Protective Effect of Water Extract of Citrus Pomace against AAPH-Induced Oxidative Stress In Vitro in Vero Cells and In Vivo in Zebrafish

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ABSTRACT: Citrus pomace (CP) is a by-product occurred during juice or other products processing. The enormous amount of CP caused serious environmental issues. However, CP is rich in a variety of bioactive compounds. In the present study, a water extract of CP (CPW) was prepared from the by-product and the in vitro and in vivo antioxidant activities of CPW were investigated. The in vitro antioxidant activities of CPW were evaluated by measuring the free radical scavenging activity and protective effects against 2,2-azobis(2-amidinopropane) hydrochloride (AAPH)-induced oxidative stress in Vero cells. CPW scavenges 1,1-diphenyl-2-picrylhydrazyl, alkyl, and hydroxyl radicals at IC50 of 0.16±0.00, 0.31±0.01, and 0.86±0.02 mg/mL, respectively. In addition, CPW improved cell viability and scavenged intracellular reactive oxygen species (ROS) in AAPH-stimulated Vero cells in a dose-dependent manner. The in vivo antioxidant activities of CPW were investigated in a model of AAPH-induced zebrafish embryos. CPW significantly improved the survival rates and reduced heart-beat rates in AAPH-stimulated zebrafish. Furthermore, the intracellular ROS and cell death levels were remarkably decreased in CPW-treated zebrafish. Therefore, the present results indicated that CPW possesses potent in vitro and in vivo antioxidant properties and could be a potential ingredient used in food, pharmaceutical, and cosmetic industries.

Keywords: citrus pomace, water extract, oxidative stress, antioxidant activity, zebrafish

INTRODUCTION

Oxidative stress is an imbalance between intracellular reactive oxygen species (ROS) accumulation and a capacity of the biological system’s scavenging ROS or repairing the resulting damage. In aerobic organisms, ROS are a series of normal products generated during normal cellular metabolic processes such as hydrogen peroxide (H2O2), hypochlorous acid (HClO), and free radicals (1-3). In general, cells maintain a balance between ROS generation and scavenging under the normal physiological conditions (4). However, this balance can be broken by many natural factors including disease, aging, and personal habits such as smoking or drinking. An excess of ROS accumulation in cells can damage the cellular organelles and essential macromolecules including DNA, lipids, and proteins, which lead to various physiological disabilities as well as result in metabolic disorders (5-7).

Antioxidants are the molecules that inhibit the oxidation of other molecules. Both synthetic and natural antioxidants are able to scavenge free radicals and to inhibit oxidation processes (8). Natural compounds from plants and algae possess remarkably high antioxidant activity with low side effects. Therefore, investigating natural antioxidants has become a focus of functional food and pharmaceutical science. Currently, finding cheap, abundant, and renewable resources of antioxidant compounds that originate from the residues of agricultural industries has attracted more attention (9).

Citrus (Citrus unshiu) pomace (CP) is the residue remaining after citrus fruits are processed for juice or other products. Nearly 50% of citrus fruit material is disposed of during industrial citrus juice processing, and the enormous amount of CP causes serious environmental issues (10). However, CP is rich in a variety of phenols and flavonoids that possess various bioactivities including anti-
oxidant, anti-inflammatory, and anti-cancer (10-13). Therefore, investigating the bioactivities of CP will help utilize industry waste more efficiently and alleviate environmental pollution.

Our previous studies had investigated antioxidant effects of CP extracts processed by far-infrared irradiation, heat, and super-heated steam (14,15). However, the antioxidant activities of water extracts of CP has not been investigated so far. The objective of the present study is to evaluate the protective effect of water extract of CP against 2,2-azobis(2-amidinopropane) hydrochloride (AAPH)-induced oxidative stress in vitro in Vero cells and in vivo in zebrafish.

MATERIALS AND METHODS

Chemicals and reagents
Gallie acid, rutin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 5,5-dimethyl-1-pyrolin N-oxide, AAPH, α-(4-pyridyl-1-oxide)-N-ter-butyl nitrotrine, dimethyl sulfoxide (DMSO), 3-(4-5-dimethyl-2yl)-2-5-diphynyltetrasolium bromide (MTT), phosphate buffered saline (PBS), and 2',7'-dichlorodihydroxflurescin diacetate (DCFH-DA) were purchased from Sigma Co. (St. Louis, MO, USA). Roswell Park Memorial Institute-1640 (RPMI-1640) medium, fetal bovine serum (FBS), penicillin/streptomycin, and trypsin-ethylenediaminetetraacetic acid were purchased from Gibco-BRL (Grand Island, NY, USA). FeSO₄·7H₂O, H₂SO₄, and H₂O₂ were purchased from Fluka Chemie GmbH (Buchs, Switzerland). All other chemicals used in this study were of analytical grade.

Preparation of water extract of CP
CP (0.1 g) was extracted with 10 mL distilled water in a shaking incubator (100 rpm) in the dark at 25°C for 24 h. Then the extracts were centrifuged at 1,000 g for 15 min to remove the residue, and then the supernatant was filtered through Whatman No. 1 filter paper. After freeze drying, the water extract of CP was obtained and was filtered through Whatman No. 1 filter paper. After 1 h incubation, the cells were treated with various concentrations (25, 50, and 100 μg/mL) of CPW and incubated for 24 h. Then, MTT solution (100 μL, 2 mg/mL in PBS) was added to each well. After 3 h incubation, the supernatants were aspirated and the formazan crystals were dissolved in DMSO. The amount of formazan was determined by measuring the absorbance at 540 nm using a microplate reader (Synergy™ HT, BioTek® Instruments, Inc., Winooski, VT, USA).

Determination of cytotoxicity of CPW on Vero cells
The free radical scavenging activity of CPW was measured using an electron spin resonance (ESR) spectrometer (JES-FA; JEOL, Tokyo, Japan). DPPH, hydroxyl, and alkyl radical scavenging activities of CPW were determined using the methods described by Heo et al. (16).

Cell culture

Monkey kidney fibroblast cells (Vero cells, ATCC® CCL-81™, American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI-1640 medium containing 10% heat-inactivated FBS, penicillin (100 unit/mL), and streptomycin (100 μg/mL) at 37°C under a humidified atmosphere containing 5% CO₂. Cells were sub-cultured every 3 days and seeded in a 24-well plate at a concentration of 1 × 10⁵ cells/well for experiments.

Determination of the effects of CPW on AAPH-induced cytotoxicity and intracellular ROS generation in Vero cells
Vero cells were seeded and incubated for 16 h, and then treated with various concentrations (25, 50, and 100 μg/mL) of CPW. After 1 h incubation, AAPH (5 mM) was introduced to the cells. The viabilities of cells stimulated with AAPH or treated with CPW were measured by the MTT assay. Cells were seeded in a 24-well plate. After 16 h incubation, the cells were treated with various concentrations (25, 50, and 100 μg/mL) of CPW and incubated for 24 h. Then, MTT solution (100 μL, 2 mg/mL in PBS) was added to each well. After 3 h incubation, the supernatants were aspirated and the formazan crystals were dissolved in DMSO. The amount of formazan was determined by measuring the absorbance at 540 nm using a microplate reader (Synergy™ HT, BioTek® Instruments, Inc., Winooski, VT, USA).

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Nuclear staining with Hoechst 33342
To measure the apoptotic bodies formation induced by AAPH, a fluorescent probe dye Hoechst 33342 was introduced to the AAPH-stimulated Vero cells. Nuclear staining was performed using the procedure described by Fernando et al. (18). The apoptotic body was observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Maintenance of zebrafish

The adult zebrafish were purchased from a commercial market (Seoul Aquarium, Seoul, Korea). The fish were kept in 3 L acrylic tanks (10 fish per tank) and maintained at 28.5°C with a 14/10-h light/dark cycle. The fish were fed TetraMin flake food (That fish place, Lancaster, PA, USA) supplemented with live brine shrimp (Artemia salina) two times a day, 6 days a week. Embryos were...
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Table 1. Total phenolic content (TPC), total flavonoid content (TFC), and free radical scavenging activities of water extract of citrus pomace (CPW)

| Sample | TPC (g/100 g GAE) | TFC (g/100 g RE) | IC50 (mg/mL) |
|--------|------------------|------------------|--------------|
| CPW    | 3.35±0.00        | 4.07±0.73        | 0.16±0.00    |

The results are expressed as the mean±SE (n=3).

GAE, gallic acid equivalent; RE, rutin equivalent; DPPH, 1-diphenyl-2-picrylhydrazyl radical scavenging activity; Alkyl, alkyl radical scavenging activity; Hydroxyl, hydroxyl radical scavenging activity.

obtained from natural spawning that was induced by turning on the light in the morning. The collection of embryos was completed within 30 min.

Application of CPW and AAPH to the embryos

The embryos were collected and transferred to individual wells on a 12-well plate (15 embryos/group) at 7~9 h post-fertilization (hpf). The embryos were incubated with CPW (25, 50, and 100 μg/mL) for 1 h and then treated with 15 mM AAPH.

Measurement of the heart-beat rate, ROS generation, and cell death in zebrafish

The heart-beat rate of both atrium and ventricle of zebrafish was recorded at 48 hpf for 1 min under the microscope. AAPH-induced intracellular ROS generation and cell death in zebrafish were analyzed with a fluorescent probe dye, DCFH-DA and acridine orange staining, respectively. The zebrafish larva was stained with the fluorescent probe dye and observed under a fluorescence microscope (Olympus, Tokyo, Japan) at 72 hpf using the ImageJ program (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All experiments were conducted in triplicate and the data were expressed as the mean±standard error (SE). A one-way ANOVA test was used to compare the mean values of each treatment. Significant differences between the means of parameters were determined by the Tukey’s test. A P<0.05 was considered significantly different. The significance differences were established as *P<0.05 and **P<0.01 compared to the AAPH-treated group and ***P<0.01 compared to control group.

RESULTS

TPC, TFC, and the free radical scavenging activities of CPW

TPC and TFC of CPW is summarized in Table 1. The phenolic and flavonoid content of CPW is 3.35±0.00 g/100 g GAE and 4.07±0.73 g/100 g RE, respectively. CPW scavenged DPPH, alkyl, and hydroxyl radicals at the half maximal inhibitory concentration (IC50) of 0.16±0.00, 0.31±0.01, and 0.86±0.02 mg/mL, respectively (Table 1). These results show that CPW possesses strong free radical scavenging activity, especially for DPPH and alkyl radicals. Based on these results, AAPH was selected as the stimulator to induce oxidative stress in Vero cells and zebrafish to evaluate the in vitro and in vivo antioxidant activities of CPW.

Cytotoxicity of CPW

The cytotoxicity of CPW in Vero cells was determined by the MTT assay. The results show that the viabilities of cells treated with different concentrations of CPW were higher than 90% (Fig. 1A). This shows that CPW was not cytotoxic under these concentrations.

Protective effects of CPW against AAPH-induced oxidative stress

The protective effect of CPW against AAPH-induced cell death and intracellular ROS generation were measured by the MTT assay and DCF-DA assay, respectively. As the results show (Fig. 1B and C), the viability of cells treated with AAPH was significantly decreased, and the intracellular ROS level was increased. However, CPW remarkably improved cell viability and reduced intracellular ROS levels in AAPH-stimulated Vero cells in a dose-dependent manner.

Protective effect of CPW against AAPH-induced apoptosis

As shown in Fig. 2, AAPH significantly induced apoptotic body formation. However, the amount of apoptotic bodies was remarkably reduced in the CPW-treated cells. Furthermore, the numbers of apoptotic in the cells were decreased as the CPW concentration increased.

Survival rate and heart-beat rate of zebrafish

The survival rate and heart-beat rate of zebrafish were measured. As shown in Fig. 3, AAPH significantly reduced the survival rate and elevated the heart-beat rate of zebrafish. However, the survival rates of zebrafish pre-treated with CPW were significantly increased in a dose-dependent manner. In addition, the heart-beat rates of
Fig. 1. Cytotoxicity and effects of water extract of citrus pomace (CPW) on 2,2-azobis(2-amidinopropane) hydrochloride (AAPH)-stimulated Vero cells. (A) Cytotoxicity of CPW on Vero cells; (B) protective effect of CPW against AAPH-induced cell death; (C) protective effect of CPW against AAPH-induced intracellular reactive oxygen species (ROS) generation in Vero cells. Cell viability was measured by MTT assay and intracellular ROS level was analyzed by DCF-DA assay. The results are expressed as the mean±SE (n=3). The significant differences were established as **P<0.01 as compared to AAPH-treated group and ##P<0.01 as compared to control group.

Fig. 2. Protective effects of water extract of citrus pomace (CPW) against 2,2-azobis(2-amidinopropane) hydrochloride (AAPH)-induced apoptotic body formation in Vero cells. Apoptotic body formation was observed under a fluorescence microscope after Hoechst 33342 staining. Apoptosis levels were measured by ImageJ software. The results are expressed as the mean±SE (n=3). **P<0.01 as compared to AAPH-treated group and ##P<0.01 as compared to control group.
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Fig. 4. The protective effect of water extract of citrus pomace (CPW) against 2,2-azobis(2-amidinopropane) hydrochloride (AAPH) induced intracellular reactive oxygen species (ROS) generation in zebrafish. (A) Zebrafish under fluorescence microscope; (B) the levels of intracellular ROS. Intracellular ROS levels were evaluated using ImageJ software. The results are expressed as the mean±SE (n=3). *P<0.05 and **P<0.01 as compared to AAPH-treated group and ##P<0.01 as compared to control group.

Effects of CPW against AAPH-induced intracellular ROS generation in zebrafish

The intracellular ROS generation of AAPH-stimulated zebrafish was analyzed by detection of DCF-DA. As shown in Fig. 4, the intracellular ROS levels of the non-treated zebrafish with AAPH was referred as 100%, and the ROS levels of AAPH-stimulated zebrafish was 242.2%. Furthermore, ROS levels of CPW-treated zebrafish were significantly decreased in a dose-dependent manner (Fig. 4B).

Effects of CPW against AAPH-induced cell death in zebrafish

In order to evaluate the protective effects of CPW against AAPH-induced cell death in zebrafish, zebrafish were dyed with acridine orange, and the fluorescent intensities were measured. The results showed that CPW remarkably and dose-dependently reduced AAPH-induced cell death in zebrafish (Fig. 5).

DISCUSSION

Research suggests that overproduction and/or accumu-
tion of ROS are associated with oxidative stress and inflammatory responses (20-24). Oxidative stress plays an important role in human chronic diseases such as obesity, cardiovascular diseases, diabetes, and neurodegenerative diseases (25-29). Therefore, a ROS scavenger that is not only effective to scavenge ROS but is also non-toxic may be an ideal candidate to help prevent these diseases. Many synthetic compounds such as butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT), and natural compounds like vitamin C and E have been studied for their antioxidant activities (30-32). The toxicity of BHA and BHT has also been reported. Some reports suggest that BHA and BHT are tumor promoters (33,34). Because of the side-effects of synthetic antioxidants, natural compounds, especially from food ingredients, are thought to be safe antioxidants.

Natural compounds such as polyphenols, flavonoids, polysaccharides, and proteins possess strong antioxidant activities (35). Lee et al. (36) separated the polyphenol compound dieckol from Ecklonia cava and evaluated its protective effects against high glucose-induced oxidative stress. Ko et al. (20) purified peptides from flounder fish and determined their protective effects against AAPH-induced oxidative stress in Vero cells.

Citrus fruits are one of the most flavorful fruits in many parts of the world. The production of citrus fruits in the world is nearly 80 million tons per year. During beverage processing, the juice yield of citrus fruits is about half of the fruit weight (38). An enormous amount of by-products like CP are produced every year. Many researches have reported that CP is rich in phenolic acids and flavonoids that are bioactive (8,38-40). Our previous study investigated the effects of a super-heated steam process on the antioxidant contents of CP and evaluated the antioxidant activity of CP (14). However, the protective effects of water extract of CP against AAPH-induced oxidative stress in vitro in Vero cells and in vivo in zebrafish has not been reported. Therefore, we investigated the antioxidant activity of CPW in vitro in Vero cells and in vivo in zebrafish.

In the present study, CP was extracted with distilled water. The in vitro antioxidant activity of CPW was evaluated by measuring the free radical scavenging activities using ESR spectroscopy, and the protective effects against AAPH-induced oxidative stress in Vero cells were measured. The in vivo antioxidant activity of CPW was investigated in AAPH-stimulated zebrafish embryos. CPW contained 3.35±0.00 g/100 g GAE phenolic content and 4.07±0.73 g/100 g RE flavonoid content, and scavenged DPPH, alkyl, and hydroxyl radicals at the IC50 of 0.16±0.00, 0.31±0.01, and 0.86±0.02 mg/mL, respectively (Table 1). Our previous study reported that 70% ethanol extract of CP (CPE) contained 2.69±0.10 g/100 g GAE phenolic content and 1.27±0.46 g/100 g RE flavonoid content, respectively. In addition, CPW scavenged DPPH, alkyl, and hydroxyl radicals at the IC50 of 0.65±0.02, 0.33±0.02, and 0.67±0.01 mg/mL, respectively (14). These results show that CPW contains higher phenolic and fla-
vonoid contents and possesses stronger DPPH and alkyl scavenging activities than CPE. Further results indicated that CPW significantly improved cell viability and reduced intracellular ROS levels in AAPH-induced Vero cells in a dose-dependent manner (Fig. 1). As shown in Fig. 2, AAPH significantly induced apoptotic body formation in Vero cells. However, CPW remarkably reduced AAPH-induced apoptotic body formation in Vero cells in a dose-dependent manner.

Zebrafish is a popular model in toxicological and pharmacological studies. Zebrafish stimulated with AAPH was successfully used to evaluate the antioxidant activity in our previous studies (4,19,41). Thus, we selected AAPH-stimulated zebrafish to evaluate the in vivo antioxidant activity of CPW. As shown in Fig. 3, the survival rate was decreased by 46.67%, and the heart-beat rate was increased by 20.62% in the AAPH-stimulated zebrafish compared to the control group. The survival rates were improved by 6.66, 16.66, and 30%, and the heart-beat rates were decreased by 3.27, 8.68, and 16.15% in the zebrafish treated with 25, 50, and 100 µg/mL of CPW, respectively. Furthermore, AAPH significantly induced intracellular ROS generation and cell death in zebrafish (Fig. 4 and 5). However, the intracellular ROS levels and cell death were remarkably decreased in CPW-treated zebrafish in a dose-dependent manner. These results showed that CPW possesses protective effects against AAPH-induced oxidative stress in vivo and zebrafish.

In conclusion, CP is rich in phenolic and flavonoid compounds and possesses potent antioxidant activities for improving cell viability, reducing intracellular ROS level, and decreasing the apoptotic body formation in AAPH-stimulated Vero cells, as well as improving survival rate, reducing heart-beat rate, and decreasing intracellular ROS levels and cell death in AAPH-stimulated zebrafish. These results suggest that CP possesses in vitro and in vivo antioxidant properties and may be a potential ingredient that could be used in the food, pharmaceutical, and cosmetic industries.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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