Food Chemistry 138 (2013) 950–955

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Protective effect of marine algae phlorotannins against AAPH-induced oxidative stress in zebrafish embryo

Min-Cheol Kang [a,1], Seon Heui Cha [b,1], W.A.J.P. Wijesinghe [a], Sung-Myung Kang [a], Seung-Hong Lee [c], Eun-A. Kim [a], Choon Bok Song [a], You-Jin Jeon [a,d,*]

[a] Department of Marine Life Sciences, Jeju National University, Jeju 690-756, Republic of Korea
[b] School of Biology and Parker H. Petit Institute of Bioengineering and Bioscience, Georgia Institute of Technolgy, GA 30332, United States
[c] School of Medicine, Jeju National University, Jeju 690-756, Republic of Korea
[d] Marine and Environmental Research Institute, Jeju National University, Jeju 695-814, Korea

ARTICLE INFO

Article history:
Received 3 August 2012
Received in revised form 29 October 2012
Accepted 2 November 2012
Available online 12 November 2012

Keywords:
Zebrafish
In vivo model
Marine polyphenol
Oxidative stress
Phlorotannins

ABSTRACT

In this study the protective effect of phlorotannins, including phloroglucinol, eckol, dieckol, eckstolonol and triphloroethol A, isolated from brown algae *Ecklonia cava* was investigated against AAPH-induced oxidative stress toxicity in zebrafish embryos. Zebrafish embryos were exposed to AAPH and compared with other groups that were co-exposed with phlorotannins until 2-days post-fertilisation. All phlorotannins scavenged intracellular ROS and prevented lipid peroxidation and reduced AAPH-induced cell death in zebrafish embryos. Negative changes in morphological phenomena, such as pericardial oedema, yolk sac oedema, and growth retardation in zebrafish embryos exposed to AAPH were not observed in groups exposed to phlorotannins. These results clearly indicate that phlorotannins possess prominent antioxidant activity against AAPH-mediated toxicity and might be potential therapeutic agents for treating or preventing several diseases implicated with oxidative stress. This study provides a useful tool for examining the protective effect of antioxidants against AAPH-induced oxidative stress in an alternative *in vivo* model.

1. Introduction

Biological properties of natural products have attracted the attention of scientists for a long time and *in vivo* studies of particular biological activities have been demonstrated in various animal models (Kang et al., 2012). A model organism should carry technical and practical advantages for studying specific biological processes, effects and mechanisms. Recently, numerous advantages of the zebrafish model system have been shown in *in vivo* studies and zebrafish (*Danio rerio*) has emerged as a highly useful vertebrate model organism (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). Development in zebrafish is very similar to the embryogenesis in higher vertebrates, including humans. Zebrafish are well suitable for functional genomics studies (Driever et al., 1996). For a vertebrate model system, zebrafish offer the unique characteristics of being simple to maintain and also viable for the assessment of drugs and/or small molecules as well as cell studies (Choi et al., 2007).

Human diseases resulting from oxidative stress can be caused by free radicals and natural antioxidants can act as free radical scavengers (Chang, Wu, & Chiang, 2007). Free radical-mediated oxidative stress and antioxidants are widely discussed in many current research areas (Valko et al., 2007). Free radicals can cause damage of tissue by reacting with other chemicals in the body. Therefore, looking for functional materials that possess antioxidant activity has become a hot research subject (Lindmark-Mansson & Akesson, 2000).

Marine organisms have been proven to be a rich source of structurally novel and biologically active secondary metabolites (Heo et al., 2008). Marine algae have many phytochemicals with various bioactivities including antioxidant, anti-inflammatory and anticancer (Lordan, Ross, & Stanton, 2011). Marine algae are also known to be rich in vitamins, minerals, polysaccharides, proteins, and polyphenols (Kuda, Tsunekawa, Goto, & Araki, 2005).

Ecklonia cava (class, Phaeophyceae; family, Lessoniaceae; order, Laminariales, *E. cava) is* a brown alga which grows abundantly in the sub-tidal regions of Jeju Island, Korea. *E. cava* harbours a richer supply of total phenolic compounds, including phlorotannins, than other brown seaweeds (Heo, Park, Lee, & Jeon, 2005; Kim et al., 2006). These phenolic secondary metabolites, such as phloroglucinol (1,3,5-trihydroxybenzene), eckol (a trimer of phloroglucinol),

* Corresponding author at: Department of Marine Life Sciences, Jeju National University, Jeju 690-756, Republic of Korea. Tel.: +82 64 754 3475; fax: +82 64 756 3493.
E-mail address: Youjinj@jejunu.ac.kr (Y.-J. Jeon).
[1] These authors equally contributed to this work.

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http://dx.doi.org/10.1016/j.foodchem.2012.11.005

6,6-bieckol (a hexamer), dieckol (a hexamer), phlorofucofuroeckol (a pentamer) and triphlorethol-A, have been associated with a variety of biological activities (Kang et al., 2006). Recently, an expanding body of evidence revealed that *E. cava* has radical scavenging, matrix metalloproteinase inhibitory, antioxidative, anti-inflammatory, immunomodulatory, HIV-1 reverse transcriptase, and anti-asthmatic activities (Ahn et al., 2008; Jung et al., 2009).

Nevertheless, relatively little research regarding the *in vivo* efficacy of *E. cava* phlorotannins has been conducted thus far. This study evaluated the potential protective efficacy of five phlorotannins, dieckol (DK), eckstolonol (ES), eckol (EK), triphloroethol A (TA), and phloroglucinol (PG), on AAPH-induced oxidative stress in zebrafish embryo as an alternative *in vivo* model.

2. Materials and methods

2.1. Materials

The marine brown alga *E. cava* was collected along the coast of Jeju Island, Korea, between October 2007 and March 2008. The samples were washed three times with tap water to remove salt, epiphytes, and sand attached to the surface. They were then carefully rinsed with fresh water, and maintained in a medical refrigerator at −20 °C. Thereafter, the frozen samples were lyophilised and homogenised using a grinder prior to extraction.

2.2. Isolation of phlorotannins from E. cava

The phlorotannins were isolated as previously described by Ahn et al. (2007) with slight modifications. Briefly, the dried *E. cava* powder (500 g) was extracted three times with 80% MeOH and then filtered. The filtrate was evaporated at 40 °C to obtain the methanol extract. The extract was then suspended in distilled water and partitioned with ethyl acetate. The ethyl acetate fraction was mixed with celite. The mixed celite was dried and packed into a glass column, and eluted sequentially with hexane, methylene chloride, diethyl ether, and methanol. The diethyl ether fraction was further purified by Sephadex LH-20 column chromatography using a stepwise gradient chloroform/methanol (2/1 → 0/1) solvents system. The phloroglucinol, eckol, triphloroethol A, eckstolonol and dieckol were purified by high-performance liquid chromatography (HPLC) using a Waters HPLC system (Waters Corporation, Milford, MA) equipped with a Waters 996 photodiode array detector and C18 column (J'sphere ODS-H80, 150 × 20 mm, 4 μm; YMC, Kyoto, Japan) by stepwise elution with methanol-water gradient (UV range: 230 nm, flow rate: 0.8 ml/min). Finally, the purified compounds were identified by comparing their ^{1}H and ^{13}C NMR data with the literature. The chemical structures of the phlorotannins are indicated in Fig. 1.

2.3. Origin and maintenance of zebrafish

Adult zebrafish were obtained from a commercial dealer (Seoul Aquarium, Korea) and 10 fish were kept in a 3-L acrylic tank under the following conditions: 28.5 °C, with a 14/10 h light/dark cycle. Fish were fed three times a day, 6 days a week, with Tetramin flake food supplemented with live brine shrimps (*Artemia salina*). Embryos were obtained from natural spawning that was induced in the morning by turning on the light. Collection of embryos was completed within 30 min.

2.4. Waterborne exposure of embryos to phlorotannins and AAPH

From approximately 3 hour post-fertilisation (3 hpf), embryos (group = 25 embryos) were transferred to individual wells of a 24-well plate and maintained in embryo medium containing 1 ml of vehicle (0.1% DMSO) or 50 μM phlorotannins for 1 h. Then embryos were treated with 25 mM AAPH (2,2′-azobis-2-methyl-propanimidamide dihydrochloride) or co-treated with AAPH and phlorotannins for up to 120 hours post-fertilisation (120 hpf).

2.5. Measurement of heartbeat rate

The heartbeat rate of both atrium and ventricle were measured at 35 hpf to determine the sample toxicity (Choi et al., 2007). Counting and recording of atrial and ventricular contractions were performed for 3 min under a microscope, and results were presented as the average heartbeat rate per min.

2.6. Estimation of intracellular ROS generation and image analysis

Generation of ROS in zebrafish embryos was analysed using an oxidation-sensitive fluorescent probe dye, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA was deacetylated intracellularly by nonspecific esterase, which was further oxidised to the highly fluorescent compound dichlorofluorescein (DCF) in the presence of cellular peroxides (Rosenkranz et al., 1992). At 3–4 hpf, the embryos were treated with 50 μM phlorotannins and, 1 h later, 25 mM AAPH was added to the plate. After treating with 25 mM AAPH for 6 h, the embryo medium was changed and the embryos developed up to 2 dpf. The embryos were transferred into 96-well plates and treated with DCFH-DA solution (20 μg/ml), and the plates were incubated for 1 h in the dark at 28.5 °C. After incubation, the embryos were rinsed in fresh embryo medium and anaesthetised before visualisation. Individual embryo fluorescence intensity was quantified using a spectrofluorometer (Perkin–Elmer LS-5B, PerkinElmer Life And Analytical Sciences, Inc., Waltham, MA) and the images of stained embryos were observed using a fluorescence microscope, which was equipped with a CoolSNAP-Pro colour digital camera (Olympus, Tokyo, Japan).

2.7. Lipid peroxidation inhibitory activity and image analysis

Lipid peroxidation was measured to assess membrane damage according to a method described by Wang, Gong, and Zhou (2008). Morphological evaluation of the embryos was performed with diphenyl-1-pyrenylphosphine (DPPP; Dojindo Laboratories, Kumamoto, Japan) which is a fluorescent probe for detection of cell membrane lipid peroxidation. DPPP is non-fluorescent, but it becomes fluorescent when oxidised. At 3–4 hpf, the embryos were treated with 50 μM phlorotannins and, 1 h later, 25 mM AAPH was added to the plate. After treating embryos with 25 mM AAPH for 6 h, the embryo medium was changed and the embryos developed up to 2 dpf. The embryos were transferred into 96-well plates and treated with DPPP solution (25 μg/ml), and the plates were incubated for 1 h in the dark at 28.5 °C. After incubation, the embryos were rinsed in embryo medium and anaesthetised before visualisation. Individual embryo fluorescence intensity was quantified using a spectrofluorometer (Beckman DTX 800, Beckman Coulter, Inc., Fullerton, CA) and image of embryos were observed using a fluorescence microscope, which was equipped with a CoolSNAP-Pro colour digital camera (Olympus).

2.8. Measurement of oxidative stress-induced cell death in zebrafish embryo

Cell death was detected in live embryos using acridine orange staining, a nucleic acid selective metachromatic dye that interacts with DNA and RNA by intercalation or electrostatic attractions. Acridine orange stains cells with disturbed plasma membrane permeability so it preferentially stains necrotic or very late apoptotic