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Induction of Quinone Reductase, an Anticarcinogenic Marker Enzyme, by Extract from *Chrysanthemum zawadskii* var. latilobum K.

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Abstract

Induction of NAD(P)H:(quinone-acceptor) oxidoreductase (QR) which promotes obligatory two electron reduction of quinones and prevents their participation in oxidative cycling and thereby the depletion of intracellular glutathione, has been used as a marker for chemopreventive agents. Induction of phase II enzyme is considered to be an important mechanism of cancer prevention. In our previous study, we assessed the quinone reductase QR-inducing activities of 216 kinds of medicinal herb extracts in cultured murine hepatoma cells, BPRc1 and hepa1c1c7 cells. Among the 216 herbal extracts tested in that study, extracts from *Chrysanthemum zawadskii* showed significant induction of QR. In this study, we examined QR-inducing activity of solvent fractions of the herbal extract. The dichloromethane fraction of the herb showed the highest QR induction among the samples fractionated with four kinds of solvents with different polarity. The fraction also significantly induced the activity of glutathione S-transferase (GST), one of the major detoxifying enzymes, at 4 μg/mL and 2 μg/mL in hepa1c1c7 and BPRc1 cells, respectively. In conclusion, dichloromethane-soluble fraction of *Chrysanthemum zawadskii* which showed relatively strong induction of detoxifying enzymes merits further study to identify active components and evaluate their potential as cancer preventive agents.

Key words: *Chrysanthemum zawadskii*, quinone reductase, glutathione S-transferase, cancer prevention, hepa1c1c7, BPRc1

INTRODUCTION

Induction of phase II enzymes such as quinone reductase (QR; NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2)), and glutathione S-transferase (GST) is closely associated with the prevention of chemical carcinogenesis (1). Accordingly screening for QR inducers from natural products has drawn much attention and diverse chemical agents such as oxidizable diphenols, isothiocyanates, and even hydrogen peroxide have been identified as QR inducers (2). The induction of phase II enzyme genes is regulated by their *cis*-acting antioxidant response element (ARE) or electrophile responsive element (EpRE) and xenobiotic responsive element (XRE). Transcriptional factor Nrf2 binds to and regulates transcription through the ARE/EpRE after heterodimerizing with one of the small Maf proteins (3).

QR induction through the XRE involves a ligand bound aromatic hydrocarbon receptor, AHR. This intracellular membrane of the PAS (Per, Arnt, Sim) family of proteins translocates to the nucleus upon binding to Arnt (4). The AHR/Arnt dimer interacts with the DNA sequences known as XREs to regulate QR expression. Both QR (NQO1) and CYP1A1 genes can be induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) and polycyclic aromatic hydrocarbons, while the induction of QR is largely dependent on the ability of bifunctional inducers such as dye, Sudan I and β-naphthoflavone to first undergo conversion to oxidative labile metabolites through a functional P450-dependent mechanism. Induction of phase I enzymes is generally believed to be harmful to cells since that enzymatic system is involved in bioactivation of procarcinogens into ultimate carcinogens (5). For instance, it has been known that benzo(a)pyrene is converted into ultimate carcinogen by the action of phase I enzyme (5). However, it seems that some compounds induce only QR without affecting the expression of phase I enzymes, and these are called

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monofunctional inducers. Accordingly, BPRc1 cells, a mutant defective in *Ah* receptor function, may be a good model system for screening the substances that induce phase II enzyme alone.

It is expected that there is an enormous variety of sources of QR inducers in the plant kingdom. Our previous study demonstrated that methanol extract of *Chrysanthemum zawadskii* was one of the strongest QR inducers among 216 kinds of herbal extracts tested (6). This study was to further characterize the active components in the herbal extract through fractionation according to polarity.

MATERIALS AND METHODS

Materials

All cell culture reagents and fetal bovine serum were obtained from Gibco BRL (Gaitherburg, MD, USA). Hepa1c1c7 and BPRc1 cells were from American Type Culture Collection (Rockville, MD, USA). *Chrysanthemum zawadskii* was kindly supplied by Euisung Medicinal Herb Experimental Station, Euisung, Kyungpook. All other chemicals were of reagent grade.

Preparation of herbal extract

A hundred grams of dry root of *C. zawadskii* was extracted with 1000 mL methanol at room temperature overnight, and the extract was evaporated *in vacuo* to give a residue. The ten grams of dry extract was suspended in 250 mL water and successively fractionated using hexane, dichloromethane, and butanol. Each fraction was evaporated to dryness below 40°C, and stored at -70°C, and redissolved in dimethylsulfoxide (DMSO) prior to use.

Cell culture

Hepa1c1c7 and its mutant (BPRc1) cells were plated at a density of 3×10^5 or 5×10^5 cells/plate in 10 mL of alpha-modified minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS). The cell culture was performed in a humidified incubator in 5% CO_2 at 37°C. Cells were cultured for 48 hrs, followed by exposure to various concentrations of herbal fractions for another 24 hrs.

Enzyme assays

QR activity was measured by a spectrophotometric assay in which the rate of reduction of 2,6-dichlorophenolindophenol was monitored at 600 nm (7). Briefly, cells were plated, grown, and exposed to different concentrations of herbal extract for 24 hr before being harvested. The cells were washed with ice-cold 0.15 M KCl-10 mM potassium phosphate (pH 7.4), removed from the plates by scraping with a rubber policeman, and disrupted for 5 sec using an ultrasonic cell disrupter (50W, Kontes, Vineland, NJ, USA). Cell homogenates were centrifuged at 12,000 rpm for 5 min in a microcentrifuge (VS-15000CFN11, Vision, Seoul, Korea). QR activity was assayed by measuring the rate of oxidation of 2,6-dichlorophenolindophenol at 600 nm in the assay system containing 25 mM Tris-HCl (pH 7.4), 0.7 mg crystalline bovine serum albumin at pH 7.4, 0.01% Tween 20, 5 μM FAD, 0.2 mM NADH, 0 or 10 μM dicoumarol, and 200 μL cell extract in a final volume of 3.0 mL. The QR induction was expressed as the ratio of the QR activity (2,6-dichlorophenolindophenol reduced/min/mg protein) in the presence and absence of the assay sample.

GST activity was assayed by the method described by Habig et al. (8), with 1-chloro-2,4-dinitrobenzene as the substrate.

Statistical analysis

Statistical significance of enzyme activity data was tested by analysis of variance, followed by Duncan's multiple range test, using SPSS software (SPSS Inc, Chicago, IL, USA). $p<0.05$ was the accepted level for statistical difference among treatment groups.

RESULTS AND DISCUSSION

A number of previous studies have suggested that induction of phase II detoxification enzymes such as QR is an important mechanism for cancer prevention (1-4). This study evaluated QR and GST induction activities of four solvent fractions from *Chrysanthemum zawadskii*. Dichloromethane-soluble fraction at the concentration of 8 μg/mL induced QR activity by 2.2-fold in BPRc1 cells, in which the enzyme is induced by monofunctional inducers only. The other samples including hexane, butanol, and water-soluble fractions did not cause any QR induction in the same cell model system (Table 1). Dichloromethane-soluble fraction of the herb also resulted in a dose-dependent increase of the enzyme activity in both BPRc1 and hepa1c1c7 cells (Fig. 1 and 2), suggesting

Table 1. Induction of QR activity by solvent fractions of *Chrysanthemum zawadskii* in BPRc1 cells[1)]

(unit: nmoles DCPIP reduced/min/mg protein)

Solvent fraction	QR activity
Control	124.8±9.9
Hexane	100.6±36.4
Dichloromethane	274.7±7.6**
Butanol	121.9±25.0
Water	119.9±18.6

[1)]BPRc1 cells (5×10^5 cells per plate) cultured in α-MEM for 48 hrs were exposed to each fraction (8 μg/mL) for 24 hrs prior to enzyme assay.

**Significantly different from control, $p<0.01$.

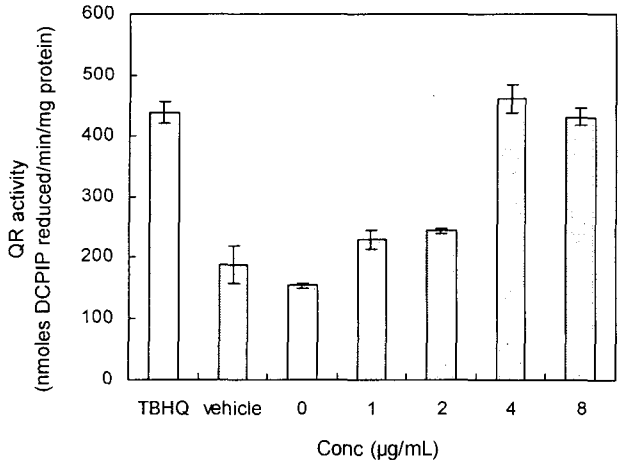

Fig. 1. QR inducing-activity of dichloromethane fraction of *Chrysanthemum zawadskii* in hepa1c1c7 cells. Cells (3×10^5 cells per plate) cultured in α-MEM for 48 hrs were exposed to various concentrations of each fraction (8 μg/mL) for 24 hrs prior to enzyme assay. *tert*-Butyl hydroquinone (TBHQ, 20 μM) was used as a positive control.

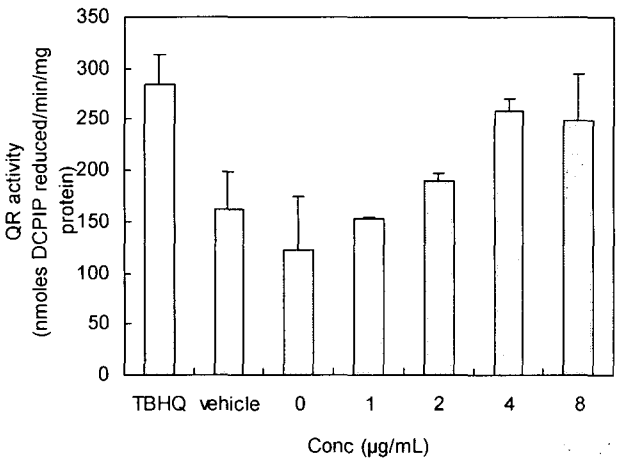

Fig. 2. QR inducing-activity of dichloromethane fraction of *Chrysanthemum zawadskii* in BPRc1 cells. Cells (5×10^5 cells per plate) cultured in α-MEM for 48 hrs were exposed to various concentrations of each fraction (8 μg/mL) for 24 hrs prior to enzyme assay. *tert*-Butyl hydroquinone (TBHQ, 20 μM) was used as a positive control.

that the sample contain monofunctional QR inducer(s). As monofunctional inducers have the least potential to bioactivate procarcinogens into ultimate carcinogens, they are considered to be good cancer preventive candidates. Furthermore, the potential of the sample to induce QR was comparable to TBHQ (20 μM), a well-known QR inducer.

Exposure to the dichloromethane fraction resulted in significant induction of GST activity in hepa1c1c7 (4 μg/mL) and BPRc1 (2 μg/mL) cells. Although GST induction by the dichloromethane fraction did not show a typical dose-response pattern, there was general tendency

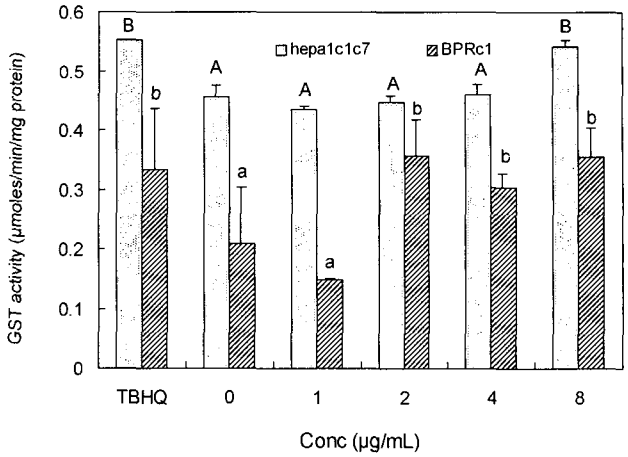

Fig. 3. GST inducing-activity of the dichloromethane fraction of *Chrysanthemum zawadskii* in hepa1c1c7 and BPRc1 cells. Cells (5×10^5 cells per plate for BPRc1 cells and 3×10^5 cells per plate for hepa1c1c7 cells) cultured in α-MEM for 48 hrs were exposed to various concentrations of each fraction (8 μg/mL) for 24 hrs prior to enzyme assay. *tert*-Butyl hydroquinone (TBHQ, 20 μM) was used as a positive control.

for GST to be proportionately enhanced with increasing concentrations of the sample. A slight decrease in GST activity at the highest concentration (4 μg/mL) in BPRc1 might be caused by the cytotoxicity of the sample.

The herb, *Chrysanthemum zawadskii* commonly known as Gu-Jul-Cho in Korea, has been used in traditional medicine to treat pneumonia, bronchitis, cough, common cold, pharyngitis, bladder-related disorders, gastroenteric disorders, and hypertension (9). Linarin is the main active compound in the herb and is recently reported to contain anticancer activity (10). However, our previous study showed that linarin did not have the potential to induce QR in BPRc1 cells, even though it caused significant QR induction in hepa1c1c7 cells (11). Therefore, there should be other compounds than linarin responsible for QR induction in *Chrysanthemum zawadskii.*

In conclusion, the dichloromethane fraction of *Chrysanthemum zawadskii* induced phase II enzymes in both BPRc1 and hepa1c1c7 cells and thus merits further study to isolate the enzyme inducer(s) and evaluate its cancer preventive activity in animal model.

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