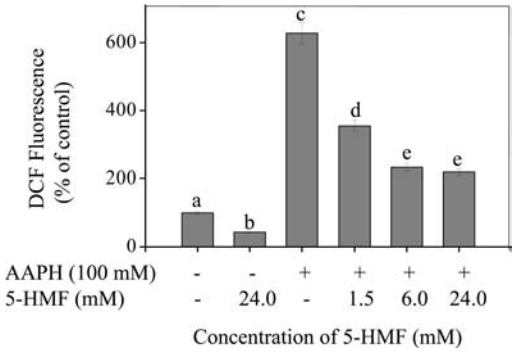


**Figure 5.** Changes of ROS production in erythrocytes. A 20% erythrocyte suspension was incubated with PBS (control) or pretreated with different concentrations (1.5−24.0 mM) of 5-HMF for 20 min and then co-incubated with or without 100 mM AAPH at 37 °C for 2 h. Values expressed are means ± SD of triplicates. A significant difference between treatments is indicated at $p < 0.05$.

SW480, and HeLa cell lines were chosen to investigate the *in vitro* cytotoxicity of 5-HMF. The results are shown in Table 2. It could be concluded that 5-HMF exhibited stronger inhibitory effects on the selected cancer cells than the normal cells, particularly, as shown by the $IC_{50}$ values of 0.58 mM for A375 cells, 2.13 mM for HK-2, and 3.71 mM for L02. The A375 cells were selected to examine the antiproliferative activity of 5-HMF.

As shown in Figure 7A, the A375 cells' viability decreased at concentrations ranging from 0.2 to 6.4 mM. With a 5-HMF concentration of 0.2 mM, the level of growth-inhibitory effect on A375 cells was 83.53 ± 0.313% and was reduced by 50% at the concentration of 0.58 mM. However, at concentrations of 3.2 and 6.4 mM, the cell viabilities were 6.13 ± 0.012% and 4.67 ± 0.008%, respectively, indicating that there was no significant difference between them. The inhibitory effects on the growth of A375 cells were dose-dependent. The morphological changes on A375 cells were attenuated by different concentrations of 5-HMF, given in Figure 7B. With increasing concentration of 5-HMF, the number of cells decreased gradually and the cells' morphology become round by comparison with control cells. This result was consistent with the detection of cell viability.

**5-HMF Induces Apoptosis and G0/G1 Cell Cycle Arrest in A375 Cells.** The possible causes of inhibition of the proliferation of cancer cells are induction of apoptosis or cell cycle arrest or a combination of these two modes.[36] To further detect the mechanism of cell death by 5-HMF, flow cytometry, a fast and accurate means to detect the physicochemical properties of individual cells, was used to examine the DNA distribution. As shown in Figure 8, with increasing doses of 5-HMF, the sub-G1 phase and G0/G1 phase increased to some extent. For the sub-G1 phase, there was no difference between the concentration of 0.625 mM and the control. Meanwhile, at concentrations of 1.250 and 2.500 mM, the sub-G1 peak increased from 1.2% (control) to 4.7% and 11.5%, respectively. However, the sub-G1 peak only reached 11.5% at the concentration of 2.5 mM, which indicated weak apoptosis induced by 5-HMF. It can be concluded that 5-HMF induces human melanoma A375 cell apoptosis and G0/G1 cell cycle arrest.

**Detection of Cell Apoptosis by TUNEL and DAPI Costaining Assay.** A375 cell apoptosis induced by 5-HMF

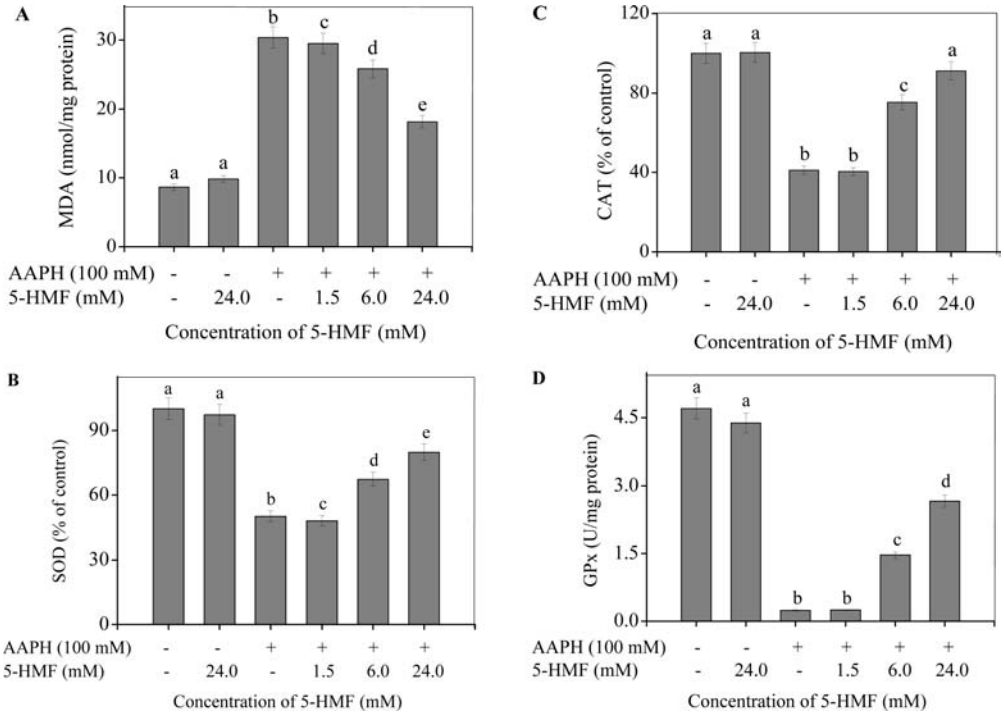


**Figure 6.** Changes in MDA content (A) and enzyme activities of SOD (B), CAT (C), and GPx (D) in erythrocytes. A 20% erythrocyte suspension was incubated with PBS (control) or pretreated with different concentrations (1.5−24.0 mM) of 5-HMF for 20 min and then co-incubated with or without 100 mM AAPH at 37 °C for 2 h. Values expressed are means ± SD of triplicates. A significant difference between treatments is indicated at $p < 0.05$.

**Table 2. $IC_{50}$ Values of 5-HMF against Human Cancer and Normal Cells[a]**

| cells | $IC_{50}$ (mM) |
|---|---|
| HK-2 | 2.13 ± 0.021 |
| L02 | 3.71 ± 0.027 |
| A549 | 1.14 ± 0.032 |
| A375 | 0.58 ± 0.012 |
| HepG-2 | 1.03 ± 0.029 |
| MCF-7 | 1.44 ± 0.002 |
| SW480 | 1.00 ± 0.014 |
| HeLa | 2.87 ± 0.190 |

[a]The cells were treated with different concentrations of 5-HMF and incubated for 72 h. Cell viability was determined by the MTT assay. All data were obtained from three independent experiments and presented as the means ± SD.

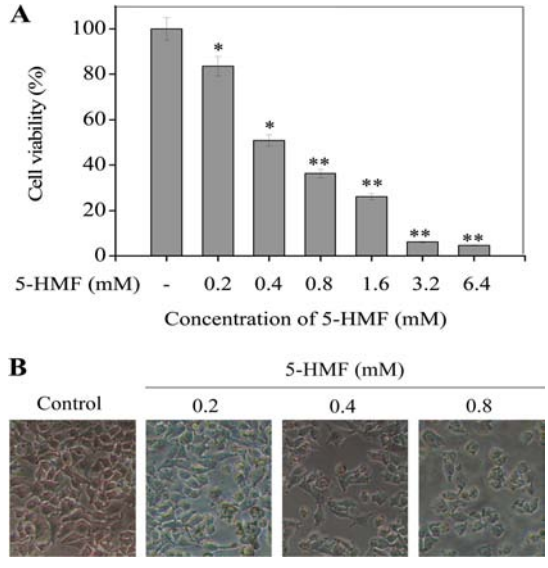


**Figure 7.** Protective effects of 5-HMF in A375 cells. (A) Dose-dependent cytotoxic effects of 5-HMF on A375 cells. The cells were treated with different concentrations of 5-HMF and incubated for 72 h. Cell viability was determined by the MTT assay. All data were obtained from three independent experiments and are presented as the means ± SD. $p < 0.05$ and $p < 0.01$ vs control group. (B) Morphological changes of A375 cells as examined by phase-contrast microscopy (magnification, 200×). The images shown here are representative of four independent experiments with similar results.

was further verified by a TUNEL and DAPI staining assay. DNA fragments can be stained by TUNEL specifically and produce green fluorescence. The complete DNA and debris can be confirmed by DAPI staining, which produces blue fluorescence. As shown in Figure 9, with an increased concentration of 5-HMF, the more cells with green fluorescence and the stronger fluorescence for single cells were observed, indicating DNA fragments were formed after A375 cells were treated with 5-HMF, and the higher the concentration of 5-HMF, the more the DNA fragments. According to DAPI staining, both nuclei with corrugated edges and chromatin condensation and marginalization were found. These were more obvious at high concentrations of 5-HMF. When the images from TUNEL and DAPI staining were overlapped, the green fluorescent and blue fluorescent of chromatin condensation or apoptotic bodies were at the same position. The results showed that 5-HMF promoted apoptosis of A375 cells in a dose-dependent manner.

In conclusion, 5-HMF exhibited antioxidant activity by scavenging ABTS and DPPH and dose-dependent inhibition of

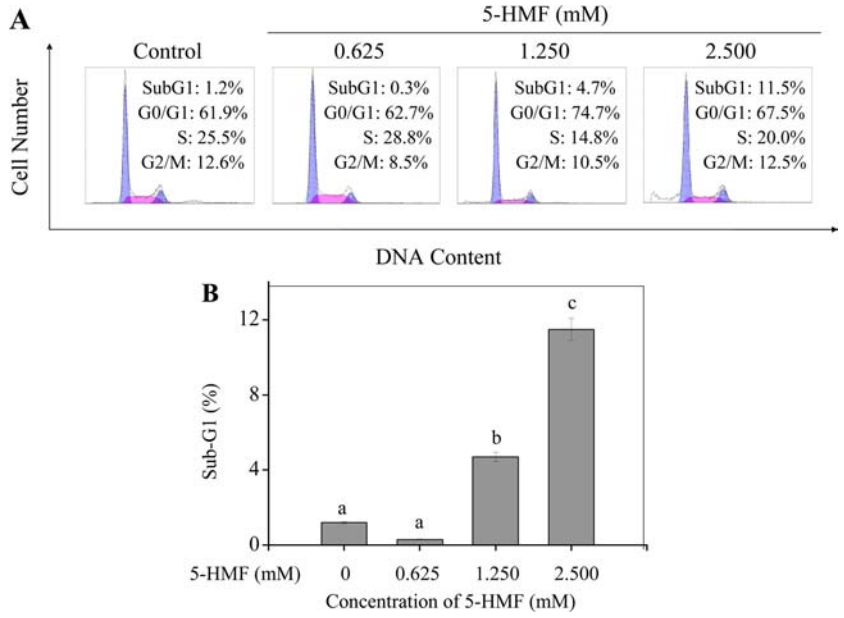


**Figure 8.** Effects of 5-HMF on cell cycle distribution of A375 cells. (A) 5-HMF-induced apoptosis and G0/G1 cell cycle arrest in A375 cells. Cells treated with different concentrations of 5-HMF for 72 h were collected and stained with PI after fixation with 70% ethanol. Following flow cytometry, cellular DNA histograms were analyzed by the MultiCycle software. Apoptotic cells with hypodiploid DNA content were measured by quantifying the sub-G1 peak. Each value represents the mean of three independent experiments. (B) Quantitative analysis of 5-HMF-induced apoptotic cell death by measuring the sub-G1 cell population. Bars with different characters (a, b, c) are statistically different at the $p < 0.05$ level.

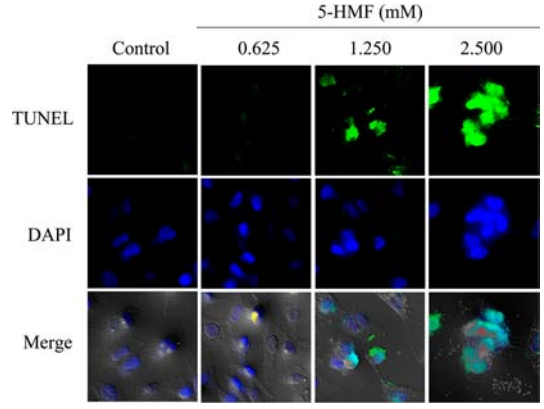


**Figure 9.** DNA fragmentation and nuclear condensation in A375 cells induced by 5-HMF as detected by a TUNEL−DAPI costaining assay (magnification, 200×). Cells were cultured with 0.625−2.500 mM 5-HMF for 24 h. The apoptotic percentages, as calculated by dividing the TUNEL-positive cell number by the total cell number (DAPI-positive) within the same area, are listed in the parentheses.

AAPH-induced hemolysis. In the hemolysis assay, the reduction of ROS and MDA concent and the increase in enzyme activities of SOD, CAT, and GPx were found in erythrocytes pretreated with 5-HMF, indicating the protection of erythrocytes by 5-HMF via the inhibition of ROS generation. This was also verified by observation of the morphological changes of erythrocytes using atomic force microscopy. In the MTT assay, 5-HMF showed higher antiproliferative activity on human melanoma A375 cells than other common human cells. Further investigation on the mechanism revealed 5-HMF could induce apoptosis of human melanoma A375 cells and G0/G1 cell cycle arrest. Meanwhile, the 5-HMF-induced A375 cell apoptosis was further characterized by a TUNEL and DAPI costaining assay. In short, antioxidant and antiproliferation activities were observed in this study.

The structural features of 5-HMF may contribute to its antioxidant and antiproliferative activities. As shown in Figure 1, the structure contains a hydroxyl group that could donate a hydrogen ion and some electron-withdrawing groups such as double bonds, an aldehyde group, and an oxygenated furan ring.[37] 5-HMF protects the erythrocytes from oxidative damage by scavenging free radicals, reducing the MDA content, and increasing the enzyme activities of SOD, CAT, and GPx. Moreover, 5-HMF could break intracellular homeostasis by scavenging intracellular ROS and interact with DNA via the aldehyde group, which finally inhibits the proliferation of cancer cells through induction of apoptosis and cell cycle arrest.[4] As previously reported, the tolerable daily intake (TDI) of 5-HMF (132 mg/person per day) is far higher than the currently available assessments of dietary intake, indicating that 5-HMF exhibits no health risk.[38] Our findings suggested that 5-HMF could be developed as a novel natural antioxidant with potential applications in cancer chemoprevention.

## AUTHOR INFORMATION

### Corresponding Authors

*E-mail: jysu@scut.edu.cn. Tel: (+86) 20 87113252. Fax: (+86) 2087113252.

*E-mail: tchentf@jnu.edu.cn.

### Author Contributions

§L. Zhao and J. Chen contributed equally to the work.

### Funding

This work was supported by the 973-Plan (2012CB720800), National Natural Science Foundation of China (Nos. 31101278, 31000781, and 31130042), Research Fund for the Doctoral Program of Higher Education of China (20110172120033), and the National Science and Technology Support Program (2012BAD37B01).

### Notes

The authors declare no competing financial interest.

## REFERENCES


(1) Xu, Q.; Li, Y. H.; Lv, X. Y. Investigation on influencing factors of 5-HMF content in Schisandra. *J. Zhejiang Univ. Sci. B* **2007**, *8*, 439−445.

(2) Lee, Y. C.; Shlyankevich, M.; Jeong, H. K.; Douglas, J. S.; Surh, Y. J. Bioactivation of 5-hydroxymethyl-2-furaldehyde to an electrophilic and mutagenic allylic sulfuric aicd ester. *Biochem. Biophys. Res. Commun.* **1995**, *3*, 996−1002.

(3) Capuano, E.; Fogliano, V. Acrylamide and 5-hydroxymethylfurfural (HMF): A review on metabolism, toxicity, occurrence in food and mitigation strategies. *LWT−Food Sci. Technol.* **2011**, *44*, 793−810.

(4) Louise, J. K. D.; Leif, B.; Björn, E. H. Evaluation of the DNA damaging effect of the heat-induced food toxicant 5-hydroxymethylfurfural (HMF) in various cell lines with different activities of sulfotransferases. *Food Chem. Toxicol.* **2009**, *47*, 880−884.

(5) Janzowski, C.; Glaab, V.; Samini, E.; Schlatter, J.; Eisenbrand, G. 5-Hydroxymethylfurfural: assessment of mutagenicity, DNA-damaging potential and reactivity towards cellular glutathione. *Food Chem. Toxicol.* **2000**, *38*, 801−809.

(6) Fu, Z. Q.; Wang, M. Y.; Cai, B. Q. Discussion of 5-hydroxymethylfurfural (5-HMF) in Chinese native medicine research present situation. *Chin. Arch. Trad. Chin. Med.* **2008**, *3*, 508−510.

(7) Chi, W.; Zhang, C. B.; Cao, Y. H.; Guo, L. Y. Investigation of the restriction on the formation of 5-HMF. *Spec. J. Pharm. People's Mil. Surg.* **1998**, *2*, 101−104.

(8) Pamplona, R.; Bellmunt, M. J.; Portero, M.; Riba, D.; Prat, J. Chromatographic evidence for amadori product formation in rat liver aminophospholipids. *Life Sci.* **1995**, *9*, 873−879.

(9) Li, M. M.; Wu, L. Y.; Zhao, T.; Xiong, L.; Huang, X.; Liu, Z. H.; Fan, X. L.; Xiao, C. R.; Gao, Y.; Ma, Y. B.; Chen, J. J.; Zhu, L. L.; Fan, M. The protective role of 5-HMF against hypoxic injury. *Cell Stress Chaperones* **2011**, *16*, 267−273.

(10) Abdulmalik, O.; Safo, M. K.; Chen, Q. K.; Yang, J. S.; Brugnara, C.; Ohene-Frempong, K.; Abraham, D. J.; Asakura, T. 5-Hydroxymethyl-2-furfural modifies intracellular sickle haemoglobin and inhibits sickling of red blood cells. *Br. J. Hamaetol.* **2005**, *128*, 552−561.

(11) Yamada, P.; Nemoto, M.; Shigemori, H.; Yokota, S.; Isoda, H. Isolation of 5-(hydroxymethyl)furfural from Lycium chinense and its inhibitory effect on the chemical mediator release by basophilic cells. *Planta Med.* **2011**, *77*, 434−440.

(12) Wang, M. Y.; Zhao, F. M.; Peng, H. Y.; Lou, C. H.; Li, Y.; Ding, X.; Yu, X. Y.; Yang, G. M.; Xu, D. Q.; Jiang, L. H.; Zhang, X.; Ye, L. H.; Cai, B. C. Investigation on the morphological protective effect of 5-hydroxymethylfurfural extracted from wine-processed Fructus corni on human L02 hepatocytes. *J. Ethnopharmacol.* **2010**, *130*, 424−428.

(13) Ding, X.; Wang, M. Y.; Yao, Y. X.; Li, G. Y.; Cai, B. C. Protective effect of 5-hydroxymethylfurfural derived from processed Fructus Corni on human hepatocyte L02 injured by hydrogen peroxide and its mechanism. *J. Ethnopharmacol.* **2010**, *128*, 373−376.

(14) Gu, H.; Jiang, Y. B.; Jiang, H. Y.; Xu, D. Q.; Yu, J. T.; Ding, X.; Zhao, F. M.; Zhan, Z.; Wang, M. Y. Effect of 5-hydroxymethyl furfural on BCL-2 and NF-*k*B gene expression of apoptotic rat hippocampal neurons injured by $H_2O_2$. *J. Chin. Med. Mater.* **2011**, *11*, 1753−1756.

(15) Changha, L.; Jeyong, Y. UV direct photolysis of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) in aqueous solution: kinetics and mechanism. *J. Photochem. Photobiol. A: Chem.* **2008**, *197*, 232−238.

(16) Zhang, H. B.; Chen, T. F.; Jiang, J.; Wong, Y. S.; Yang, F.; Zheng, W. J. Selenium-containing allophycocyanin purified from selenium-enriched *Spirulina platensis* attenuates AAPH-induced oxidative stress in human erythrocytes through inhibition of ROS generation. *J. Agric. Food Chem.* **2011**, *59*, 8683−8690.

(17) Nikolaos, N.; Maria, T. Observations on the estimation of scavenging activity of phenolic compounds using rapid 1,1-diphenyl-2-picrylhydrazyl (DPPH·) tests. *JAOCS* **2002**, *79*, 1191−1195.

(18) Sharma, O. P.; Bhat, T. K. DPPH antioxidant assay revisited. *Food Chem.* **2009**, *4*, 1202−1205.

(19) Wu, H. C.; Chen, H. M.; Shiau, C. Y. Free amino acids and peptides as related to antioxidant properties in protein hydrolyzates of mackerel (Scomber austriasicus). *Food Res. Int.* **2003**, *9/10*, 947−957.

(20) Miki, M.; Tamai, H.; Mino, M.; Yamamoto, Y.; Niki, E. Free radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by alpha-tocopherol. *Arch. Biochem. Biophys.* **1987**, *258*, 373−380.

(21) Miele, L.; Gabrieli, M. L.; Forgione, A.; Vero, V.; Gallo, A.; Capristo, E.; Gasbarrini, G.; Grieco, A. Oxidative stress in metabolic syndrome and nonalcoholic steatohepatitis. Is it possible a role for vitamins in clinical practice? *Recenti Prog. Med.* **2006**, *97*, 1−5.

(22) Ferder, L.; Inserra, F.; Martinez-Maldonado, M. Inflammation and the metabolic syndrome: Role of angiotensin II and oxidative stress. *Curr. Hypertens. Rep.* **2006**, *8*, 191−198.

(23) Cheung, L. M.; Cheung, P. C. K.; Ooi, V. E. C. Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chem.* **2003**, *81*, 249−255.

(24) Chen, T.; Wong, Y. S. Selenocystine induces caspase-independent apoptosis in MCF-7 human breast carcinoma cells with involvement of p53 phosphorylation and reactive oxygen species generation[J]. *Int. J. Biochem. Cell Biol.* **2009**, *41*, 666−676.

(25) Flohe, L.; Gunzler, W. A. Assays of glutathione peroxidase. *Methods Enzymol.* **1984**, *105*, 114−121.

(26) Bannister, J. V.; Calabrese, L. Assays for superoxide dismutase. *Methods Biochem. Anal.* **1987**, *32*, 279−312.

(27) Katarzyna, K. V.; Krystyna, O.; Eugene, J. K.; Czeslaw, M.; Katarzyna, W. S.; Anna, K. Free radical activity and antioxidant defense mechanisms in patients with hyperthyroidism due to Graves' disease during therapy. *Clin. Chim. Acta* **2000**, *300*, 107−117.

(28) Lick, H. Catalase. In Methods of Enzymatic Analysis.Bergemeuer, H. U., Ed.; Verlag Chemie, 1963; pp 885−888.

(29) Chen, T.; Zheng, W.; Wong, Y. S.; Yang, F. Mitochondria-mediated apoptosis in human breast carcinoma MCF-7 cells induced by a novel selenadiazole derivative. *Biomed. Pharmacother.* **2008**, *62*, 77−84.

(30) Chen, T. F.; Wong, Y. S. In vitro antioxidant and antiproliferative activities of selenium-containing phycocyanin from selenium-enriched *Spirulina platensis*. *J. Agric. Food Chem.* **2008**, *56*, 4352−4358.

(31) Sugihara, T.; Rawicz, W.; Evans, E. A.; Hebbel, R. P. Lipid hydroperoxides permit deformation-dependent leak of monovalent cation from erythrocytes. *Blood* **1991**, *77*, 2757−2763.

(32) Jia, Z.; Misra, H. P. Reactive oxygen species in in vitro pesticide induced neuronal cell (SH-SY5Y) cytotoxicity: role of NFkappaB and caspase-3. *Free Radical Biol. Med.* **2007**, *42*, 288−298.

(33) Fernandes, J. C.; Eaton, P.; Nascimento, H.; Belo, L.; Rocha, S.; Vitorino, R.; Amado, F.; Gomes, J.; Santos-Silva, A.; Pintado, M. E.; Malcata, F. X. Effects of chitooligosaccharides on human red blood cell morphology and membrane protein structure. *Biomacromolecules* **2008**, *9*, 3346−3352.

(34) He, J.; Lin, J.; Li, J.; Zhang, J. H.; Sun, X. M.; Zeng, C. M. Dual effects of Ginkgo biloba leaf extract on human red blood cells. *Basic Clin. Pharm. Toxicol.* **2009**, *104*, 138−144.

(35) Pansarasa, O.; Bertorelli, L.; Veeehiet, J.; Felzani, G.; Marzatieo, F. Age-dependent changes of antioxidant activities and markers of free radical damage in human skeletal muscle. *Free Radical Biol. Med.* **1999**, *27*, 617−622.

(36) Fan, C. D.; Jiang, J.; Yin, X.; Wong, K. H.; Zheng, W. J.; Chen, T. F. Purification of selenium-containing allophycocyanin from selenium-enriched Spirulina platensis and its hepatoprotective effect against t-BOOH-induced apoptosis. *Food Chem.* **2012**, *134*, 253−261.

(37) Li, Y.-X.; Li, Y.; Qian, Z.-J.; Kim, M.-M.; Kim., S.-K. In vitro antioxidant activity of 5-HMF isolated from marine red alga Laurencia undulata in free radical mediated oxidative systems. *J. Microbiol. Biotechnol.* **2009**, *19* (11), 1319−1327.

(38) Zaitzev, A. N.; Simonyan, T. A.; Pozdnyakov, A. L. Hygienic standardization of oxymethylfurfurol in food products. *Vopr. Pitan.* **1975**, *1*, 52−55.