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Quinone Reductase Induction Activity of Phlorotannins Derived from *Eisenia bicyclis* in Hepa1c1c7 Cells

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Abstract

To assess the feasibility of phlorotannins from *Eisenia bicyclis* as cancer chemopreventative agents, we tested whether they induced quinone reductase (QR) in Hepa1c1c7 cells. The ethyl acetate (EtOAc) soluble fraction obtained from *E. bicyclis* exhibited a QR induction activity in Hepa1c1c7 cells. Successive column chromatography of the active EtOAc fraction resulted in the isolation of four phlorotannins. Their structures were elucidated using one- and two-dimensional nuclear magnetic resonance spectroscopic techniques and characterized as phloroglucinol (**1**), dioxinodehydroeckol (**2**), dieckol (**3**), and fucofuroeckol-A (**4**). Among these compounds, fucofuroeckol-A (**4**) showed moderate QR induction activity, and dioxinodehydroeckol (**2**) exhibited potent QR induction potency with 2.05 ± 0.04 fold induction at a concentration of 50 μM compared to the dimethyl sulfoxide solvent-treated control cells. However, phloroglucinol (**1**) and dieckol (**3**) exerted no detectable QR induction activity in Hepa1c1c7 cells. These results suggest that dioxinodehydroeckol could serve as a useful cancer chemopreventive chemical.

Key words: *Eisenia bicyclis*, Quinone reductase, Phlorotannins, Dioxinodehydroeckol, Fucofuroeckol-A

Introduction

Quinone reductase (QR), one of the representative phase II enzymes, is a flavoprotein that catalyzes the reduction and detoxification of electrophilic quinones and quinone derivatives. QR has been shown to protect mammalian cells from redox cycling, oxidative stress, and neoplasia by converting toxic quinines to hydroquinones and inducing other detoxifying enzyme such as glutathione-, glucuronidyl-, and sulfotransferase enzymes in response to xenobiotics, antioxidants, oxidants, heavy metals, and radiation (Talalay et al., 1995; Song et al., 1999; Jaiswal, 2000). Therefore, upregulation of QR is thought to be a useful biomarker of phase II metabolic activity and carcinogenic elimination (Moon et al., 2006). Measuring the QR induction activity in cultured Hepa1c1c7 cells is a simple screening method for detecting the capacity of compounds to act as potential chemopreventors (Prochaska, 1994). Hepa1c1c7 cells are a suitable model for studying chemoprotection because they maintain many characteristics of normal tissue including the capacity for carcinogen activation and metabolism in response to environmental, nutritional, and hormonal factors (De Long et al., 1986).

Eisenia bicyclis (Kjellman) Setchell is a perennial brown alga belonging to the family Laminariaceae distributed throughout the coastal areas of Korea and Japan. It is consumed as a foodstuff, along with *Laminaria japonica*, *Undaria pinnatifida*, and *Ecklonia stolonifera*. Many beneficial bioactivity effects have been studied in *E. bicyclis*, such as antioxidation, antidementia, anti-inflammation, and diabetic complication treatment effects (Whitaker and Carlson, 1975;

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Nakamura et al., 1996; Okada et al., 2004; Jung et al., 2010; Yoon et al., 2011). In particular, phlorotannins, the major metabolites of *Eisenia* and *Ecklonia* species, are polyphenolic compounds that have a variety of positive physiological effects such as antioxidation, antidementia, antihyperlipidemic, and angiotensin-converting enzyme I and tyrosinase inhibitory activities (Kang et al., 2004; Jung et al., 2006, 2010; Yoon et al., 2008, 2009).

Many polyphenols including flavonoids from terrestrial plants have been shown to modulate the phase II enzyme detoxification pathway, a mechanism implicated in their chemopreventive action (Lampe, 2003; Chen and Blumberg, 2008; Yang and Liu, 2009). However, the phase II enzyme activity of phlorotannins from *E. bicyclis* remains unclear.

In the present study, we investigated the QR induction activity of phlorotannins derived from *E. bicyclis* in Hepa1c1c7 cells.

Materials and Methods

Materials

Dried leafy thalli of *E. bicyclis* were purchased from Ullengdomall (Ullengdo, Korea) in March 2008 and stored in a freezer at -20°C until use. 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fetal bovine serum (FBS), digitonin, glucose 6-phosphate, menadione, nicotinamide adenine dinucleotide phosphate (NADP), flavin adenine dinucleotide (FAD), albumin from human serum, glucose 6-phosphate dehydrogenase, sodium dodecyl sulfate, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Alpha minimal essential medium (α-MEM), penicillin/streptomycin, and other materials required for culturing of cells were purchased from Gibco BRL/Life Technologies (Grand Island, NY, USA). All other reagents were of the highest commercial grade available.

Extraction and isolation of phlorotannins

The dried powder of *E. bicyclis* (3.0 kg) was extracted with methanol (MeOH, 10 L) three times at 70°C. The MeOH extract (624.3 g) was partitioned successively with organic solvents to yield dichloromethane (CH_2Cl_2, 170.5 g), ethyl acetate (EtOAc, 90.4 g), and *n*-butanol (*n*-BuOH, 100.8 g) fractions, in addition to a H_2O residue (262.6 g). The EtOAc fraction (90.4 g) of *E. bicyclis* was subjected to column chromatography over a Sephadex LH-20 with MeOH, yielding 10 subfractions (EF01-EF10) based on thin layer chromatographic analysis. The Sephadex LH-20 column chromatography of fraction 2 (EF02, 1.1 g) was conducted with MeOH to isolate compound **1** (520.0 mg). Compound **2** (55 mg) was purified from fraction 3 (EF03, 630 mg), with RP-18 (20-50% aqueous MeOH, gradient). EF06 (3.7 g) was separated over RP-18 eluting with 30-60% aqueous MeOH (gradient) to give five subfractions (EF0601-EF0605). EF0603 was further purified over Sephadex LH-20 (MeOH) to afford compound **3** (670 mg). The RP-18 column chromatography of EF07 (1.6 g) using 40-60% aqueous MeOH led to the isolation of compound **4** (35 mg).

Cell culture and cytotoxicity determination

Hepa1c1c7 cells (mouse hepatoma cells; American Type Culture Collection, Manassas, VA, USA) were cultured and maintained in α-MEM (Gibco BRL/Life Technologies) containing 100 μg/mL penicillin streptomycin and 10% FBS, and maintained at 37°C under a humidified atmosphere with 5% CO_2.

Cytotoxicity levels of the MeOH extract and its solvent soluble fractions of *E. bicyclis* and compounds isolated from the active EtOAc fraction on the above cell line were measured using the MTT method as described by Mosmann (1983) with slight modifications. Cell lines were cultured in 96-well plates at a density of 5×10^3 cells/well. After 24 h, cells were washed with fresh medium and were treated with different concentrations of samples. After incubation for 48 h, cells were washed two times with phosphate-buffered saline, and 100 μL of MTT solution (1 mg/mL) was added to each well. After 4 h, the MTT solution was removed from each well by suction, and then 100 μL of dimethyl sulfoxide (DMSO) was added to solubilize the formazan salt. The optical density was measured at 540 nm using a UV microplate reader (Tecan Austria GmbH, Groedig, Austria). Relative cell viability was calculated compared to the untreated group.

QR induction assay

The QR induction bioassay was modified from a method described previously (Prochaska and Santamaria, 1988; Zhang et al., 1992). Hepa1c1c7 murine hepatoma cells were grown in α-MEM without nucleosides or deoxyribonucleosides and supplemented with 10% FBS at 37°C in an atmosphere of 5% CO_2 in a humidified incubator. Cultured Hepa1c1c7 cells were plated in 12-well plates and decanted after 24 h incubation. Fresh medium and test samples dissolved in 10% DMSO were serially introduced. The final DMSO concentration in the medium was less than 0.5%. The cells were incubated for an additional 48 h. Sulforaphane was used as a positive control. The medium was removed, the cells were lysed with 50 μL of 0.8% (w/v) digitonin in 2 mM EDTA at pH 7.6 and incubated for 10 min at 37°C. The plates were then agitated on a shaker (120 rpm) for 10 min at room temperature. A 200 μL aliquot of reaction solution (7.5 mL of 0.5 M Tris HCl buffer, pH 7.4; 100 mg of albumin from human serum; 1 mL of 1.5% Tween 20 solution; 0.1 mL of 7.5 mM FAD; 1 mL of 150 mM glucose 6-phosphate; 90 μL of 50 mM NADP; 300 U of yeast glucose 6-phosphate dehydrogenase; 45 mg of MTT; 150 μL of 50

mM menadione [as an oxidant] in acetonitrile; and 140.16 mL distilled water added to a total volume of 150 mL) was added to lysed cells. Menadione solution was added just before the mixture was dispensed into the microtiter plates. Readings were made in triplicate for each sample at 590 nm. Total protein concentrations were determined in a duplicate set of plates using crystal violet staining and subsequently scanned at 490 nm (Prochaska et al., 1992). The specific activity of QR is defined as nmol MTT blue formazan reduced per min and per mg protein (De Long et al., 1986). Induction was expressed as the ratio of the specific activity of QR in the presence and absence of the test sample.

$$\text{Specific activity} = \frac{\text{Absorbance change of MTT/min} \times 3{,}345 \text{ nmol/mg of protein}}{\text{Absorbance of crystal violet}}$$

Here, 3,345 nmol/mg is the ratio of the proportionality constant determined for crystal violet and the extinction coefficient of MTT.

$$\text{Fold of induction (ratio)} = \frac{\text{Specific activity of the sample-treated group}}{\text{Specific activity of the DMSO-treated control group}}$$

Results and Discussion

Cancer chemoprevention has been recognized as the administration of chemicals or dietary components to interdict, suppress, or reverse the development of cancer in normal or preneoplastic tissue (Sporn and Newton, 1979; Wattenberg, 1985). As a new strategy for cancer chemoprevention, the induction of phase II drug-metabolizing enzymes like QR is a principal mechanism of protection against chemical stress and the initiation of carcinogenesis (Talalay et al., 1995). QR induction blocks the generation of mutagenic quinone metabolites and diminishes the covalent conjugation of oxygenated metabolites to microsomal proteins (Prochaska et al., 1985), and this enzyme maintains the capacity of the cells to survive the stress of oxidative metabolites (Talalay and Benson, 1982; Ysern and Prochaska, 1989; Forrest et al., 1990; Merk et al., 1991; Dicker and Cederbaum, 1993).

In the present study, we investigated the QR induction activities of the MeOH extract and its organic solvent fractions derived from *E. bicyclis* in Hepa1c1c7 cells. The cell viability of the tested extract and fractions were measured using Hepa1c1c7 cells, and these results are presented in Fig. 1. Among these, the MeOH extract, EtOAc, and H_2O fractions showed no significant cytotoxicity in the concentration range of 62.5-250 µg/mL, whereas the CH_2Cl_2 and *n*-BuOH fractions exhibited cytotoxicity on Hepa1c1c7 cells. As shown in Fig. 2, the EtOAc fraction resulted in a maximum of 1.75 ± 0.05 fold induction over the control in inducing QR activity in

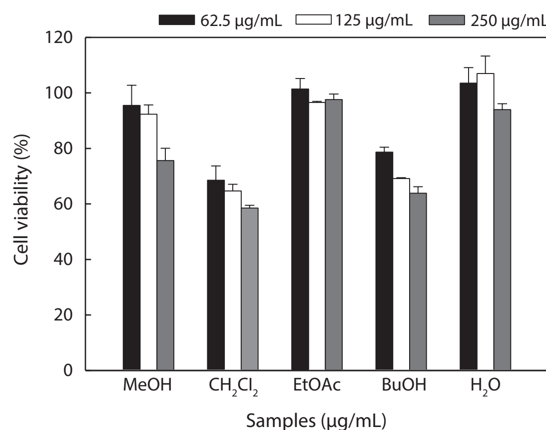

Fig. 1. The cell viability of the MeOH extract and its organic solvent fractions on Hepa1c1c7 cells.

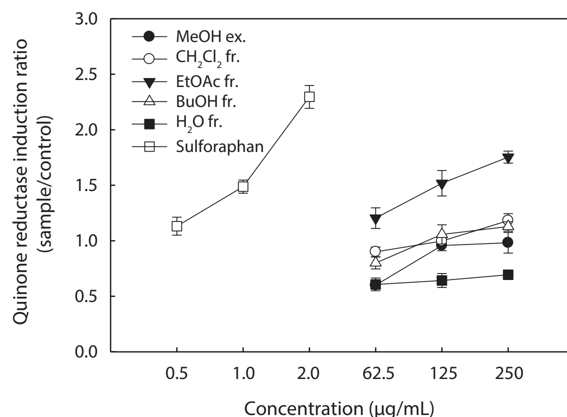

Fig. 2. Relative quinone reductase induction activity of the MeOH extract and its organic soluble fractions of *Eisenia bicyclis* in Hepa1c1c7 cells.

Hepa1c1c7 cells at a concentration of 250 µg/mL. However, the CH_2Cl_2 and *n*-BuOH fractions showed weak QR induction activities despite low cell viability at concentrations in the range of 62.5-250 µg/mL.

These results suggested that the active ingredients of *E. bicyclis* were included in the EtOAc fraction. Therefore, the most active EtOAc fraction was subjected to further chemical analysis, with repeated column chromatography over silica gel, Sephadex LH-20, and RP-18 gel, which led to the isolation of four phlorotannins. The structure identification of these compounds was verified by one-dimensional (^{1}H- and ^{13}C-nuclear magnetic resonance) and two-dimensional (heteronuclear multiple quantum correlation and heteronuclear multiple bond correlation) spectroscopic analyses, and by comparisons to published data (Kang et al., 2004). The chemical structure of the compounds was identified as phloroglucinol (**1**), dioxinodehydroeckol (**2**), dieckol (**3**), and fucofuroeckol-A (**4**) (Fig. 3).

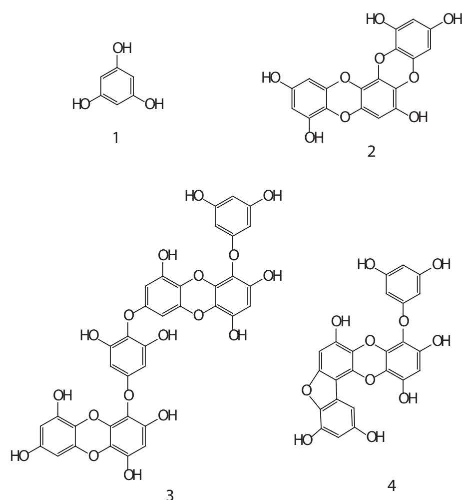

Fig. 3. The structure of compounds isolated from the EtOAc fraction of *Eisenia bicyclis*. **1**, phloroglucinol; **2**,dioxinodehydroeckol; **3**, dieckol; **4**, fucofuroeckol-A.

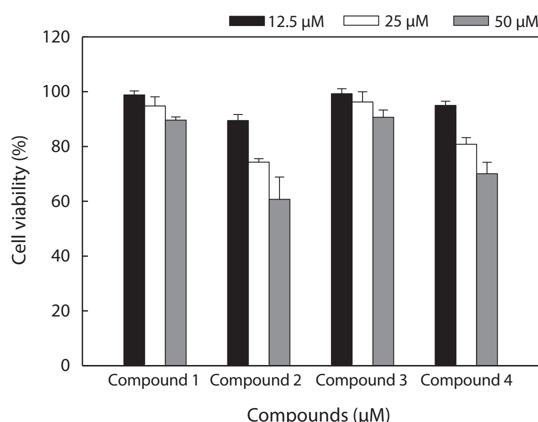

Fig. 4. The cell viability of phlorotannins derived from the EtOAc fraction of *Eisenia bicyclis* on Hepa1c1c7 cells.

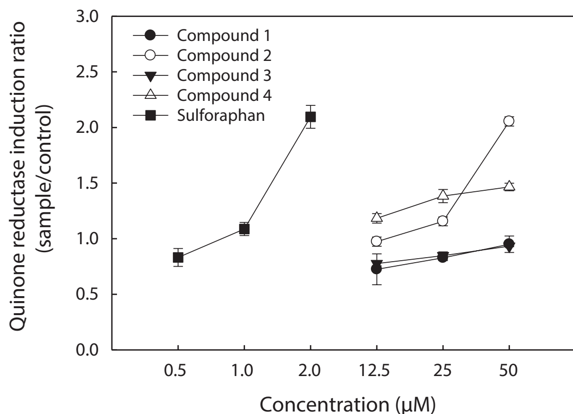

Fig. 5. Relative quinone reductase induction activity of phlorotannins isolated from the EtOAc fraction of *Eisenia bicyclis* in Hepa1c1c7 cells.

The cell viability and QR induction activity of the isolated compounds were also assessed as shown in Figs. 4 and 5, respectively. Of these, dioxinodehydroeckol and fucofuroeckol-A exhibited cytotoxicity on Hepa1c1c7 cells, whereas phloroglucinol (**1**) and dieckol (**3**) did not show cytotoxicity in the concentration range of 62.5-250 μg/mL.

Moreover, dioxinodehydroeckol (**2**) exhibited the most potent QR induction activities in Hepa1c1c7 cells, with a 2.05 ± 0.04 fold induction at 50 μM, compared to the DMSO treated control cells; however, it was lower than that of sulforaphane, which was used as a positive control. Fucofuroeckol-A (**4**) showed moderate QR induction activity with 1.46 ± 0.03-fold induction at 50 μM. Phloroglucinol (**1**) and dieckol (**3**) exerted no detectable QR induction activity in Hepa1c1c7 cells.

QR induction properties of phlorotannins were similar to those of representative QR active compounds derived from terrestrial organisms—quercetin, genistein, and resveratrol—which showed weak cytotoxicity on Hepa1c1c7 cells and exhibited potent QR induction activity (Yang and Liu, 2009). Although phlorotannins showed slightly lower QR induction activity than quercetin, genistein, and resveratrol, they were comparable (Yang and Liu, 2009). Sulforaphane, which was employed as a positive control, is known to have significant antitumor and anticarcinogenic enzyme induction effects (Zhang and Talalay, 1994; Zhang et al., 1994).

In conclusion, the EtOAc fraction from *E. bicyclis* and phlorotannins derived from the active EtOAc fraction showed potent QR induction activities in Hepa1c1c7 cells. Further studies are needed to explore the action mechanism of phlorotannins derived from *E. bicyclis* as chemoprevention/therapeutic agents.

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