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Original Article

Induction of Anticarcinogenic Enzymes by Dichloromethane-soluble Fraction of *Physalis alkekengi var. francheti Hort.* in Mouse Hepatoma Cells

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Abstract *Physalis alkekengi var. francheti Hort.* is known as an insecticide and traditional remedy for liver related diseases. Therefore, this study investigated the chemopreventive effects of extracts and several solvent fractions (*n*-hexane, dichloromethane, *n*-butanol, water) of *Physalis alkekengi var. francheti Hort.* First, their cytotoxicity and NQO1 activity were measured using an MTT assay, plus a quinone reductase [NAD(P)H dehydrogenase (quinone); NAD(P)H: (quinone acceptor) oxidoreductase, EC 1.6.99.2]-inducing activity assay was performed using cultured murine hepatoma cells (Hepa1c1c7) and its mutant cells(BpRc1). The reduction of electrophilic quinones by NQO1 is an important detoxification pathway and major mechanism of chemoprevention. When compared with the other solvent soluble fractions with different polarities, the dichloromethane fraction of *Physalis alkekengi var. francheti Hort.* showed a higher NQO1-inducing activity that was also dose-dependent. Moreover, the dichloromethane fraction of *Physalis alkekengi var. francheti Hort.* induced ARE-luciferase activities in HepG2-C8 cells that were generated by transfecting the ARE-luciferase gene construct, suggesting the Nrf2-ARE-mediated induction of anti-oxidative enzymes. In conclusion, the dichloromethane-soluble fraction of *Physalis alkekengi var. francheti Hort.* showed a relatively strong induction of detoxifying enzymes, thereby meriting further study to identify the active components and evaluate their potential as cancer preventive agents.

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Introduction

Physalis alkekengi L. (Solanaceae) is a popular plant in traditional Korean and Chinese folk medicine, and has been reported to have many ethnopharmacological properties, including antifungal, anti-cough, anti-inflammatory, analgesic, and febricide activities. Although the accumulation of alkaloids is characteristic to Solanaceae species, which makes the roots and aerial parts of *P. alkekengi* toxic, its fruit is indeed edible. Therefore, this paper investigates the antioxidant potential and phase 2 detoxifying enzyme-inducing activity of the *P. alkekengi* fruit. The dried fruit was extracted using 80% methanol, and its cancer preventive potential assessed by investigating its effect on the levels of phase 2 detoxifying enzymes.

NQO1, a typical anticarcinogenic marker enzyme, is expressed by activation of an antioxidant response element (ARE), a cis-element bound by a transcriptional activator Nrf2, similar to other detoxifying phase 2 enzymes. Therefore, the induction of NQO1 not only protects against quinone-mediated cytotoxicity, but also acts as a potential mechanism in the prevention of chemical carcinogenesis (Mehta & Pezzuto, 2002). An antioxidant response element (ARE) is present in the promoter region of genes encoding for phase 2 detoxification/antioxidant enzymes, such as heme oxygenase-1 (HO-1), NADPH quinone: oxidoreductase (EC 1.6.99.2) (NQO1, QR), and glutathione *S*-transferase. In unstressed states, Nrf2 is present in the cytoplasm in association with Kelch-like ECH-associated protein 1 (Keap1). Disturbance of the interaction between Nrf2 and Keap1, including covalent or oxidative modification of the cysteine thiols in Keap1 by electrophiles or oxidative stress, results in Nrf2 release and its translocation into the nucleus. The binding of Nrf2 to the ARE

sequence in genes encoding phase 2/antioxidant enzymes causes transcriptional activation of the relevant genes, promoting the removal of reactive oxygen species (ROS) or toxic chemicals. Many natural compounds, such as curcumin, caffeic acid phe-nethyl ester (CAPE), and sulphoraphane, are known to act as electrophiles in Nrf2/ARE activation (Balogun et al., 2003; Dinkova-Kostova et al., 2002; Prochaska et al., 1985; Zhang et al., 1992).

P. alkekengi var. francheti Hort. was previously found to induce the NQO1 enzyme and some other phase 2 detoxifying enzymes in a dose-dependent manner, suggesting its potential to exert anti-carcinogenic activity.

Materials and Methods

Materials

All the cell culture reagents and fetal bovine serum were obtained from Hyclone (Logan, UT, USA). The Hepa1c1c7 and BpRc1 cells were from the American Type Culture Collection (Rockville, MD, USA). All the other chemicals were of reagent grade.

Cell culture

The Hepa1c1c7 and its mutant (BpRc1) cells lacking ARNT (A*h* receptor nuclear transporter), which is only responsive to monofunctional NQO1 inducers, were plated at a density of 3×10^5 and 5×10^5 cells per 100 mm plate (Nunc, Rochester, NY) in 10 mL of α-MEM supplemented with 10% FBS, respectively. The HepG2-C8 cell line established in Dr. Kong's lab at Rutgers, The State University of New Jersey, by transfecting human hepatoma HepG2 cells with a pARE-TI-luciferase construct was used for the reporter assay (Kim et al., 2003). The HepG2-C8 cells were maintained in modified DMEM supplemented with 10% FBS, GlutaMax (Gibco No. 35050-061), and 0.5 mg/mL neomycin. The cells were normally incubated for 3~4 days in a humidified incubator in 5% CO_2 at 37°C.

Preparation of samples

The *P. alkekengi var. francheti Hort.* was obtained from the Daegu Medicinal Herb Market located in Daegu and extracted using ten volumes of methanol at 28°C for 24 h, followed by rotary evaporation to dryness. Next, the dried extract was suspended in water and separated using different solvents (*n*-hexane, dichloromethane, and *n*-butanol) (Figure 1). Each solvent soluble fraction was then evaporated under a rotary vacuum evaporator (Eyela, Rikakikai, Japan). In this experiment, the efficiency of the total methanolic extract was 39.5%. The fractions partitioned sequentially by *n*-hexane, dichloromethane, *n*-butanol and water soluble fraction accounted for 6.6%, 1.6%, 1.6%, and 5.4%, respectively, per 100 g dry sample (Table 1).

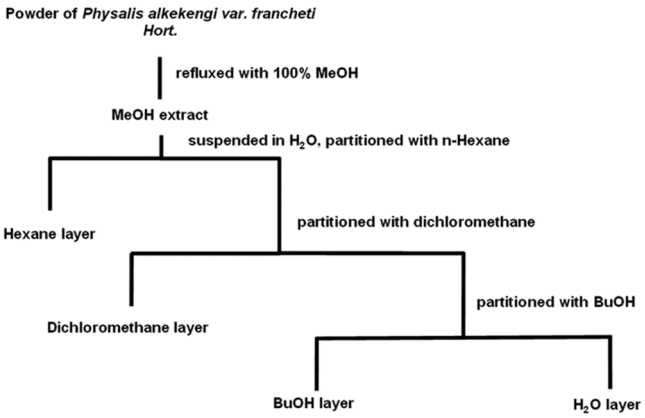

Figure 1. Extraction scheme of *Physalis alkekengi var. francheti Hort.*

Table 1. Extraction efficiency of several solvent fractions

Solvent fraction	Extraction efficiency (%)
Methanol extract	39.5
Hexane-soluble fraction	6.6
Dichloromethane-soluble fraction	1.6
Butanol-soluble fraction	1.6
Water-soluble fraction	5.4

MTT cytotoxicity assay

The Hepa1c1c7 or BpRc1 cells were plated at a density of 5×10^3 cells/well (200 μL) in 96-well plates that had been pre-incubated for 4 h. After incubating the cells for 72 h, a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) working solution was added to each well (200 μg/mL), and the plates were incubated at 37°C for another 4 h. Thereafter, the wells were completely removed and 150 μL of dimethylsulfoxide (DMSO) was added to each well to dissolve the crystalline formazan product. The absorbance in each well was measured at 540 nm using a TECAN sunrise plate reader (Tecan Trading AG, Switzerland). The treatment toxicity was quantified as the treatment mean absorbance as a percentage of the control mean absorbance, yielding a relative measure of the remaining viable, metabolically active cells after exposure.

Quinone reductase activity assay

The QR activity was measured using a spectrophotometric assay, where the rate of reduction of 2,6-dichlorophenolindophenol was monitored at 600 nm according to the modified method described by Benson and coworkers (1980). Briefly, the Hepa1c1c7 and BpRc1 cells were plated at a density of 3×10^5 and 5×10^5 cells/plate, respectively, in 10 mL α-MEM supplemented with 10% FBS. The cell culture was performed in a humidified incubator in 5% CO_2 at 37°C The cells were cultured for 48 h, followed by exposure to various sample concentrations for another 24 h before being harvested. The cells were then washed with ice cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4), collected from the plates by scraping with a

rubber policeman, and disrupted for 2 seconds 7 times using an ultrasonic cell distruptor (50W, Kontes, Vineland, NJ, USA). The cell homogenates were then centrifuged at 10,500×g for 5 min in a microcentrifuge (VS-15000CFN11; Vision, Seoul, Korea). The NQO1 activity was assayed by measuring the rate of oxidation of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm for 2 min in an assay system containing 250 mM Tris-HCl (pH 7.4), 70 mg bovine serum albumin, 0.1% Tween 20, 500 μM FAD, 2 mM NADH, and 200 μL of the cell extract in a final volume of 3.0 mL. The QR induction was expressed as 2,6-dichlorophenolindophenol reduced/min/mg protein. Plus, the protein concentration was determined using the modified method of Lowry (Peterson et al., 1977).

Assay of reporter gene activity

The HepG2-C8 cells were plated in 6-well plates at a density of 5×10^5 cells/well. After 16 h of incubation, the cells were cultured in fresh modified DMEM with high glucose containing 0.5% FBS for 12 h before the sample treatment. After culturing the cells for another 16 h in the presence of various sample concentrations, the cells were collected and the luciferase activity determined according to the protocol provided by the manufacturer (Promega Corp., Madison, WI) (Kim et al., 2003). Briefly, after the sample treatment, the cells were washed twice with ice-cold PBS and harvested in a reporter lysis buffer. The homogenates were then centrifuged at 12,000×g for 2 min at 4°C. Thereafter, 20 μL of the supernatant was assayed for its luciferase activity using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). The luciferase activity was normalized against the protein concentration.

Western blot analysis

This was performed on cytosolic fractions prepared from the cultured cells to estimate the level of detoxifying enzymes according to a previously described protocol (Kim et al., 2004; Im et al., 2007). The primary antibodies, including anti-NQO1, anti-HO1, anti-γ GCS, anti-β-tubulin, anti-Nrf2, and anti-SAM68, and the horseradish peroxidase-conjugated secondary antibody anti-goat or anti-rabbit IgG were all from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Statistical analysis

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

Results

Extraction rate of sample according to experimental setting

The dry fruit of *P. alkekengi var. francheti Hort* was powdered and extracted using pure methanol. The extraction efficiency was 39.5% on a dry basis. The extract was further fractionated using *n*-hexane, dichloromethane, *n*-butanol, and water, consecutively, with yields of 6.6, 1.6, 1.6, and 5.4%, respectively.

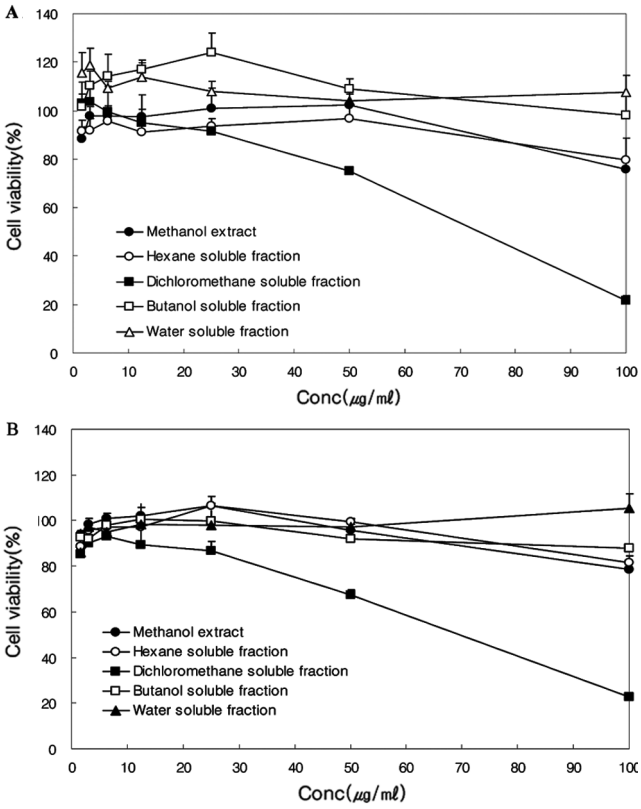

Figure 2. Antiproliferative effects of methanol extract and solvent fractions of *Physalis alkekengi var. francheti Hort.* (PA) against Hepa1c1c7 (A) and BPRc1 (B).

Anti-proliferative effects of extract and solvent fractions from *P. alkekengi var. francheti Hort.* in mouse hepatoma cells.

To examine the growth inhibitory effects of the extract and solvent fractions from *P. alkekengi var. francheti Hort*., the cell viability of mouse hepatoma cells (Hepa1c1c7 and its mutant BpRc1) was measured after 72 h of various dose treatments with the extract and solvent fractions of *P. alkekengi var. francheti Hort.* The dichloromethane-soluble fraction of *P. alkekengi var. francheti Hort.* was found to inhibit the growth of both cell types in a dose-dependent manner, with an IC_{50} of approximately 75 μg/mL. However, the other solvent soluble fractions of *P. alkekengi var. francheti Hort.* did not exhibit any growth inhibition activity, even at the maximum dose used (100 μg/mL) (Figure 2).

Induction levels of QR using extract and solvent fractions of *P. alkekengi var. francheti Hort.* in mouse hepatoma cells.

Each solvent fraction with a different polarity (*n*-hexane, dichloromethane, *n*-butanol, and water) was assayed for its ability to induce NQO1 in the BpRc1 cells. Among the solvent fractions,

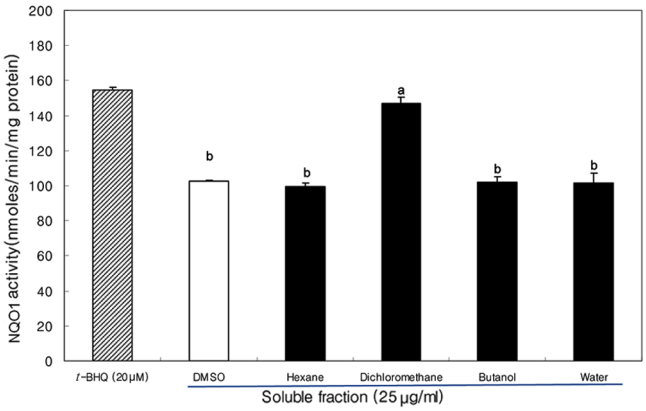

Figure 3. Induction of NQO1 activitiy by several solvent fractions of *Physalis alkekengi var. francheti Hort.* in BpRc1cells.

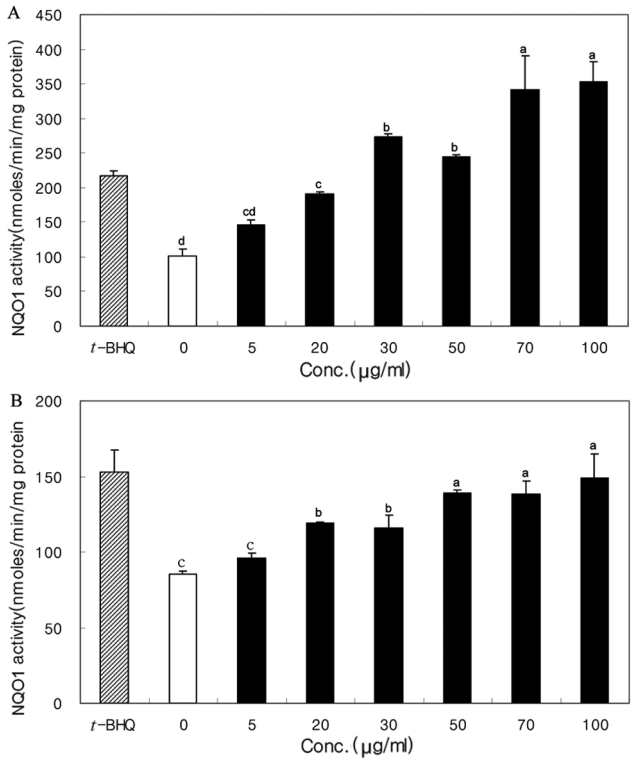

Figure 4. Induction of NQO1 activities by dichloromethane fraction of *Physalis alkekengi var. francheti Hort.* in Hepa1c1c7 (A) and BpRc1 (B) cells.

only the dichloromethane-soluble fraction showed a stronger NQO1 induction (Figure 3). In addition, the dichloromethane-soluble fraction also exhibited a dose-dependent NQO1 inducing activity in both the Hepa1c1c7 and mutant BpRc1 cells (Figure 4).

Effect of extract and solvent fractions of *P. alkekengi var. francheti Hort.* on pARE-TI-Luciferase expression

This study investigated whether the induction of NQO1 was mediated via an antioxidant response element (ARE) in the respective genes using HepG2-C8 cells transfected with a pARE-TI-luciferase construct. Thus, the HepG2-C8 cells were exposed to the dichloromethane-soluble fraction at final concentrations of 25 and 50 μM for 16 h. The ARE-luciferase activity of the HepG2-C8 cells exposed to the dichloromethane-soluble fraction (100 μg/mL) of PA increased 3.6-fold, while it increased 15.6-fold when exposed to 20 μM tert-butyl hydroquinone (*t*BHQ), a known NQO1 inducer (Figure 5).

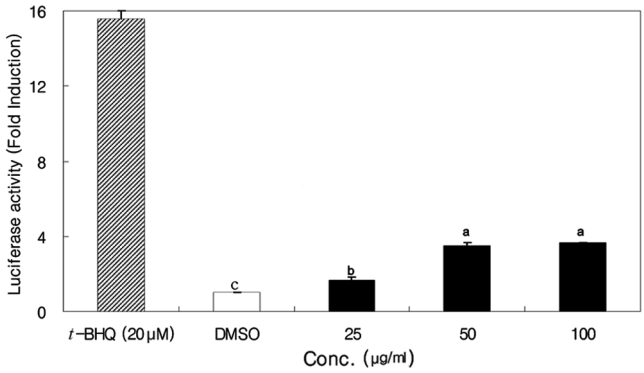

Figure 5. Induction of ARE-luciferase activities by dichloromethane fraction of *Physalis alkekengi var. francheti Hort.* in HepG2-C8 cells.

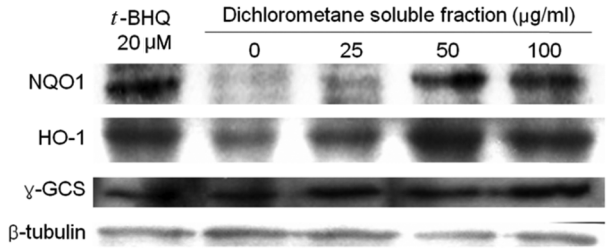

Figure 6. Expression of phase 2 detoxifying enzymes by dichloromethane-soluble fraction from methanol extract of *Physalis alkekengi var. francheti Hort.* in Hepa1c1c7 cells.

Effect of extract and solvent fractions of *P. alkekengi var. francheti Hort.* on phase 2 enzyme expression in hepa1c1c7 cells

To determine whether the dichloromethane-soluble fraction of *P. alkekengi var. francheti Hort.* affected the expression of certain phase 2 detoxifying enzymes, the hepa1c1c7 cells were exposed to the compounds at levels of 0, 25, 50, and 100 μg/mL for 24 h and a Western blot performed using antibodies against gamma-glutamylcysteine synthase (γGCS), quinone reductase (NQO1), hemeoxigenase 1 (HO-1). As shown in Figure 6, the expression of three phase 2 enzymes (NQO1, HO-1, γGCS) was up-regulated by the dichloromethane-soluble fraction in a dose dependent manner.

Discussion

The induction of phase 2 detoxifying and antioxidant enzymes by most monofunctional inducers has been reported to be mediated

by the interaction between NF-E2-related factor-2 (Nrf2) and an antioxidant response element (ARE) in the promoter region of phase 2 detoxifying and antioxidant enzyme genes. Considering that the dichloromethane fraction of the fruit extract stimulated luciferase activity in the reporter assay representing the ARE-binding activity of Nrf2, the fraction appears to liberate Nrf2 from the Nrf2-keap1 complex, and promote the nuclear migration and ARE-binding of Nrf2 to activate the transcription of antioxidant enzyme genes (Itoh et al., 1997; Kobayashi et al., 2009).

It is generally believed that the induction of NQO1 represents the anticarcinogenic potential of the sample. Therefore, there is possibility that the fruit extract may exert anticarcinogenic action through enhancing the expression of phase 2 detoxifying enzymes and thereby promoting the degradation of endogenous and exogenous carcinogens. In particular, the dichloromethane fraction showed a dose-dependent increase of NQO1 activity in both the wild-type mouse hepatoma hepa1c1c7 cells and its mutant BpRc1 cells, as shown in Figure 4. Furthermore, this increased NQO1 activity appeared to be mediated by an enhanced expression of the NQO1 protein, as shown in Figure 6. The protein levels of heme oxygenase-1 (HO-1) and gamma-glutamylcysteine synthetase (γGCS) were also increased by the dichloromethane fraction of the fruit extract. It is already well established that phase 2 detoxifying enzymes are regulated by the Nrf2 signaling pathway. That is, Nrf2 is present in a heterodimeric complex form with the Keap1 protein in the cytosol of cells in an unstressed condition. However, Nrf2 is released from the complex under stressful conditions, such as the entry of certain phytochemicals and oxidative stress, migrates into the nucleus, and binds with the ARE sequence in the promoter region of phase 2/antioxidant enzyme genes, activating their transcription. This study also confirmed that the dichloromethane fraction of the fruit extract stimulated the nuclear translocation of Nrf2 in a dose-dependent manner, suggesting that the sample regulated the phase 2 enzyme levels through the Nrf2 signaling pathway (Figure 7).

P. alkekengi (Bladder cherry, Chinese lantern, Japanese lantern, or Winter cherry; Japanese: hōzuki) is a relative of *P. peruviana* (Cape Gooseberry), easily identifiable by the larger bright orange to red papery covering over its fruit, which resembles Chinese lanterns. *P. alkekengi* varieties are grown for the decorative value of their brilliantly colored, swollen calyces. Its sepals represent rich sources of two important xanthophylls: zeaxanthin and β-cryptoxanthin (Pintea et al., 2005; Deineka et al., 2008). β-cryptoxanthin is one of the xanthophylls with provitamin A activity, a fact that gives it a greater biological importance and application perspectives (Pintea et al., 2005). The functional role of lutein/zeaxanthin in the human macula, including supporting evidence from epidemiological studies that higher consumption of these two carotenoids is associated with a lower risk of age-related macular degeneration, makes the topics of photo-protection and eye disease prevention by these pigments active areas of

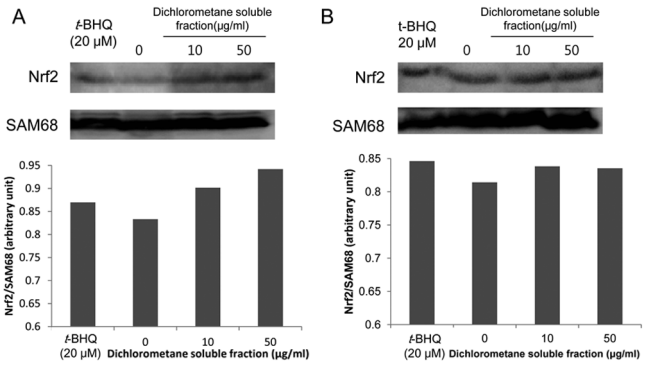

Figure 7. Translocation into nucleus of Nrf2 by dichloromethane soluble fraction from methanol extract of *Physalis alkekengi var. francheti Hort.* in Hepa1c1c7 and BpRc1 cells.

investigation (Rózanowska et al., 2010). However, it is not yet clear whether zeaxanthin and β-cryptoxanthin are responsible for the NQO1-inducing activity of the fruit extract.

Thus, when taken together, the current data demonstrated that the dichloromethane fraction derived from *P. alkekengi* induced QR in cultured cells, suggesting its potential as a cancer preventive agent.

Acknowledgments

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