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Induction of Detoxifying Enzyme by Sesquiterpenes Present in *Inula helenium*

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Running title: Induction of QR by sesquiterpenes

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Abstract

Our previous study showed that methanol extract of *Inula helenium* had potential to induce detoxifying enzymes such as quinine reductase (QR) and glutathione S-transferase activity. In this study we further fractionated the methanol extract into hexane, dichloromethane, butanol, and water-soluble fractions according to polarity. Hexane fraction showed the highest QR-inducing activity and also induced glutathione S-transferase in dose-dependent manner. Its potential to induce the reporter activity suggested the ARE-mediated mechanism of action in the induction of phase 2 detoxifying enzyme. The injection of hexane fraction of *Inula helenium* into mouse caused a significant increase of QR activity in liver, kidney, small intestine, and stomach. Sesquiterpenes, isolated from hexane soluble fraction, appeared to be major components responsible for QR induction. Among seven compounds tested in this study, alantolactone, isoalantolactone, and 5-epoxy alantolactone significantly induced QR activity in Hepa1c1c7 and BPRc1 cells. In conclusion, sesquiterpenes including alantolactone, isoalantolactone and 5-epoxyalantolactone present in *Inula helenium* merit further evaluation as cancer preventive agents.

Key words: sesquiterpenes, alantolactone, isoalantolactone, 5-epoxyalantolactone, quinone reductase, *Inula helenium*, cancer prevention

Introduction

Phytochemicals have drawn much attention due to their various bioactive activities including cancer prevention. There are two broad types of mechanisms which apply to cancer chemoprevention: antimutagenic and antiproliferative (1). Some antimutagens can enhance conjugation or binding of activated carcinogens to glutathione or other molecules to inactivate them and facilitate their removal. Quinone reductase (QR) has been used as a prototype for anti-carcinogenic phase 2 enzymes because of its widespread distribution in mammalian systems, large amplitude of inducer response and ease of measurement in murine hepatoma cells (2-4). In our previous study where more than 200 plant extracts were screened, methanol extract of *Inula helenium* was found to contain significantly high capacity to induce QR activity (5). We further

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fractionated the methanol extract of the plant and found that hexane fraction was most active in inducing detoxifying enzymes. A previous study also showed that sesquiterpenes present in the hexane fraction had various anti-tumor potential including anti-proliferative activity (6). In this study the potential of sesquiterpenes isolated from *Inula helenium* extract to induce detoxifying enzymes as an anti-carcinogenic biomarker and in vivo efficacy of sample extract in QR induction were evaluated.

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). All other chemicals were of reagent grade.

Inula helenium, widely used for treating microbial infection in Korean traditional medicine, was obtained from Uisung Herb Experimental Station (Uisung, Kyungpook).

Cell culture

Hepa1c1c7 and its mutant (BPRc1) cells were plated at density of 3×10^5 and 5×10^5 cells per 100 mm plate in 10 mL of α-MEM supplemented with 10% FBS, respectively. The HepG2-ARE-C8 cell line established in Dr Kong's lab by transfecting human hepatoma HepG2 cells with pARE-TI-luciferase construct was used for reporter assay (7). HepG2-C8 cells were maintained in modified F-12 medium supplemented with 10% FBS, 1.17 g/liter sodium bicarbonate, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% essential amino acids, and 0.1% insulin. Cells were normally starved overnight in 0.5% FBS-containing medium before treatment. The cells were normally incubated for 3~4 days in a humidified incubator in 5% CO_2 at 37°C. Cells were cultured for 48 hrs, exposed to various concentrations of sample for another 24 hrs, followed by biochemical assays.

Animal Experiment

ICR male mice weighing 33-36 g obtained from Daehan Experimental Animal Center Co. (Eumsung, Korea) were used. They were maintained at 22 (± 2 °C)

under a 12:12 h light/dark cycle and allowed free access to tap water and laboratory food (Chow, Samyang, Korea). To study the effect of repeated exposure, groups of five animals were administered vehicle or hexane fraction of *Inula helenium* extract (5 mg/kg) 3 times a week by i.p. injection. On the seventh day, the animals were sacrificed for examination of enzyme activities and GSH levels in their tissues (11). The stomach, small intestine, kidneys, lungs, and liver were immediately removed and stored at -70 °C until analysis. For enzyme analyses, a known weight of tissue was immediately homogenized in 4 volumes of buffer (0.15 M KCl and 0.25 M phosphate buffer, pH 7.25). The homogenates were centrifuged at 10,500g at 4 °C for 15 min, and then the resulting supernatant was centrifuged at 100,000g at 4 °C for 60 min to obtain a cytosolic fraction. Cytosolic fractions were stored at -70°C until analyses. Protein concentrations were measured according to the method of Lowry (12) using bovine serum albumin as the standard.

Biochemical assays

QR activity was measured by a spectrophotometric assay in which the rate of reduction of 2,6-dichlorophenolindophenol was monitored at 600 nm (8). Glutathinone-S-transferase activity was assayed by the method described by Habig (9), with 1-chloro-2,4-dinitrobenzene as a substrate. Total cellular GSH was determined by rate measurements in a recycling assay (10). In brief, cells were grown for 24 h in 96-well plates (10,000 per well for hepa1c1c7 and BPRc1 cells), exposed to serial dilutions of sesquiterpenes for 24 h, and finally lysed in 50 µl of 0.08% digitonin. One half of the wells were used for protein determination. The other half received 50 µl of ice-cold metaphosphoric acid (50 g/liter) in 2 mM EDTA to precipitate cellular protein. After 10 min at 4°C, plates were centrifuged at 1,500 × g for 15 min and 50 µl of the resulting supernatant fractions was transferred to the corresponding wells of a parallel plate. To each of these wells, 50 µl of 200 mM sodium phosphate buffer, pH 7.5, containing 10 mM EDTA, was added and GSH content was measured by rate measurements in a recycling assay. tert-butylhydroquinone (TBHQ, 20uM or 3.3 ug/ml), a known QR inducer, was used as a positive control.

Assay of reporter gene activity.

HepG2-C8 cells were plated in 6-well plates at a density of 10^5 cells/well (7). After overnight incubation, cells were cultured in fresh F-12 containing 0.5%

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FBS for 12 h before drug treatment. The luciferase activity was determined according to the protocol provided by the manufacturer (Promega Corp., Madison, WI). Briefly, after drug treatment, cells were washed twice with ice-cold PBS and harvested in reporter lysis buffer. The homogenates were centrifuged at 13,000 g for 5 min at 4°C. A 10 μl supernatant was assayed for luciferase activity using TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Luciferase activity was normalized against protein concentration.

Western blot

This was performed on cytosolic fractions prepared from cultured cells to estimate the level of QR according to a protocol described previously (4). Electrophoresis was carried out in a Bio-Rad Mini-Protein II Cell apparatus (Bio-Rad Co., Hercules, CA) using a discontinuous buffer system. Electroblotting was performed in a semi-dry blotting unit (Trans-BlotR SD Blotting Kit, Bio-Rad). Aliquots (about 3 μg of protein) of cytotolic fraction of cells was loaded onto 10% SDS-polyacrylamide gel, and transferred onto polyvinylidene difluoride (PVDF) membranes in transfer buffer (48 mM Tris, 39mM glycine, 20% methanol). The membrane was blocked with blocking buffer containing 5% skim milk in 0.1% Tween 20 in PBS (PBS/T). The antibody against QR (200 g/mL), a kind gift from Dr Anil Jaiswal at Baylor College of Medicine, was diluted by 1:1000 with dilution buffer (5% skim milk in PBS/T), added to the membrane, and incubated for 4 hrs at room temperature. After washing 6 times with PBS/T, blots were incubated in washing buffer (PBS/T) with a secondary antibody for 1 hr at room temperature, followed by washing 8 times with PBS/T. Washed blots were incubated with horseradish peroxidase-conjugated secondary antibody anti-rabbit IgG which was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and exposed onto a film. Antibody against rat β-tubulin was used as a loading control. For measuring the expression of Nrf2 protein, the PVDF membrane was blocked in 5% nonfat milk solution for 1 h at room temperature, then incubated overnight at 48C with an anti-Nrf2 primary antibody (1:1000 dilution).

Isolation and identification of sesquitepenes from Inula heleium

The root of the plant was freeze-dried and powdered, and subjected to methanol extraction. After removal of the solvent in rotary evaporator below 40^{o}C, the residue was partitioned with n-hexane, ethylacetate, butanol, and

water sequentially. Isolation and identification of sesquiterpenes from hexane fraction were performed as described previously (Konishi et al., 2003).

Statistical analysis

Statistical significance of enzyme activity and GSH content data was tested by analysis of variance, followed by Duncan's multiple range test, using SPSS software (SPSS Inc, Chicago, IL, USA). $P \leq 0.05$ means statistical difference among treatment groups.

Results

Effect of *Inula helenium* extract on phase 2 enzyme activity

Our preliminary study demonstrated that methanol extract of *Inula helenium* strongly induced quinone reductase activity, one of well-known biomarkers for chemopreventive potential. We further fractionated methanol extract according to polarity using hexane, dichloromethane, butanol, and water as solvent. Among them hexane fraction showed the strongest QR-inducing activity, increasing QR activity by 2 –fold at 2 μg/mL in BPRc1 cells which lacks ARNT and are used for screening monofunctional inducers (Fig. 1). Hexane fraction of *Inula helenium* extract also induced the enzyme activity in a dose-dependent manner in both hepa1c1c7 and BPRc1 cells as shown in Fig 3. A slight drop in QR activity at the concentration of 4 μg/mL might be caused by toxicity of the extract. Hexane-soluble fraction also induced glutathione S-transferase activity in both hepa1c1c7 and BPRc1 cells in a dose-dependent manner while induction rate was more prominent in wild type murine hepatoma cells than its mutant BPRc1 cell line (Fig.4). Meanwhile, intracellular glutathione level was not significantly modulated by hexane fraction of the methanol extract of the plant (Fig. 5). Meanwhile, QR activities in the stomach, small intestine, and liver showed significant induction in mouse injected i.p. with hexane fraction (5 mg/kg, 3 times a week) as shown in Table 1.

Dose-Response of on the Expression of pARE-TI-Luciferase

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We investigated the expression of ARE-mediated gene expression via the activity of the luciferase reporter gene in the cytosol of the HepG2-C8 cells. Hexane fraction from *Inula helenium* extract was added to HepG2-C8 cells at the indicated concentrations as described in Fig. 6. As shown in Fig. 6, the luciferase activity was increased in a dose-dependent manner in the range of 0.5 to 2 ug/ml. However, at the concentration higher than 4 ug/ml luciferase activity was dropped to control level probably due to cytotoxic effect of the sample. This result suggests that QR induction by the sample was mediated via interaction between Nrf2 and ARE-sequence.

Induction of QR by sesquiterpenes isolated from *Inula helenium*

Seven kinds of sesquiterpenes were previously isolated and identified as cytotoxic components present in *Inula helenium* (6). As hexane soluble fraction showed strong QR induction activity and cytotoxicity in this study, we attempted to evaluate whether these compounds induce QR or not. As shown in Fig. 7 & 8, 5α-epoxyalantolactone, isoalantolactone, and alantolactone showed strong potential to induce QR activity. These compounds induced QR activity by more than 2-fold at 5 μM while 20 μM TBHQ, a positive control, caused 2-fold induction of the enzyme, suggesting that three sesquiterpenoid compounds are much more potent QR inducers than TBHQ. Furthermore, compounds appear to be monofunctional inducer since they induced QR in BPRc1 cells which lack ARNT (13).

Discussion

Our preliminary study showed that methanol extract of *Inula helenium* (Compositae) has strong potential to induce quinone reductase. In this study we further fractionated the methanol extract using hexane, dichloromethane, butanol, and water, and evaluated QR-inducing activity of each fraction. Hexane-soluble fraction caused the highest induction of the enzyme. As hexane-soluble fraction of *Inula helenium* contains some sesquiterpenes which have potential to regulate redox state in cells (14), we tested the QR-inducing activity of sesquiterpenes isolated from the plant. Three compounds, 5-epoxyalantolactone, alantolactone, and isoalantolactone induced significantly

QR activity, compared to the other sesquiterpenes. Sesquiterpenes used in this study were previously shown to contain strong anti-proliferative activity against MK-1, HeLa, and B16F10 (6). Furthermore, sesquiterpenes reported to have strong cytotoxicity also showed relatively significant QR inducing activity. Thus, sesquiterpenes with high QR induction potential may generate high levels of free radicals, which was accompanied by robust redox cycling, oxygen consumption and induction of apoptosis (15). QR gene expression is induced in response to antioxidants, xenobiotics, oxidants, heavy metals, UV light, and ionizing radiation. Also QR induction is a kind of an electrophile and /or oxidative stress induced cellular defense mechanism that includes induction of many genes. Although the mode of action of *Inula helenium* extract in increasing QR activity, the component(s) appears to interact with ARE sequence of QR gene to activate its transcription and thereby enhance the enzyme activity. And major components responsible for QR induction in the herbal extract, especially in hexane fraction, appear to be sequiterpenes. However, it is still not clear whether the sesquiterpenes could be absorbed in gastrointestinal tract because oral feeding did not induce QR significantly although i.p. injection was effective.

In conclusion, sesquiterpenes present in *Inula helenium*, in particular, alantolactone and isoalantolactone, appear to be strong QR inducing agents, suggesting that sesquiterpene components present in the herb have good ant-carcinogenic potential.

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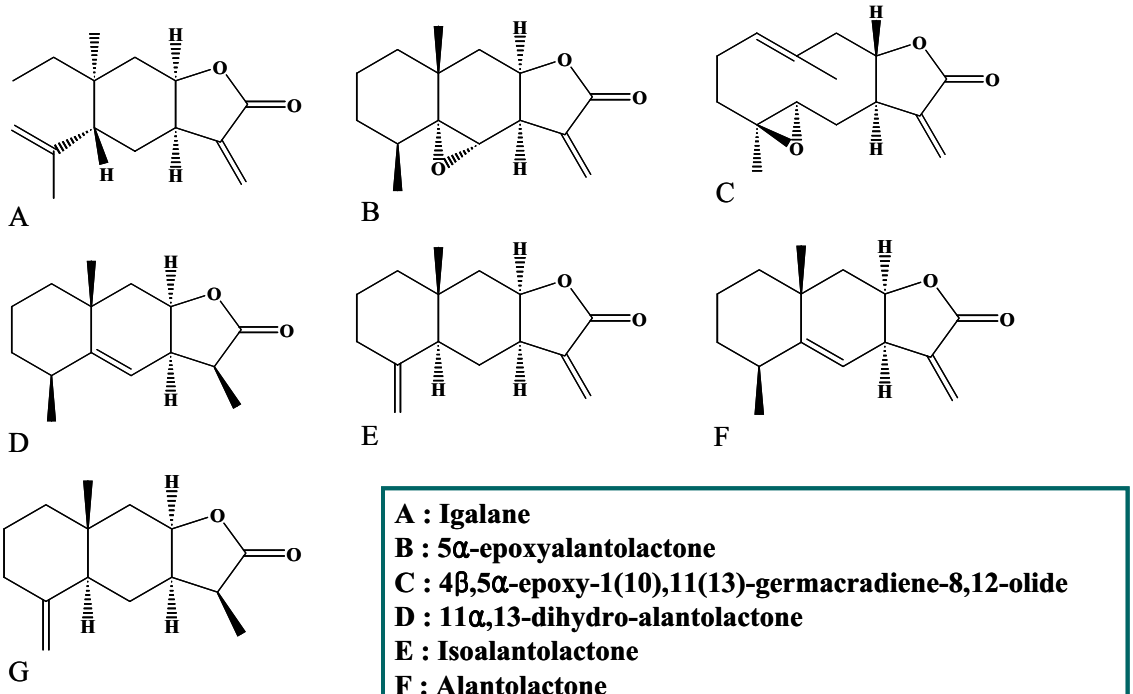

Fig. 1. Structure of sequiterpenes used in this study

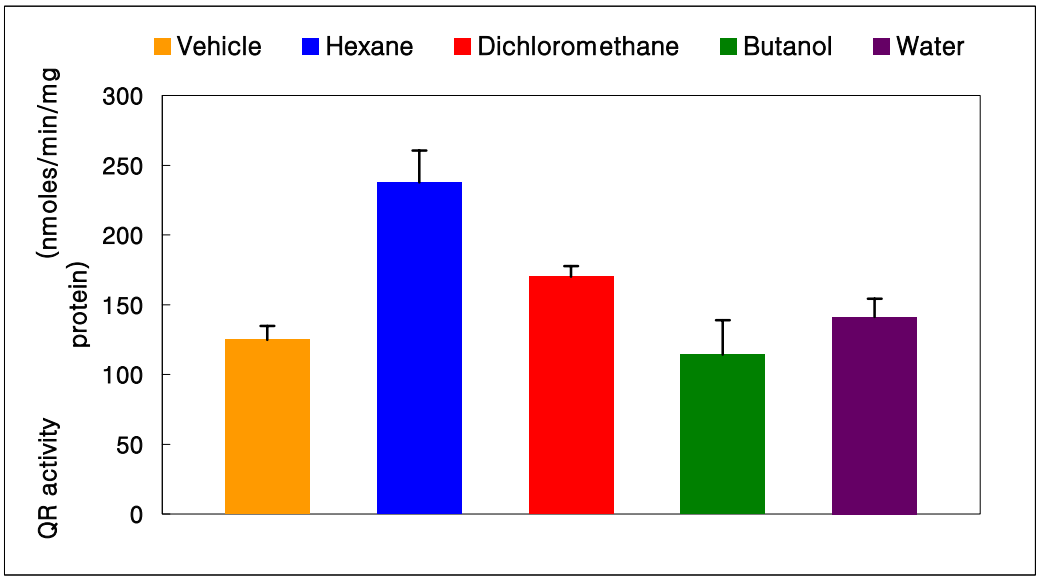

Fig. 2. QR activities of solvent fractions from methanolic extract of *Inula helenium* in Hepa1c1c7 cells and BPRc1 cells.

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Table 1. Change of QR activities in mice organs by injection of hexane fraction of methanolic extract of *Inula helenium*

	QR activity (nmoles DCPIP reduced/min/mg protein)				
	liver	**lung**	**kidney**	**small intestine**	**stomach**
Control	21.9±2.90	56.99 ± 11.23	119.30 ± 7.52	110.87 ± 13.64	216.99 ±160.81
hexane fraction (5mg/kg)	27.02 ±3.86*	63.42 ±8.08	114.76 ± 10.52	127.99 ± 11.06*	805.93 ± 232.22*

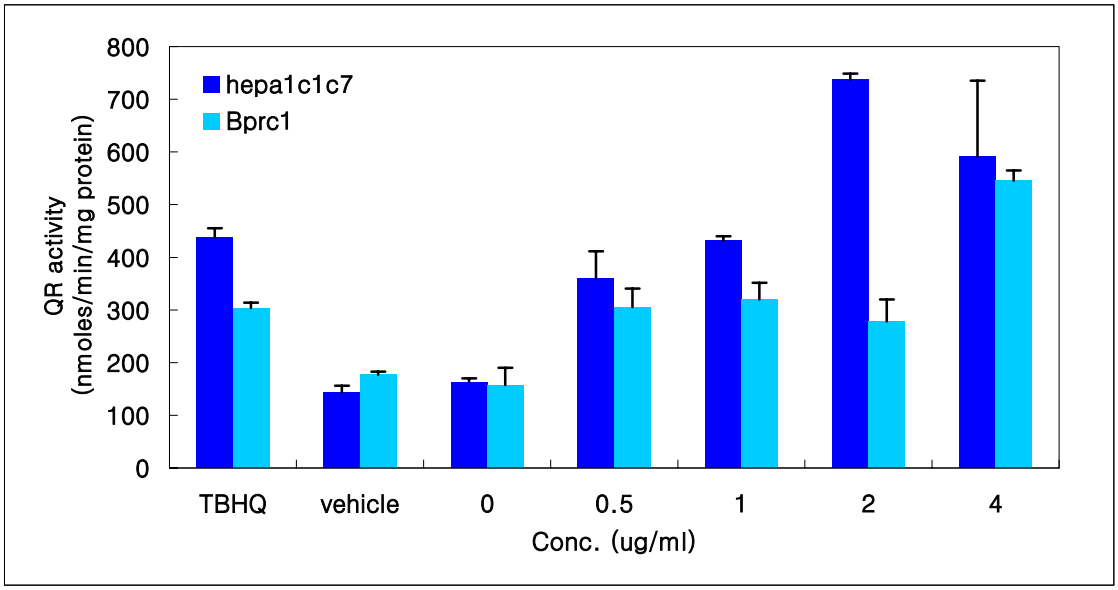

Fig. 3. QR activities in mouse hepatoma cells exposed to various concentrations of hexane fraction of methanolic extract of *Inula helenium.*

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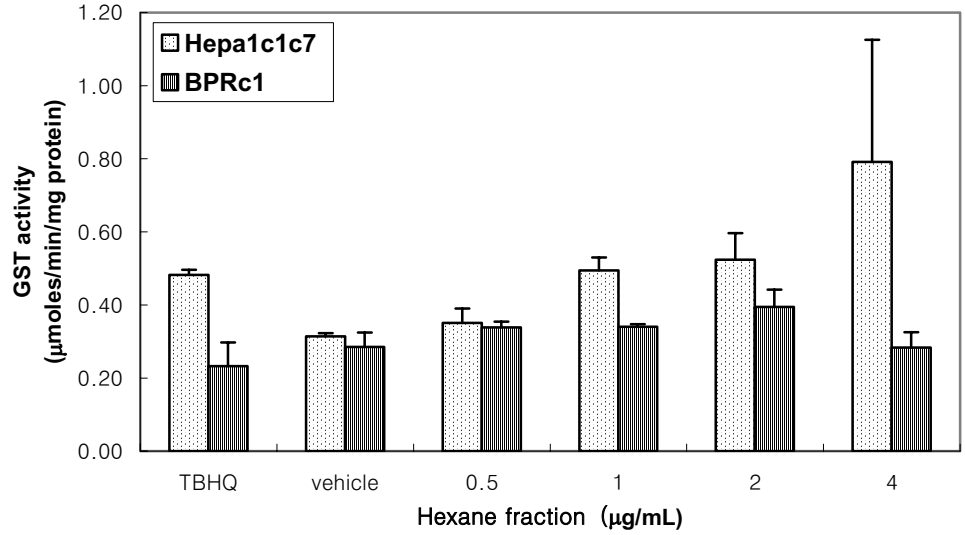

Fig. 4. GST activities in mouse hepatoma cells exposed to various concentrations of hexane fraction of methanolic extract of *Inula helenium.*.

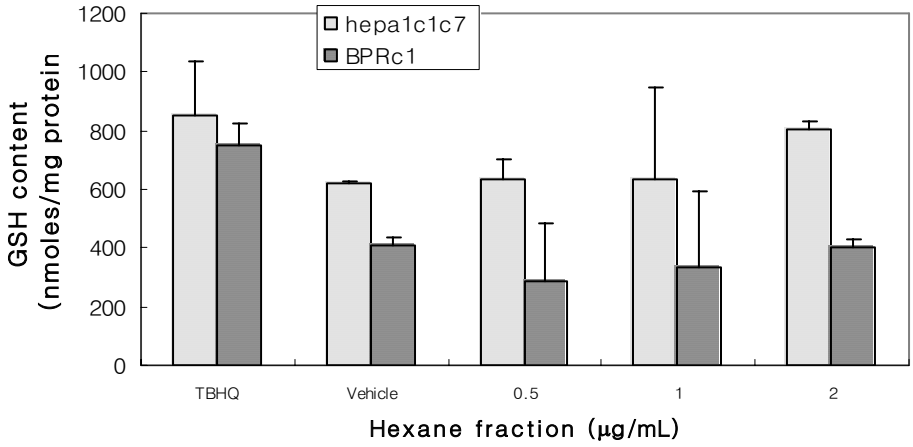

Fig. 5. GSH contents in mouse hepatoma cells exposed to various concentrations of hexane fraction of methanolic extract of *Inula helenium*.

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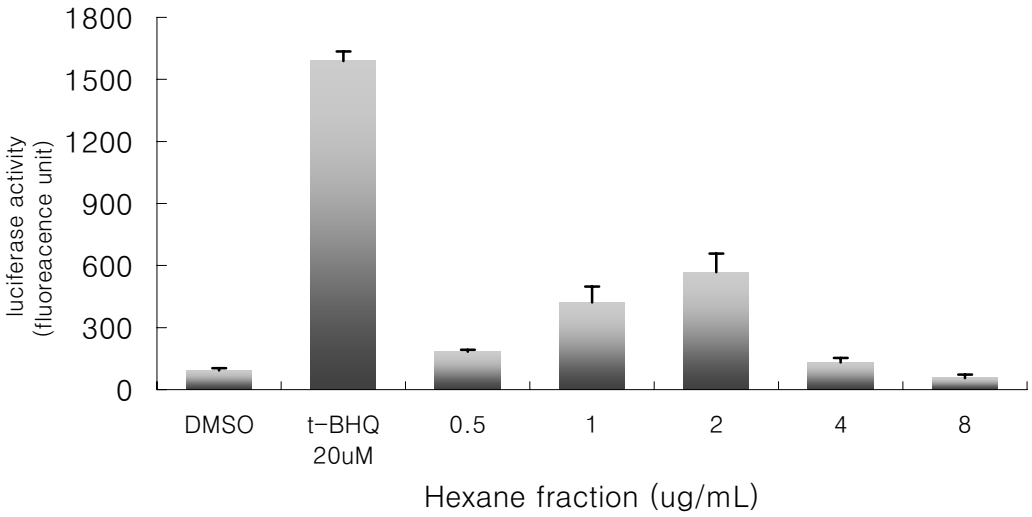

Fig. 6. Induction of ARE-luciferase by hexane fraction of methanolic extract of *Inula helenium* in HepG2-C8 cells.

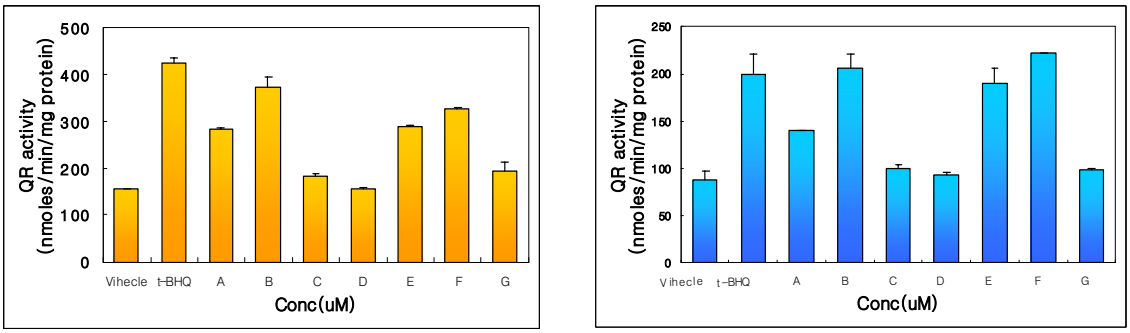

Fig. 7. Change of QR activity by sesquiterpenes isolated from *Inula helenium* in murine hepatoma cell lines. A, ignalane; B, 5-α-epoxyalantolactone; C, 4β,5α-epoxy-1(10),11(13)-germacradiene-8,12-olide; D, 11 α ,13-dihydroalantolactone; E, isoalantolactone; F, alantolactone; G, 11 α ,13-dihydro-isoalantolactone

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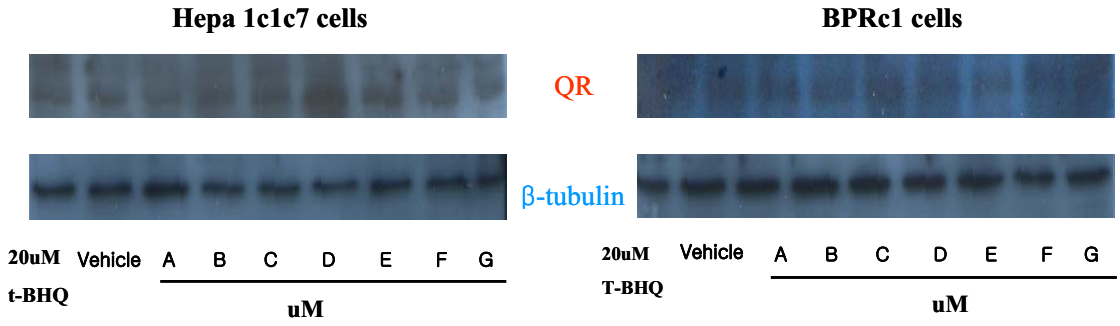

Fig. 8. Change of QR expression by sesquiterpenes isolated from *Inula helenium* in murine hepatoma cell lines.