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Protective effects of raspberry on the oxidative damage in HepG2 cells through Keap1/Nrf2-dependent signaling pathway

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ABSTRACT

The aim of the present study was to explore the protective effects of raspberry and its bioactive compound cyanidin 3-*O*-glucoside against H_2O_2-induced oxidative stress in HepG2 cells. We established a model of oxidative stress in HepG2 cells induced by H_2O_2 and examined the protein expression of Keap1/Nrf2. The antioxidant activity of raspberry extract was carried out measuring the level of reactive oxygen species (ROS), and the changes of phase II detoxification elements such as GSH level and CAT activity. Also the expression of proteins related to the Keap1/Nrf2 signaling was tested. The results revealed that raspberry extract significantly reduced the ROS levels in oxidative injured cells, increased GSH content and CAT activity, and activated the expression of proteins Keap1, Nrf2, HO-1, NQO1, and γ-GCS. These results taken together indicated that raspberry treatment could ameliorate H_2O_2-induced oxidative stress in HepG2 cells via Keap1/Nrf2 pathway.

1. Introduction

Reactive oxygen species (ROS) are involved in a spectrum of physiological and pathological processes and for its normal homeostasis the cellular redox system is necessary (Medzhitov, 2008). Low physiological levels of ROS regulate cellular signal transduction and play an important role in normal cell proliferation. However, excessive existence of ROS can break the cell imbalance between antioxidants and oxidants, which can lead to oxidative stress and may cause lipid peroxidation and seriously damage to DNA and proteins (Liang et al., 2013; Qi et al., 2017). Scientific evidence have shown that oxidative stress is involved in the etiology of chronic diseases like hyperglycaemia, hypertension, cardiovascular diseases, inflammation, neurodegenerative disorders and cancer (Pitocco et al., 2013; Zinellu et al., 2017; Khan et al., 2016; Breimer, 1990). Therefore, modulation of oxidative stress and antioxidant defenses are considered to be important in the maintenance of people's health through modulating oxidative processes *in vivo* (Poprac et al., 2017).

Oxidative stress may be caused by the stimulation of the external environment, which causes the alteration in the oxidation/antioxidation homeostasis, leading to a stress response in the body. Under normal conditions, the system involved in the generation and removal of ROS is in a state of dynamic equilibrium. Due to the stimulation of the external environment or to changes at the cell level, ROS production increases and the body may experience oxidative stress accordingly. When oxidative stress is established, the ROS content in a particular tissue may be relatively high; being difficult for the organism's scavenging capacity to reach an equilibrium state, thereby leading to a pathological estate

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(Poprac et al., 2017; Sies, 2017).

The epoxy chloropropane Kelch sample related protein-1 (Keap1)-nuclear factor erythroid-2 related factor (Nrf2)/antioxidant response element (ARE) signal pathway is important in cellular antioxidant response. The induction of phase II metabolic enzymes and antioxidant proteins/enzymes offer cellular protection under oxidative stress. The pathway is regulated by interactions between Nrf2 and the cytosolic repressor protein Keap1. In case of oxidative stress, Nrf2 detaches from Keap1 and translocates to the nucleus. This interaction leads to the increase in the transcription of cytoprotective genes that poses one or more AREs in their promoter regions. By regulating the basal and inducible expression of this diverse set of genes, Keap1-Nrf2-ARE signal pathway influences cell and organism sensitivity to oxidative insults (Bellezza et al., 2018; Clarke et al., 2016).

In the last years, the close relationship between diet and human health has led us to investigate the bioactive phytochemicals present in fruits and vegetables (Cao et al., 2018; Zhao et al., 2018, 2019; Dragan et al., 2019). Berry fruits (mainly blueberry, blackberry, raspberry, and strawberry) are among the best known dietary sources of bioactive compounds. Numerous studies demonstrated that berry fruits exhibit a wide range of biological activities including antioxidant, anti-carcinogenic, anti-aging and vasodilatory properties (Bowen-Forbes et al., 2010; Kim et al., 2017; de Sá et al., 2014). The most significant health benefits of berry fruits are attributed to polyphenols, such as flavonoids, phenolic acids and tannins (Bobinaitė et al., 2012).

Rubus coreanus Miq. is one of the most popular berries, known as Korean blackberry, bokbunja, and Korean bramble. Native to Korea, China, and Japan this berry has been traditionally used for the treatment of liver and kidney diseases (Chae et al., 2014). Hypoglycaemic, anti-osteoporosis, antioxidant, and anti-inflammatory properties have been also described (Lee et al., 2011). Raspberry is well known as a rich source of antioxidants such as anthocyanins and flavonoids.

According to all-above mentioned, the aim of the present study was to investigate the protective effects of raspberry, and its bioactive compound cyanidin 3-*O*-glucoside, against H_2O_2-induced oxidative stress in HepG2 cells.

2. Materials and methods

2.1. Chemicals and reagents

Folin-Ciocalteu reagent, 6-carboxy-2′,7′-dichlorofluorescein diacetate (DCF-DA), quercetin (≥98%) and rutin (> 98%) were purchased from Solarbio Science Technology Co., Ltd. (Beijing, China). Gallic acid (98%) was purchased from Xirong Science Co., Ltd. (China). 2,2-Diphenyl-1,2,4,6-trinitrophenyl-hydrazyl (DPPH) was purchased from TCI Development Co., Ltd., (Shanghai, China). Cyanidin 3-*O*-glucoside (98%) was acquired from Desite Biotechnology Co., Ltd. (Chengdu, China). T-AOC, GSH and CAT kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China). Water was purified using MING-CHE 24UV Water Purification System (Millipore, France). The antibodies and horseradish peroxidase conjugated anti-goat secondary antibody were purchased from Biotech Bioengineering (Shanghai, China).

2.2. Sample preparation

Raspberry fruits were purchased from Yantai, Shandong Province in China (37°27′35.90″N 121°27′10.98″E). Plant species authentication was performed by Prof. Dr. Chen Gongxi, executive director of Chinese national medical association-Jishou University-where a voucher specimen was deposited under FP-0192. Raspberry samples were frozen at −80 °C overnight, then, the frozen sample was freeze-dried using a freeze dryer operated at −40 °C until all moisture was removed. The freeze-dried sample was ground into powder using a grinder, passed through a 40 mesh sieve, and dry stored. Using a flash extractor (Beijing Inlake Technology Development Co., Ltd. Peking, China) at a voltage of 150 V and extraction time of 63 s, samples were extracted with 65% (v/v) ethanol solution at room temperature. The suspension was centrifuged at 3000 × *g*, 4 °C for 10 min, and the supernatant was collected in a clean Petri dish, stored at −80 °C overnight, then freeze-dried at −40 °C for 12 h, collected, and stored at −20 °C. The raspberry extract (RE) dissolved in DMSO at the concentration from 312.5 to 5000 μg/mL for the following assay experiments.

2.3. Total polyphenols content assay

The total polyphenols content (TPC) was determined according to the Folin-Denis method as described by Teng et al. (2013) with some modifications. Appropriately diluted raspberry extract (RE) (100 μL) was reacted with 500 μL deionized water, 250 μL of 0.1 mol/L Folin-Ciocalteu reagent and 400 μL of 7.5% Na_2CO_3. After maintaining the sample at room temperature for 20 min, 1 mL of distilled water was added before measuring the visible absorbance at 760 nm. The TPC were calculated by calibration curves using gallic acid as a standard and expressed as milligram gallic acid equivalents (GAE) per gram dry weight (mg GAE/g dw). All samples were analyzed in triplicate.

2.4. Total flavonoid content assay

The total flavonoid content (TFC) was determined as reported previously (Teng et al., 2013). Appropriately diluted extract (70 μL) was reacted with 430 μL deionized water, 50 μL of 5% $NaNO_2$ reagent and 50 μL of 10% $Al(NO_3)_3{\cdot}9H_2O$ were added, and then kept still for 6 min at room temperature. Fifty μL of 1 mol/L NaOH, 1 mL of distilled water was added before measuring the visible absorbance at 510 nm. The TFC were calculated by calibration curves using rutin as a standard and expressed as milligram rutin equivalents (RT) per gram dry weight (mg RT/g dw). All samples were analyzed in triplicate.

2.5. Total anthocyanin content assay

The total anthocyanins content (TAC) was determined using the pH differential method described by Teng et al. (2013). Samples were appropriately diluted with 0.025 M potassium chloride (pH 1.0) and 0.4 M sodium acetate (pH 4.5) until the absorbance at 530 nm was less than 1.2. Then, the mixture was equilibrated for 15 min before recording the absorbance at 530 nm and 700 nm. Distilled water was used as blank for calibration. The TAC were calculated by calibration curves using cyanidin 3-*O*-glucoside as a standard and expressed as milligram cyanidin 3-*O*-glucoside equivalents (CGE) per gram dry weight (mg CGE/g dw). All samples were analyzed in triplicate.

2.6. HPLC analysis

Anthocyanin presented in RE was analyzed by HPLC according to the method described by Teng et al. (2013) and Li et al. (2016) using Agilent 1260 series HPLC system (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was performed on a ZORBAX SB-C18 column (4.6 × 150 mm, 5 μm, Agilent, USA) at room temperature. The mobile phase consisted of 0.1% phosphoric acid (phosphoric acid/water, v/v) (A) and acetonitrile (B) with a gradient elution of 92-80% A at 0–15 min. The flow rate of mobile phase was 0.80 mL/min, and the injection volume was 10 μL, and the peak was detected at 510 nm. All samples were filtered through a 0.45 μm micropore membrane before injection.

2.7. Cytotoxicity assay

RE's stock solutions were prepared in DMSO. HepG2 cells were grown in 24 multi-well plates at 5×10^4 cells/well and cultured for 24 h at 37 °C with 5% CO_2. HepG2 cells were incubated with hydrogen

peroxide (H_2O_2) or RE solutions for 6, 12 and 24 h at various concentration, and the RE solutions and H_2O_2 final concentration in cell culture ranged from 25 to 400 μg/mL, respectively. About 50 μL of 5 mg/mL MTT solution was added to each well and incubated further for 4 h at 37 °C; the supernatant was discarded and 500 μL DMSO was added to lyse the cells on a gyratory shaker. The absorbance was recorded at 450 nm by the microplate reader. The results were expressed as mean of cell survival normalized to control (without treatment).

2.8. Intracellular ROS assay

DCF-DA was used to evaluate intracellular ROS. Briefly, HepG2 cells were incubated in 24 multi-well plates at 5×10^4 cells/well and cultured for 24 h at 37 °C with 5% CO_2, then treated with or without the indicated 50 μL of 40 μM H_2O_2 (final concentration in cell culture 4 μM). After 6 h, cells were treated with 50 μL various concentrations of RE solutions (final concentration in cell culture from 50 to 200 μg/mL) for 12 h. Afterwards, the compound-treated cells were stained with 50 μL 100 μM of DCFH-DA (final concentration in cell culture 10 μM) for 30 min, washed triplicate with PBS, and then fluorescence intensity was measured with a 24 multi-well plates fluorometer (Molecular Devices, SpectraMax M5, CA, USA) at excitation and emission wavelengths of 485 nm and 535 nm, respectively.

2.9. Intracellular GSH assay

GSH assay was referred to the kit instructions: HepG2 cells were grown in 6 multi-well plates at 2×10^5 cells/well, after the culture treatment according to the above method, the cells were collected and added the cell lysates, then centrifuged for 10 min to collect the supernatants. Subsequently, 100 μL of the samples were added to the sample well. The samples were then mixed and incubated at room temperature for 5 min. The OD was measured at 405 nm, and the concentration of GSH was calculated.

2.10. Intracellular CAT assay

CAT assay was referred to kit instructions: Five μL of the test samples were added to the sample well, 5 μL of the deionized water was added to the blank-control well. The samples were then mixed, and colorimetric spectrophotometry was used to measure the absorbance of each tube at 405 nm. Then CAT viability was calculated.

2.11. Western blotting

HepG2 cells were cultured in 6-wells plate and treated with or without samples; cell lysate was extracted with ice-cold RIPA buffer containing a protease inhibitor cocktail and phenylmethylsulfonyl fluoride (PMSF) for 30 min. The cell lysates were collected and centrifuged at 12000 × *g* at 4 °C for 15 min and drain the supernatant. Protein concentrations were determined using protein assay reagent purchased from Biotech Bioengineering Co., Ltd. (Shanghai, China). Equal amounts of protein from each sample were separated with 12% SDS-PAGE gels and then the proteins were separated by electrophoresis. The gels were then transferred onto PVDF transfer membranes from Yu Wei Biological Technology Instrument Co., Ltd. (Guangzhou, China) at 300 mA for 60 min. The membranes were blocked with 5% BSA in TBST (Tris-buffered saline with Tween 20) for 60 min. Then, they were incubated with primary antibodies at a dilution of 1:1000 anti-Keap1, anti-Nrf2, anti–HO–1 Heme oxygenase (1), anti-NQO1 NAD(P)H dehydrogenase [quinone] (1) and anti-γ-GCS (Gamma-glutamyl cysteine synthetase) overnight at 4 °C. After five times washing in TBST and 3 min for each membrane was incubated with horseradish peroxidase (HRP) conjugated goat-anti-rabbit IgG secondary antibody (dilution at 1:5000) in blocking solution for 45 min at room temperature on a shaker and washed five times. The enhanced chemiluminescence (ECL) substrate, purchased from Biotech Bioengineering Co., Ltd (Shanghai, China) was used to detect the protein signal by using a Gel Catcher 3400 (Shanghai, China). β-Actin was used as protein-loading controls.

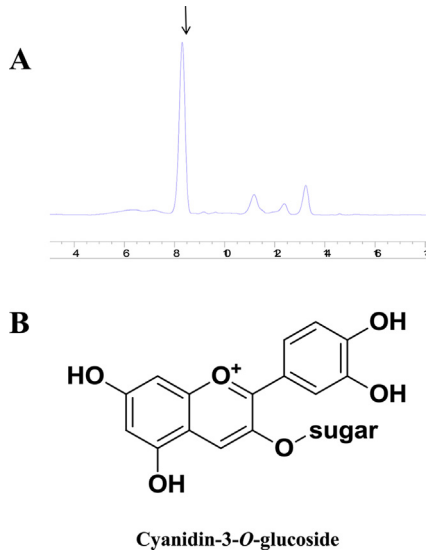

Fig. 1. HPLC profile of RE (raspberry extract) and structure of CGE (cyaniding 3-*O*-glucoside). (A) HPLC profile of the ethanol extract of raspberry; (B) Structure of CGE.

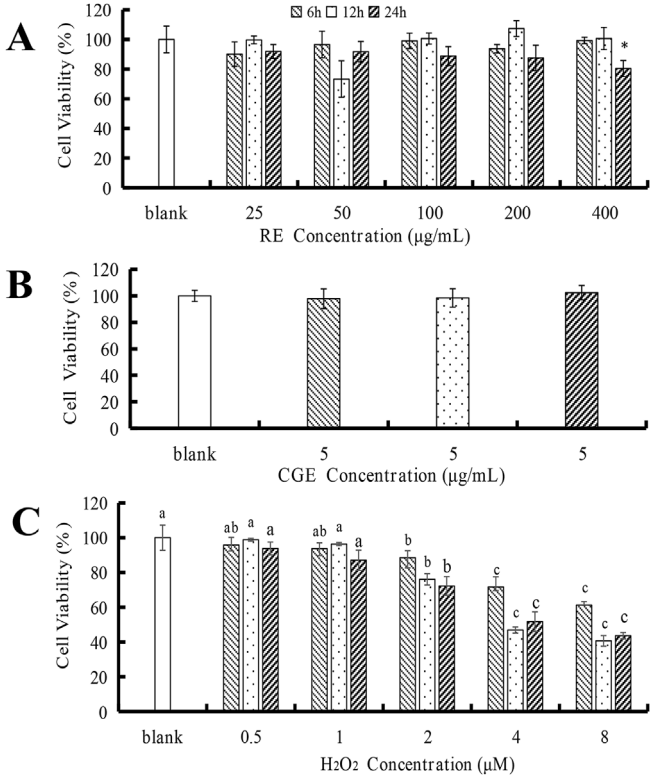

Fig. 2. Cytotoxicity in HepG2 cells. Cells were plated at a density of 24 multi-well plates at 5×10^4 cells/well for 24 h and incubated with tested compounds for 4, 12 and 24 h and viability was measured by MTT assay. The values for each compound concentration tested represent the average (mean ± SD; n = 3); (A) HepG2 cells were incubated with RE at various concentration; (B) HepG2 cells were incubated with CGE at various concentration; (C) HepG2 cells were incubated with H_2O_2 at various concentration. The values for each column are mean values ± SD (n = 3). For each parameter, columns not sharing superscript letters are statistically different ($P < 0.05$). CGE: Cyaniding-3-*O*-glucoside. RE: Raspberry extract.

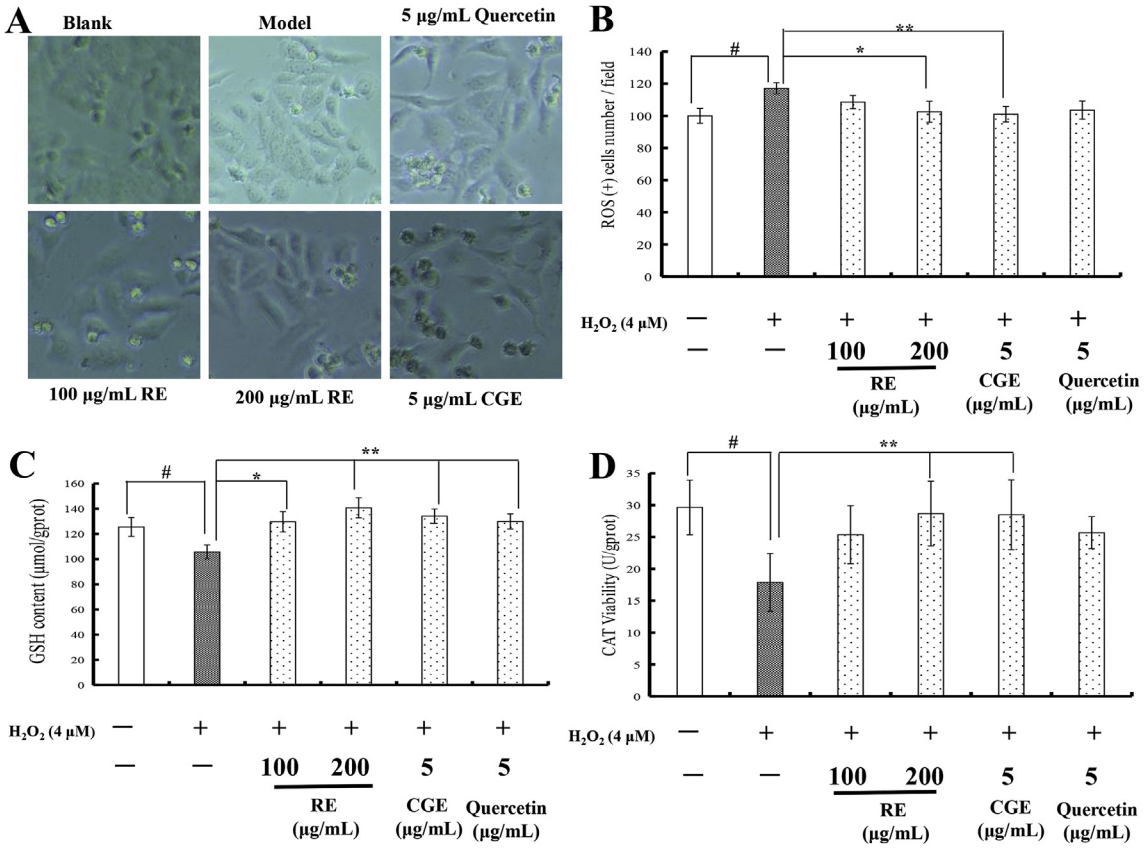

Fig. 3. Cellular antioxidant effects of RE and CGE. After induced by H_2O_2, (A) Cell morphology caused by adding different concentrations of compounds. (B) Changes in intracellular ROS levels caused by the addition of different concentrations of compounds. (C) Changes in intracellular GSH content caused by the addition of different concentrations of compounds. (D) Changes in intracellular CAT activity caused by the addition of different concentrations of compounds. The values for each column are mean values ± SD (n = 3). $^{*}P < 0.05$. $^{\#}P < 0.05$. $^{++}P < 0.001$. $^{\#\#}P < 0.001$. CAT: Catalase CGE: Cyaniding-3-*O*-glucoside. GSH: Reduced glutathione. RE: Raspberry extract. ROS: Reactive oxygen species.

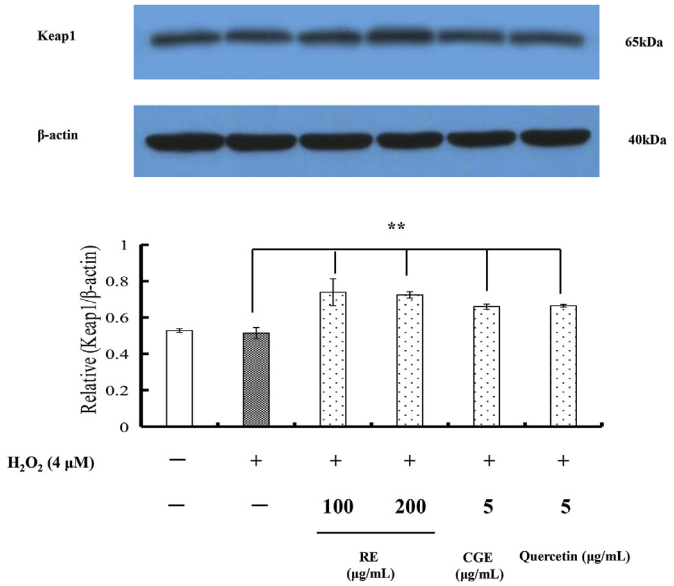

Fig. 4. Characterization of RE and CGE-mediated protein expression of antioxidant Keap1/Nrf2 pathway. Whole cell lysates were harvested and analyzed with antibodies to Keap1 by addition of RE and CGE, Keap1expression was analyzed by Western blotting in cultured HepG2 cells. Cells were treated with various concentrations of RE and CGE for 12 h. The test was repeated three times, and representative blots are shown. Data shown are representative of three independent experiments. Values of each column are mean values ± SD (n = 3). $^{**}P < 0.01$. CGE: Cyaniding-3-*O*-glucoside. RE: Raspberry extract.

2.12. Statistical analysis

Data were expressed as mean ± SD. One-way (Tukey's test) or two-way ANOVA tests was used for data analysis. The differences between the mean values were considered to be statistically significant once $P \leq 0.05$, which was performed *via* IBM SPSS Statistics 20.0 for Windows (SPSS Inc., Chicago, IL).

3. Results

3.1. Phytochemicals

TPC, TFC and TAC of RE were 10.286 ± 0.396 mg GAE/g dw, 15.833 ± 2.89 mg RT/g dw, and 2.467 ± 0.035 mg CGE/g dw, respectively. The RE was preliminarily and systematically identified using an HPLC consists of UV detector. A major peak (33.59%) appeared at the retention time around 8 min was detected as cyanidin 3-*O*-glucoside (Fig. 1).

3.2. Cytotoxic effect of RE and CGE on HepG2 cells

To evaluate the effects of RE and CGE on the cytotoxicity of HepG2 cells, the cells were treated for 6, 12 and 24 h with a series of concentrations of RE ranged from 50 to 400 μg/mL. CGE at 5 μg/mL dose was used a comparative control. As shown in Fig. 2A, RE did not show cytotoxicity of HepG2 cell. Compared to the control, except the treatment for 24 h by 400 μg/mL of samples, RE caused nearly a 20% inhibition of cell growth, and the remaining groups showed that RE had no toxic effect on HepG2 cells. CGE did not show inhibition of HepG2

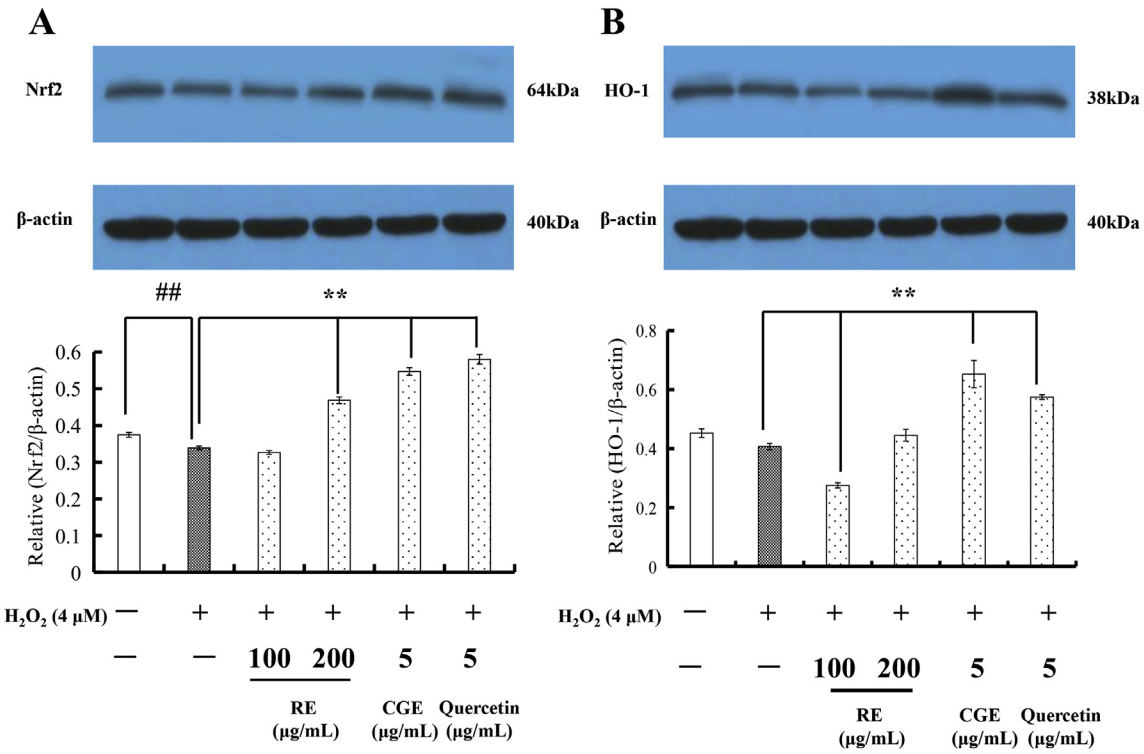

Fig. 5. Functional characterization of RE and CGE-mediated protein expression of antioxidant Keap1/Nrf2 pathway. Whole cell lysates were harvested and analyzed with antibodies to Nrf2, HO-1 by addition of RE and CGE, Nrf2 (A), HO-1 (B) expression was analyzed by Western blotting in cultured HepG2 cells. Cells were treated with various concentrations of RE and CGE for 12 h. The test was repeated three times, and representative blots are shown. Data shown are representative of three independent experiments. Values of each column are mean values ± SD (n = 3). $^{**/\#\#}P < 0.01$. CGE: Cyaniding-3-*O*-glucoside. HO-1: Heme oxygenase 1. Nrf2: Nuclear factor erythroid-2 related factor. RE: Raspberry extract.

cell.

Hydrogen peroxide (H_2O_2) is an important ROS that easily permeates cell membranes and reacts with intracellular iron ions; therefore, it has become an important tool to evaluate the oxidative injury of cells (Gachou et al., 1999). To evaluate the effects of H_2O_2 on the proliferation of HepG2 cells, the cells were treated with a series of concentrations of H_2O_2 from 0.5 to 8 μM for 6, 12 and 24 h. As shown in Fig. 2C, H_2O_2 showed cytotoxicity of HepG2 cell in a dose- and time-dependent manner. Compared with the control group, the cells were incubated with 4 μM of H_2O_2 for 6 h and the cell viability was close to 70%.

3.3. Effect of RE and CGE on cellular ROS, GSH and CAT

Quercetin can regulate the expression of related genes and proteins in the Nrf2 pathway and protect oxidative damage of cells (Nishimura et al., 2017; Granado-Serrano et al., 2012; Xu et al., 2016; Ramyaa and Padma, 2014). Therefore, quercetin was used as a positive control in this section.

CAT and GSH are the primary barriers minimizing the radical oxygen cascade and removing cytotoxic peroxides in mammalian systems (Zhang et al., 2016). Therefore, to investigate the effect of RE on hydrogen peroxide-induced HepG2 cells and oxidative damage, we performed ROS clearance tests and determination of changes in intracellular GSH level and CAT activities in HepG2 cells.

As expected, treatment of cells with RE and CGE also blocked the increase of intracellular ROS generation, at the same time, the activity of intracellular GSH and CAT increased. After induced by H_2O_2, cell morphology changed significantly by adding different concentrations of samples (Fig. 3A). Compared with the blank group, the ROS level (Fig. 3B) in the model group was significantly increased, while, the GSH (Fig. 3C) content and CAT (Fig. 3D) activity were significantly decreased ($P < 0.05$). On the other hand, the ROS level in the RE treated cells decreased to 102.55–108.55% (Fig. 3B), while, the GSH content and the CAT activity increased to 129.69–140.73 μmol/g protein (Figs. 3C) and 25.36–28.68 U/g protein (Fig. 3D), respectively.

3.4. Effect of RE and CGE on protein expression of Keap1/Nrf2

To further investigate the possible role of the RE and CGE involved in regulating Keap1/Nrf2 signaling pathways, we further examined the target genes at protein expression levels of Keap1/Nrf2 signaling pathways. Western blotting assays showed in Fig. 4, compared with the blank group, the protein expression levels of Keap1, Nrf2, HO-1, NQO1, and γ-GCS in the model group decreased. The expression levels of Keap1 (Fig. 4), Nrf2 (Fig. 5A), HO-1 (Fig. 5B), NQO1 (Fig. 6A), and γ-GCS (Fig. 6B) in the 200 μg/mL RE-treated cells were approximately 43.58%, 38.40%, 9.43%, 18.56%, and 11.24% higher than those in the model group, respectively. The CGE treatment group was about 40.86, 28.35, 60.34, 24.77, and 13.03% higher than the model group, respectively. All tested samples had significant differences. Taken together, results represented above clearly indicated that RE and CGE activated Keap1/Nrf2 signaling pathways leading to enhanced expression of its target protein implicated in protection against oxidative-caused damage in HepG2 cells.

4. Discussion

The data generated during this investigation strongly support therapeutic efficacy of raspberry that exerts cytoprotection during H_2O_2-induced injury in HepG2 cells. Published literature reveals a direct correlation between regular consumption of fruit and prevention of oxidative stress-related diseases (Zamora-Ros et al., 2016). Interestingly, raspberry is one such fruit which is a rich source of flavonoids,

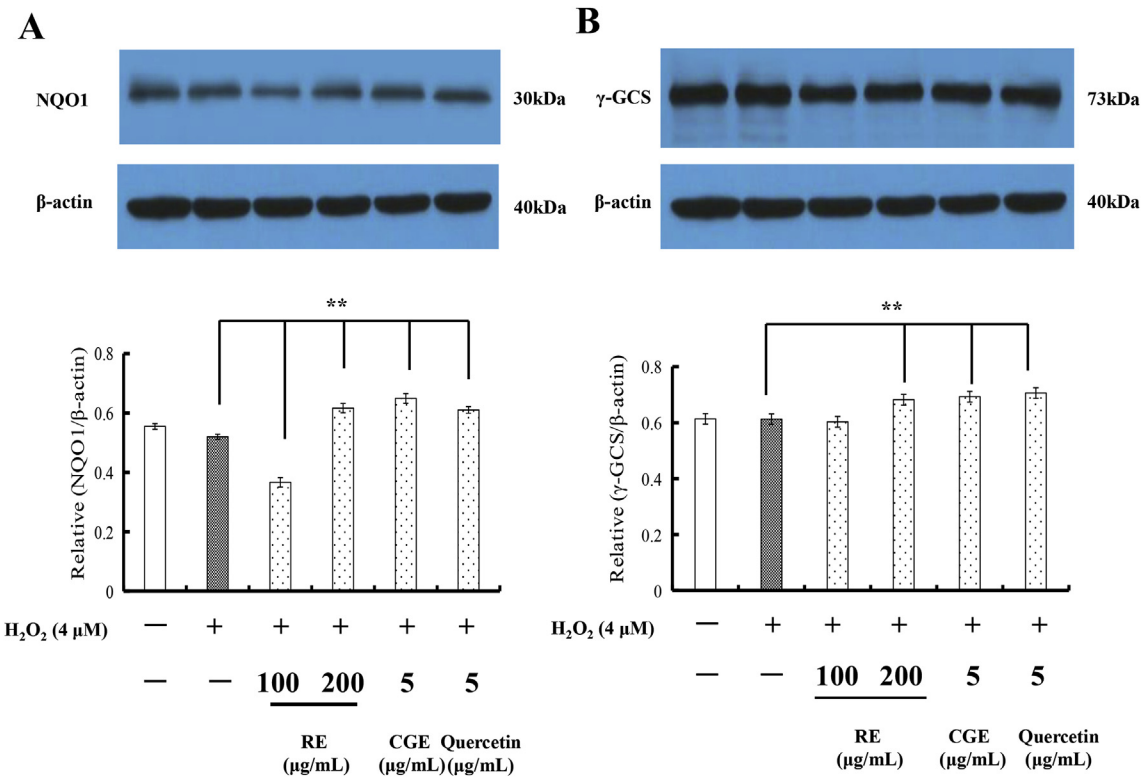

Fig. 6. Functional characterization of RE and CGE-mediated protein expression of antioxidant Keap1/Nrf2 pathway. Whole cell lysates were harvested and analyzed with antibodies to NQO1, γ-GCS by addition of RE and CGE, NQO1 (A), γ-GCS (B) expression was analyzed by Western blotting in cultured HepG2 cells. Cells were treated with various concentrations of RE and CGE for 12 h. The test was repeated three times, and representative blots are shown. Data shown are representative of three independent experiments. Values of each column are mean values ± SD (n = 3). */#$P < 0.05$ indicate significant differences compared with the control/model group. **/##$P < 0.01$. CGE: Cyaniding-3-*O*-glucoside. γ-GCS: Gamma-glutamyl cysteine synthetase. NQO1: NAD(P)H dehydrogenase [quinone] 1. RE: Raspberry extract.

polyphenols and antioxidants (Teng et al., 2013). Raspberry possesses free radical scavenging potential and antioxidant activities due to the presence of flavonol glucosides, hydroxycinnamicacid derivatives, tannins and proanthocyanidins; and hence is suggested beneficial for human health (Teng et al., 2017). In this study, we have evaluated antioxidant power of raspberry in terms of its total phenolics, anthocyanins. The examined levels of these selected parameters of raspberry corroborate with the previously published work (Teng et al., 2016) and signify a remarkable antioxidant potential. These findings are further supported by our HPLC data which demonstrated the presence of rich cyanidin 3-*O*-glucoside in raspberry (Fig. 1). Gowd et al. (2019) observed that anthocyanins presented in raspberry had a pronounced protective effect against cytotoxicity and possessed strong antioxidant activity in vitro. Our HPLC results reveal the presence of these constituents in raspberry confirming its high antioxidant potential. In fact, fruits and vegetables are rich in polyphenols. The *ortho*-phenolic hydroxyl group in is easily oxidized, and has a strong ability to capture free radicals (Xiao and Högger, 2015). This is the reason why polyphenols can scavenge free radicals and quench active oxygen. At present, there are about one thousand kinds of polyphenols present in fruits and vegetables that have been described to be good for human health (Xiao et al., 2016; Xiao, 2017, 2018; Chen et al., 2018). Many of them have been involved in the prevention of cancer, diabetes, cardiovascular diseases or some neurological disorders (Khan et al., 2019; Zhao et al., 2019). Raspberry fruit is rich in polyphenols and has a variety of biomedical properties (Peiffer, 2018). In fact, many studies showed that some bioactive substances in raspberries have inhibitory effects on cancer (Coates et al., 2007), diabetes (Noratto et al., 2017), inflammation (Lee et al., 2015), among others. This is why raspberries have attracted the focus of researchers from food technology and health areas. However, studies investigating whether the active substances in raspberry are involved in the regulation of Keap1/Nrf2 signaling pathway and the molecular mechanism of its action do not exist or are related to other related species but not the same.

Dietary polyphenols are known to exert direct antioxidant effects by ROS scavenging, but also indirect effects through the induction of endogenous protective mechanisms like the regulation of intracellular GSH concentration and the expression levels of GSH-related detoxifying enzymes (Jiménez-Aspee et al., 2016). As expected, ROS in HepG2 cells induced by H_2O_2 increased significantly, meanwhile, a decreased trend of GSH content and CAT activity were also found. After treatment with RE or CGE, intracellular ROS decreased significantly. At the same time, an increase in GSH content and CAT activity was found. Thus, RE and CGE were able to protect from hydrogen peroxide-induced damage. These results agree with those from previous studies in which positive effects of raspberries on oxidative stress protection have been described (Sun et al., 2013; Choi et al., 2016).

Also, Nrf2 is an important transcription factor regulating Phase II detoxifying enzymes and antioxidant status to combat cellular oxidative stress. Under physiological conditions, cytoplasmic chaperone molecule Keap1 binds and inhibit Nrf2 (Nguyen et al., 2009; Ma, 2013). Nrf2 and Keap1 are decoupled into the nucleus under the stimulation of external factors such as increased ROS. Nrf2 induces the expression of factors that detoxify carcinogens and repress oxidative stress, including HO-1, NQO1, and γ-GCS, and are thus an important defense mechanism (Kobayashi and Yamamoto, 2005; Sun et al., 2016). Our results showed that Keap1, Nrf2, HO-1, NQO1, and γ-GCS were reduced in HepG2 cells subjected to hydrogen peroxide-induced damage. However, the treatment with RE or CGE of H_2O_2-induced cells led to the overexpression of Nrf2, HO-1, NQO1, and γ-GCS proteins. This finding was interesting,

since it means that RE and CGE exerted antioxidant activity by direct free radical scavenging but also by modulating molecular mechanisms. In fact, by up-regulation of these proteins' expression related to Phase II detoxifying enzymes involved in Keap1/Nrf2 signaling pathway, RE and CGE effectively inhibited cell damage from H_2O_2. However, the specific mechanism of activation of the Keap1/Nrf2 pathway by RE and CGE remains unclear and needs further study.

5. Conclusion

In conclusion, the present study convincingly demonstrates role of Keap1/Nrf2 pathway during therapeutic intervention by raspberry and its bioactive compound cyanidin 3-*O*-glucoside in hydrogen peroxide-induced cell model. The presence of antioxidants (cyanidin 3-*O*-glucoside) in the raspberry offers remarkable protection against the cell damage. It is envisaged that raspberry-mediated antioxidant action, improved antioxidant enzyme activities, and attenuate ted oxidative stress through the modification of Keap1/Nrf2.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Bellezza, I., Giambanco, I., Minelli, A., Donato, R., 2018. Nrf2-Keap1 signaling in oxidative and reductive stress. BBA-Mol Cell Res. 1865, 721–733.

Bobinaitė, R., Viškelis, P., Venskutonis, P.R., 2012. Variation of total phenolics, anthocyanins, ellagic acid and radical scavenging capacity in various raspberry (Rubus spp.) cultivars. Food Chem. 132, 1495–1501.

Bowen-Forbes, C.S., Zhang, Y., Nair, M.G., 2010. Anthocyanin content, antioxidant, anti-inflammatory and anticancer properties of blackberry and raspberry fruits. J. Food Compos. Anal. 23, 554–560.

Breimer, L.H., 1990. Molecular mechanisms of oxygen radical carcinogenesis and mutagenesis: the role of DNA base damage. Mol. Carcinog. 3, 188–197.

Cao, H., Ou, J.Y., Chen, L., Zhang, Y.B., Szkudelski, T., Delmas, D., Daglia, M., Xiao, J.B., 2018. Dietary polyphenols for managing type 2 diabetes: human studies and clinical trials. Crit. Rev. Food Sci. Nutr. https://doi.org/10.1080/10408398.2018.1492900.

Chae, H.J., Yim, J.E., Kim, K.A., Chyun, J.H., 2014. Hepatoprotective effects of *Rubus coreanus* Miquel concentrates on liver injuries induced by carbon tetrachloride in rats. Nutr Res Pract 8, 40–45.

Chen, L., Teng, H., Xie, Z.L., Cao, H., Cheang, W.S., Skalicka-Woniak, K., Georgiev, M.I., Xiao, J.B., 2018. Modifications of dietary flavonoids towards improved bioactivity: an update on structure-activity relationship. Crit. Rev. Food Sci. Nutr. 58 (4), 513–527.

Choi, M.H., Shim, S.M., Kim, G.H., 2016. Protective effect of black raspberry seed containing anthocyanins against oxidative damage to DNA, protein, and lipid. J. Food Sci. Tech. Mys. 53, 1214–1221.

Clarke, J.L., Murray, J.B., Park, B.K., Copple, I.M., 2016. Roles of Nrf2 in drug and chemical toxicity. Curr Opi. Toxicol 1, 104–110.

Coates, E.M., Popa, G., Gill, C.I., McCann, M.J., McDougall, G.J., Stewart, D., Rowland, I., 2007. Colon-available raspberry polyphenols exhibit anti-cancer effects on *in vitro* models of colon cancer. J. Carcinog. 6 4.

Dragan, S., Buleu, F., Christodorescu, R., Cobzariu, F., Iurciuc, S., Velimirovici, D., Xiao, J.B., Luca, C.T., 2019. Benefits of multiple micronutrient supplementation in heart failure: a comprehensive review. Crit. Rev. Food Sci. Nutr. 59 (6), 965–981.

de Sá, L.Z.M., Castro, P.F., Lino, F.M., Bernardes, M.J., Viegas, J.C., Dinis, T.C., et al., 2014. Antioxidant potential and vasodilatory activity of fermented beverages of jabuticaba berry (*Myrciaria jaboticaba*). J Funct. Foods. 8, 169–179.

Gachou, C.M., Laget, M., Guiraud-Dauriac, H., De Meo, M., Elias, R., Dumenil, G., 1999. The protective activity of α-hederine against H 2 O 2 genotoxicity in HepG2 cells by alkaline comet assay. Mutat Res-Gen Tox En. 445, 9–20.

Granado-Serrano, A.B., Martín, M.A., Bravo, L., Goya, L., Ramos, S., 2012. Quercetin modulates Nrf2 and glutathione-related defenses in HepG2 cells: involvement of p38. Chem. Biol. Interact. 195, 154–164.

Gowd, V., Bao, T., Chen, W., 2019. Antioxidant potential and phenolic profile of blackberry anthocyanin extract followed by human gut microbiota fermentation. Food Res. Int. 120, 523–533.

Jiménez-Aspee, F., Theoduloz, C., Ávila, F., Thomas-Valdés, S., Mardones, C., von Baer, D., Schmeda-Hirschmann, G., 2016. The Chilean wild raspberry (*Rubus geoides* Sm.) increases intracellular GSH content and protects against H_2O_2 and methylglyoxal-induced damage in AGS cells. Food Chem. 194, 908–919.

Khan, M.S., Ali, T., Kim, M.W., Jo, M.H., Jo, M.G., Badshah, H., Kim, M.O., 2016. Anthocyanins protect against LPS-induced oxidative stress-mediated neuroinflammation and neurodegeneration in the adult mouse cortex. Neurochem. Int. 100, 1–10.

Khan, H., Reale, M., Ullah, H., Sureda, A., Tejada, S., Wang, Y., Zhang, Z.J., Xiao, J.B., 2019. Anti-cancer effects of polyphenols via targeting p53 signaling pathway: updates and future directions. Biotechnol. Adv. https://doi.org/10.1016/j.biotechadv.2019.04.007.

Kim, J., Cho, S.Y., Kim, S.H., Cho, D., Kim, S., Park, C.W., et al., 2017. Effects of Korean ginseng berry on skin antipigmentation and antiaging via FoxO3a activation. J Gins Res 41, 277–283.

Kobayashi, M., Yamamoto, M., 2005. Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. Antioxidants Redox Signal. 7, 385–394.

Lee, J.E., Park, E., Lee, J.E., Auh, J.H., Choi, H.K., Lee, J., Cho, S., Kim, J.H., 2011. Effects of a *Rubus coreanus* Miquel supplement on plasma antioxidant capacity in healthy Korean men. Nutr Res Pract 5, 429–434.

Lee, H.J., Jung, H., Cho, H., Hwang, K.T., 2015. Anti-inflammatory effect of black raspberry seed oil in high-fat diet-induced obese mice. J. Food Biochem. 39, 612–621.

Li, Z.H., Guo, H., Xu, W.B., Ge, J., Li, X., Alimu, M., He, D.J., 2016. Rapid identification of flavonoid constituents directly from PTP1B inhibitive extract of raspberry (*Rubus idaeus* L.) leaves by HPLC-ESI-QTOF-MS-MS. J. Chromatogr. Sci. 54, 805–810.

Liang, L., Gao, C., Luo, M., Wang, W., Zhao, C., et al., 2013. Dihydroquercetin (DHQ) induced HO-1 and NQO1 expression against oxidative stress through the Nrf2-dependent antioxidant pathway. J. Agric. Food Chem. 61, 2755–2761.

Ma, Q., 2013. Role of nrf2 in oxidative stress and toxicity. Annu. Rev. Pharmacol. 53, 401–426.

Medzhitov, R., 2008. Origin and physiological roles of inflammation. Nature 454 (7203) 428.

Nguyen, T., Nioi, P., Pickett, C.B., 2009. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. J. Biol. Chem. 284, 13291–13295.

Nishimura, K., Matsumoto, R., Yonezawa, Y., Nakagawa, H., 2017. Effect of quercetin on cell protection via erythropoietin and cell injury of HepG2 cells. Arch. Biochem. Biophys. 636, 11–16.

Noratto, G.D., Chew, B.P., Atienza, L.M., 2017. Red raspberry (*Rubus idaeus* L.) intake decreases oxidative stress in obese diabetic (db/db) mice. Food Chem. 227, 305–314.

Peiffer, D.S., 2018. Preparing black raspberry components for their use as cancer therapeutics. J. Berry Res. 8, 297–306.

Pitocco, D., Tesauro, M., Alessandro, R., Ghirlanda, G., Cardillo, C., 2013. Oxidative stress in diabetes: implications for vascular and other complications. Int. J. Mol. Sci. 14, 21525–21550.

Poprac, P., Jomova, K., Simunkova, M., Kollar, V., Rhodes, C.J., Valko, M., 2017. Targeting free radicals in oxidative stress-related human diseases. Trends Pharmacol. Sci. 38, 592–607.

Qi, Z., Ci, X., Huang, J., Liu, Q., Yu, Q., Zhou, J., et al., 2017. Asiatic acid enhances Nrf2 signaling to protect HepG2 cells from oxidative damage through Akt and ERK activation. Biomed. Pharmacother. 88, 252–259.

Ramyaa, P., Padma, V.V., 2014. Quercetin modulates OTA-induced oxidative stress and redox signalling in HepG2 cells—up regulation of Nrf2 expression and down regulation of NF-κB and COX-2. Bba-Gen subjects 1840, 681–692.

Sies, H., 2017. Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: oxidative eustress. Redox Bio 11, 613–619.

Sun, J., Zhu, H., Dong, G., 2013. Oxidization resistance *in vivo* for raspberry flavone. Eng. Times 5 455.

Sun, X., Ou, Z., Chen, R., Niu, X., Chen, D., Kang, R., Tang, D., 2016. Activation of the p62-Keap1-NRF2 pathway protects against ferroptosis in hepatocellular carcinoma cells. Hepatology 63, 173–184.

Teng, H., Lee, W.Y., Choi, Y.H., 2013. Optimization of microwave-assisted extraction for anthocyanins, polyphenols, and antioxidants from raspberry (*Rubus Coreanus* Miq.) using response surface methodology. J. Sep. Sci. 36, 3107–3114.

Teng, H., Fang, T., Lin, Q., Song, H., Liu, B., Chen, L., 2017. Red raspberry and its anthocyanins: bioactivity beyond antioxidant capacity. Trends Food Sci. Technol. 66, 153–165.

Teng, H., Chen, L., Huang, Q., Wang, J., Lin, Q., Liu, M., Song, H., 2016. Ultrasonic-assisted extraction of raspberry seed oil and evaluation of its physicochemical properties, fatty acid compositions and antioxidant activities. PLoS One 11, e0153457.

Xiao, J.B., Högger, P., 2015. Stability of dietary polyphenols under the cell culture condition: avoiding erroneous conclusions. J. Agric. Food Chem. 63 (5), 1547–1557.

Xiao, J.B., Capanoglu, E., Jassbi, A.R., Miron, A., 2016. Advance on the flavonoid C-glycosides and health benefits. Crit. Rev. Food Sci. Nutr. 56 (S1), S29–S45.

Xiao, J.B., 2017. Dietary flavonoid aglycones and their glycosides: which show better biological significance? Crit. Rev. Food Sci. Nutr. 57, 1874–1905.

Xiao, J.B., 2018. Stability of dietary polyphenols: it's never too late to mend? Food Chem. Toxicol. 119, 3–5.

Xu, X.R., Yu, H.T., Yang, Y., Hang, L., Yang, X.W., Ding, S.H., 2016. Quercetin phospholipid complex significantly protects against oxidative injury in ARPE-19 cells associated with activation of Nrf2 pathway. Eur. J. Pharmacol. 770, 1–8.

Zhang, H., Wang, J., Liu, Y., Gong, L., Sun, B., 2016. Wheat bran feruloyl oligosaccharides ameliorate AAPH-induced oxidative stress in HepG2 cells via Nrf2 signaling. J Funct

Foods 25, 333–340.
Zhao, C., Yang, C.F., Wai, S.T.C., Zhang, Y.B., Portillo, M.P., Paoli, P., Wu, Y.J., Cheang, W.S., Liu, B., Carpéné, C., Xiao, J.B., Cao, H., 2019. Regulation of glucose metabolism by bioactive phytochemicals for the management of type 2 diabetes mellitus. Crit. Rev. Food Sci. Nutr. 59 (6), 830–847.
Zhao, C., Yang, C.F., Liu, B., Lin, L., Sarker, S.D., Nahar, L., Yu, H., Cao, H., Xiao, J.B., 2018. Bioactive compounds from marine macroalgae and their hypoglycemic benefits. Trends Food Sci. Technol. 72, 1–12.
Zinellu, A., Sotgiu, E., Fois, A.G., Zinellu, E., Sotgia, S., Ena, S., et al., 2017. Blood global DNA methylation is decreased in non-severe chronic obstructive pulmonary disease (COPD) patients. Pulm. Pharmacol. Ther. 46, 11–15.
Zamora-Ros, R., Knaze, V., Rothwell, J.A., Hémon, B., Moskal, A., Overvad, K., et al., 2016. Dietary polyphenol intake in Europe: the European prospective investigation into cancer and nutrition (EPIC) study. Eur. J. Nutr. 55 (4), 1359–1375.