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Indigofera suffruticosa Mill extracts up-regulate the expression of the π class of glutathione *S*-transferase and NAD(P)H: quinone oxidoreductase 1 in rat Clone 9 liver cells

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ABSTRACT

Because induction of phase II detoxification enzyme is important for chemoprevention, we study the effects of *Indigofera suffruticosa* Mill, a medicinal herb, on the expression of π class of glutathione *S*-transferase (GSTP) and NAD(P)H: quinone oxidoreductase 1 (NQO1) in rat Clone 9 liver cells. Both water and ethanolic extracts of *I. suffruticosa* significantly increased the expression and enzyme activities of GSTP and NQO1. *I. suffruticosa* extracts up-regulated GSTP promoter activity and the binding affinity of nuclear factor erythroid 2-related factor 2 (Nrf2) with the GSTP enhancer I oligonucleotide. Moreover, *I. suffruticosa* extracts increased nuclear Nrf2 accumulation as well as ARE transcriptional activity. The level of phospho-ERK was augmented by *I. suffruticosa* extracts, and the ERK inhibitor PD98059 abolished the *I. suffruticosa* extract-induced ERK activation and GSTP and NQO-1 expression. Moreover, *I. suffruticosa* extracts, especially the ethanolic extract increased the glutathione level in mouse liver and red blood cells as well as Clone 9 liver cells. The efficacy of *I. suffruticosa* extracts in induction of phase II detoxification enzymes and glutathione content implies that *I. suffruticosa* could be considered as a potential chemopreventive agent.

1. Introduction

Indigofera suffruticosa Mill, a herbaceous plant growing in tropical and subtropical areas, belongs to the Fabaceae species. In addition to being a natural indigo dye in the textile industry, *I. suffruticosa* has been used as a folk medicine to treat bacterial and fungal infections, inflammations, and epilepsy in Brazil (Wong et al., 1999; Leite et al., 2003, 2006). Moreover, accumulating data have shown the antimicrobial, gastroprotective, and embryotoxic effects of *I. suffruticosa* extracts (Leite et al., 2004, 2006; Vieira et al., 2007; Luiz-Ferreira et al., 2011). The alkaloidal fraction and the pure alkaloid indigo acquired from *I. suffruticosa* exhibited cytotoxic activity in murine tumor cell lines (Lopes et al., 2011). The water extract of *I. suffruticosa* effectively inhibited sarcoma 180 growth in albino Swiss mice (Vieira et al., 2007).

The π class of glutathione *S*-transferase (GSTP), a phase II detoxification enzyme, belongs to the glutathione *S*-transferase (GST) superfamily which catalyzes the conjugation of glutathione with electrophilic compounds. GSTs play a crucial role in protecting cells from oxidative stress and toxic foreign chemicals (Hayes et al., 2005). Different from other isoenzymes of the GST superfam-

Abbreviations: AP-1, activator protein-1; ARE, antioxidant response element; DMSO, dimethylsulfoxide; ERK1/2, extracellular signal-regulated kinase 1/2; GPEI, GSTP enhancer I; GST, glutathione *S*-transferase; GSTP, π class of glutathione *S*-transferase; HPLC, high-performance liquid chromatography; Keap1, Kelch-like ECH-associated protein 1; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide; NQO1, NAD(P)H quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2; PARP, poly ADP-ribose polymerase; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcriptase polymerase chain reaction; TRE-like, 12-*O*-tetra-decanoyl-phorbol-13-acetated response-like.

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ily, rat GSTP is hardly expressed in the normal liver, but it is highly induced during hepatocarcinogenesis (Satoh et al., 1985; Hayes and Pulford, 1995). Given this specific expression pattern, GSTP is widely used as a reliable determinant of cancer susceptibility (Satoh et al., 1985). The importance of GSTP expression in chemoprevention was proven in two transgenic rodent models which were deficient in GSTP1 expression and were more sensitive toward chemical carcinogens in mouse skin and rat liver (Henderson et al., 1998; Nakae et al., 1998). The GSTP enhancer I (GPEI), at 2.5 kilobases upstream of the GSTP-cap site, is essential to the induction of GSTP expression. GPEI has two 12-*O*-tetra-decanoyl-phorbol-13-acetated response-like (TRE-like) elements (Okuda et al., 1989, 1990) which are similar to the DNA sequences of antioxidant response element (ARE) and Maf recognition element (Kataoka et al., 1994). Both activator protein-1 (AP-1) and nuclear factor erythroid 2-related factor 2 (Nrf2) with MafK are able to bind to GPEI and then induce GSTP expression (Oridate et al., 1994; Ikeda et al., 2004).

Nrf2, a redox-sensitive transcription factor, can drive ARE-mediated gene expression including phase II detoxification enzymes (Hayes et al., 2010; Hu et al., 2010). Data have shown that Nrf2 knockout mice ($Nrf2^{-/-}$) have low expression of phase II detoxification enzymes, such as GSTP and NAD(P)H quinone oxidoreductase1 (NQO1) induced by phenolic antioxidants. Additionally, Nrf2 knockout mice were more vulnerable to tumorigenesis induced by oxidative stress and carcinogen diesel exhaust compared with wild type control (Chan and Kan, 1999; Aoki et al., 2001).

I. suffruticosa has been shown to hold promise as an alternative anticancer agent, but the underlying mechanism was not well described (Vieira et al., 2007; Lopes et al., 2011). Data from our laboratory showed that *I. suffruticosa* extracts increased ARE activation and a Nrf2-response gene, heme oxygenase-1 expression in RAW 264.7 macrophages (Chen et al., 2013). Activation of Nrf2-ARE pathway to induce expression of phase II detoxification enzymes could biotransform the endogenous compounds and xenobiotics to be more easily excretive forms and is considered crucial in chemoprevention (Jana and Mandlekar, 2009). Additionally, tripeptide glutathione, the most abundant non-protein thiol in mammalian cells, is important against the toxicity of xenobiotics and oxidants (Ballatori et al., 2009). In this present study, we assessed the effect of *I. suffruticosa* extracts on expression of GSTP and NQO-1 and the glutathione level and further explored the possible molecular mechanisms behind these effects.

3. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless specified otherwise. The rat liver cell line, Clone 9, was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan) and C57BL/6JNarl male mice were from the National Laboratory Animal Center (Taipei, Taiwan). Fetal bovine serum was from HyClone Laboratories (Losan, UT) and RPMI 1640 media and medium supplements for cell culture were obtained from Invitrogen Corporation (Carlsbad, CA). The specific antibodies for NOQ-1and Nrf2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), while the antibodies for GSTP and β-actin were from Transduction Laboratories (Lexington, KY) and Sigma–Aldrich, respectively. The antibodies against extracellular signal-regulated kinase 1/2 (ERK1/2), phosphorylated ERK1/2 and poly ADP-ribose polymerase (PARP) were from Cell Signaling Technology Inc. (Beverly, MA). Reagents such as enzymes, cofactors, and nucleotides for reverse transcriptase polymerase chain reaction (RT-PCR) were from Promega Corp. (Madison, WI). Oligonucleotide primer sequences of NQO-1 and β-actin for RT-PCR were selected by using Primer Select (DNASTAR, Madison, WI). Real-time quantitative PCR primers and TaqMan® Universal PCR Master Mix were from Applied Biosystems (Foster City, CA). The oligonucleotide primers for RT-PCR and electrophoretic mobility shift assay (EMSA) were synthesized by MDBio Inc. (Taipei, Taiwan). Luciferase Assay System, β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer and pSV-β-galactosidase control vector were got from Promega Co.

2.2. Preparation of extractions

Commercially available, air-dried *I. suffruticosa* stems were purchased from Tainan, Taiwan and were identified by Dr. Yi-Ching Li (Department of Pharmacology, Chung Shan Medical University). Voucher specimen was kept in our laboratory, at the Department of Nutrition, Chung Shan Medical University, Taichung, Taiwan, for further reference.

The powdered air-dried *I. suffruticosa* stems were extracted with 95% ethanol (plant material: solvent, 1: 13.3, w/v) for 2 h at 40 °C with continuous stirring. After filtration through a 0.22 μm pore size membrane (type GV, Millipore Corp., Bedford, MA), the ethanolic was removed at 37 °C under reduced pressure and was stored at −20 °C.

The air-dried *I. suffruticosa* stems were boiled in Milli-Q water (5 ml/g plant material) for 1 h and then were filtered through Whatman #1 filter paper. The resulting solution was freeze-dried (FD4, Heto Lab Equipment, Denmark) to yield the water extract and was stored at −20 °C.

2.3. High performance liquid chromatography/mass spectrometry (HPLC/MS) analysis

Acid hydrolysis of *I. suffruticosa* extracts by 6 N HCl were performed before HPCL analysis of phenolic compounds in *I. suffruticosa* extracts. The phenolic compounds were identified by their retention times, compared to those of the reference standards in HPLC systems and by the mass of the selected ions. The phenolic compounds in our in-house library were used as the reference standards (Yao et al., 2011). In the HPLC/MS system, an Agilent Zorbax Eclipse XDB-C8 column (5 μm, 150 × 3.0 mm i.d.) was used. The mobile phase consisted of solvents A (10 mM ammonia acetate containing 0.5% formic acid) and B (acetonitrile containing 0.5% formic acid). The gradient system was 10–90% B (0–45 min), 90–10% B (45–50 min), and 10% B (50–60 min). The flow rate was 0.6 ml/min. Data acquisition was via selected ion monitoring. Ions representing negative species of the compounds were selected, and peak areas were measured. The calibration curves of authentic standards were linear over the concentration range of 0.005–40 μg/ml with correlation coefficients of $\geqslant$0.99.

2.4. Cell culture

The rat liver cell line, Clone 9 (passage numbers between 24 and 30) was cultured in RPMI 1640 medium supplemented with 10 mM HEPES, 1×10^5 unit/l penicillin, 100 mg/l streptomycin, and 10% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. According to the preliminary data of our laboratory, water and ethanolic extracts of *I. suffruticosa at* 1000 μg/ml and 500 μg/ml, respectively, increased GSTP expression in Clone 9 liver cells. Based on this finding, 90% confluent cells were treated with the water extract (250–1000 μg/ml) and ethanolic extract (250–500 μg/ml) of *I. suffruticosa* or phenolic compounds of *I. suffruticosa* for indicated times. Inhibition of ERK 1/2 activity by PD98059 at a concentration of 20 μM was added 1 h before *I. suffruticosa* extract treatments. Cells treated with phosphate buffered saline (PBS) and dimethylsulfoxide (DMSO) alone were used as vehicle controls of water and ethanolic extracts of *I. suffruticosa*, respectively.

2.5. Cell viability assay

After incubation with water and ethanolic extracts of *I. suffruticosa* for 24 h, cells were incubated in RPMI medium containing 0.5 mg/ml 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) for an additional 3 h. The medium was then removed and isopropanol was added to dissolve the formazan. After centrifugation at 5000*g* for 5 min, 100 μl of supernatant from each sample was transferred to 96-well plates, and absorbance was read at 570 nm in a VersaMaxTM Tunable Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA).

2.6. Western blot analysis

Cells were washed twice with cold PBS and were harvested in 150 μl lysis buffer containing 10 mM Tris–HCl, 5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 20 μg/ml aprotinin, pH 7.4. The protein content in each sample was quantified by use of the Coomassie Plus Protein Assay Reagent Kit (Pierce Chemical Co., Rockford, IL). Equal amounts of proteins were denatured and separated on SDS–polyacrylamide gels and were then transferred to polyvinylidene difluoride membranes (NewTM Life Science Product, Inc., Boston, MA). The blots were incubated sequentially with primary antibodies and horseradish peroxidase-conjugated anti-goat or anti-rabbit IgG (Bio-Rad, Hercules, CA). Immunoreactive protein bands were developed by the enhanced chemiluminescence kit (Perkin–Elmer Life Science, Boston, MA), then were visualized by luminescent image analyzer (LAS-1000 plus, Fuji Photo Film Company, Japan) and quantified by AlphaImager 2200 (Alpha Innotech Corp., San Leandro, CA). Changes of GSTP and NQO-1 protein levels were normalized to the amount of β-actin.