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The methoxyflavones in *Citrus reticulata Blanco cv. ponkan* and their antiproliferative activity against cancer cells

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ABSTRACT

The major polymethoxyflavones in the fruit (ponkan) peels of *Citrus reticulata Blanco cv. ponkan* were identified as isosinensetin, sinensetin, nobiletin and tetramethyl-*o*-scutellarein by a combined separation using high-speed countercurrent chromatography and preparative high performance liquid chromatography, and structure elucidation by electrospray ionisation mass spectrometry (ESI-MS) and ^{1}H and ^{13}C nuclear magnetic resonance (NMR). The antiproliferative activity of the four compounds against four cancer cell lines (A549, HL-60, MCF-7 and HO8910) showed that isosinensetin had a lower IC_{50} value for MCF-7 and HO8910 cancer cell lines. Determination of polymethoxyflavones in ponkan peels from different cultivation regions displayed relatively steady contents of the four compounds and a higher content of isosinensetin, which suggested that ponkan peels are excellent sources of functional polymethoxyflavones that may help prevent female cancers, such as ovarian cancer and breast cancer.

1. Introduction

The primary bioactive constituents of *Citrus* species are flavonoids and synephrine which have high content in the peels of the fruits. Three types of flavonoids occur in *Citrus* species: flavanones, flavones and flavonols (Mouly, Gaydou, & Auffray, 1998). Amongst them, polymethoxyflavones (PMFs) show chemopreventive potential in antimutagenic and antitumor properties (Li, Lambros, Wang, Goodnow, & Ho, 2007; Li et al., 2009a, 2009b; Walle, 2007). Various *Citrus* species present different composition of PMFs (Green, Wheatley, Osagie, St. A. Morrison, & Asemota, 2006; Hirata et al., 2009; Li, Pan, et al., 2007; Mizuno, Iinuma, Ohara, Tanaka, & Iwamasa, 1991). Ponkan, the fruit of *Citrus reticulata Blanco cv. ponkan*, is produced in Asia, known throughout the world as the thick skinned mandarin orange. In Taiwan ponkan has a 200-year history, originating with cultivation transplanted from China. Although many studies on PMFs from different *Citrus* species (e.g. *Citrus aurantium*, *Citrus sinensis*) have been reported and most PMFs have been confirmed by UV, IR, MS, ^{1}H NMR and ^{13}C NMR (Hirata et al., 2009; Kurowska & Manthey, 2004; Li, Lambros, et al., 2007; Li, Lo, & Ho, 2006; Li, Pan, et al., 2007; Lin et al., 2003; Manthey, Grohmann, Montanari, Ash, & Manthey, 1999; Miyazawa, Okuno, Fukuyama, Nakamura, & Kosaka, 1999; Murakami et al., 2001; Raman, Jayaprakasha, Cho, Brodbelt, & Patil, 2005; Wang, Wang, Huang, Tu, & Ni, 2007; Wang et al., 2005), there is no systematic study on PMFs from ponkan. In order to screen resources possessing high functional PMF content, the present study performs the isolation of PMFs in ponkan peels based on high-speed chromatography and preparative high performance liquid chromatography, identification of structures by electrospray ionisation mass spectrometry (ESI-MS) and ^{1}H and ^{13}C nuclear magnetic resonance (NMR), and an assay of antiproliferation of obtained PMFs against cancer cells.

2. Experimental

2.1. Materials

All solvents for extraction and separation were of analytical grade, purchased from Hangzhou Huadong Chemicals Inc., China. The dried ponkan peels were provided by Zhejiang Juda Industry Co., Ltd., China. Cell lines and the reagents for assay of antiproliferation against cancer cells were provided by Zhejiang Academy of Medical Sciences, China. Thiazolyl blue tetrazolium bromide (MTT) was purchased from Sigma (Shanghai Branch, China).

2.2. Extraction of crude PFMs

The dried ponkan peels (2.5 kg) were ground to powder (about 30 mesh) and was refluxed with 15 L of 75% (v/v) ethanol for 3 h. Then ethanol solution was concentrated at vacuum until the ethanol was eliminated. The concentrated solution was extracted with dichloromethane, and the dichloromethane fraction was

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concentrated to dryness to give 18.5 g of crude PMFs which was subjected to subsequent HSCCC separation.

2.3. Separation of crude PFMs

The separation of crude *PFMs* was performed by high-speed countercurrent chromatography (HSCCC) and preparative high performance liquid chromatography (PHPLC). The high-speed countercurrent chromatograph used in the present study was constructed at the Institute of Food and Biological Engineering, Zhejiang Gongshang University (Hangzhou, China). The apparatus was equipped with a 1200 mL column with six-layer coils made of 5.0 mm i.d. polytetrafluoroethylene (PTFE) tubing. The separation system was composed of a K-1800 Wellchrom pump (Knauer, Germany), a 150 mL sample loop made of 3 mm i.d. PTFE tubing, the high-speed countercurrent chromatograph and a B-684 collector (Büchi, Switzerland). For the HSCCC separation of the crude PFMs, the solvent system was composed of *n*-hexane–ethyl acetate–methanol–water (1:1:1:1:1.5, v/v), and the organic upper phase was used as the stationary phase. The sample solution was prepared by dissolving 5.0 g of crude PFMs in 150 mL of the mobile phase. For each separation, the coil column was first entirely filled with stationary phase. Then, the apparatus was rotated at 700 rpm and the sample solution was injected into the HSCCC system through the PTFE sample loop with the mobile phase at a flow-rate of 5.0 mL/min. The mode for HSCCC separation was "head to tail". The effluent was monitored at 330 nm by Elite UV-200 detector (Elite, Dalian, China). The HSCCC separation yielded fractions I–VI. The fraction V containing two compounds belong to PFMs was separated by PHPLC using JAI LC-9103 equipped a ODS-BP-30 column (250 × 30 mm I.D.) (JAI Inc., Japan) eluted by methanol–water (60:40, v/v).

2.4. Analytical controls and structure elucidation

2.4.1. HPLC analysis of PFMs

The HPLC system was composed of an Waters Alliance 2695 separations module (Milford, MA), a Waters Symmetry C-18 column (250 × 4.6 mm i.d., 5 μm) (Milford, MA), a 996 PDA detector (Milford, MA), a Bruker Esquire ion trap multiple mass spectrometer (Bremen, Germany) and a Millennium HPLC 2010 processing system (Waters, Milford, MA). The mobile phase composed of methanol and water with a gradient profile: 0–20 min 65% H_2O; 20–40 min 65–50% H_2O (Green et al., 2006). The flow-rate was 1 ml/min. For the analyses of PFMs in ponkan peels from various cultivation areas, one gram of freeze-dried sample was powdered and extracted twice with 100 mL of 80% methanol for 1 h at 60 °C. The extract solution was evaporated in a vacuum at 40 °C to remove methanol. Then, the residual solution was partitioned twice with isovolumetric dichloromethane. The extraction solution was evaporated to dryness. The residue was dissolved in methanol for HPLC determination. Each peak of PFMs (isosinensetin, sinensetin, tetramethyl-*o*-isoscutellarein and nobiletin) was checked as a single compound by ESI-MS.

2.4.2. Electrospray ionisation mass spectrometry (ESI-MS)

All ESI-MS experiments of the compounds obtained from our separation procedure were performed on a Bruker Esquire ion trap multiple mass spectrometer (Bremen, Germany) in positive ionisation mode analysing ions up to *m*/*z* 2200. ESI-MS parameters (positive mode): capillary, −4500 V; end plate, −4000 V; cap exit, +90 V; cap exit offset, +60 V; skim 1, +30 V; skim 2, +10 V. Drying gas was nitrogen (gas flow 7.0 L/min, 330 °C), and nebuliser pressure was set to 34.5 kPa.

2.4.3. Nuclear magnetic resonance (NMR) analysis

^1H, ^{13}C, and DEPT 90/135 NMR spectra were recorded in [$^2H_1$] chloroform ($CDCl_3$) on a Bruker Avance 500 II (Karlsruhe, Germany) with 500 MHz for ^1H measurements and 125 MHz for ^{13}C measurements, respectively.

2.5. Assay of antiproliferation against cancer cells

The percentage of growth inhibition was determined by using a MTT assay to measure viable cells (Zhang et al., 2003). A total of 2.5×10^3 cells/well was seeded onto a 96-well plate for 24 h, treated with various concentrations of isosinensetin (ISNT), sinensetin (SNT), tetramethyl-*o*-isoscutellarein (TISL) and nobiletin (NBL), and incubated for an additional 3 days at 37 °C. Subsequently, 10 μl of MTT at a concentration of 5 mg/ml was added to each well, and cells were incubated for an additional 5 h. The supernatant was aspirated, and 100 μl of DMSO were added to the wells to dissolve any precipitate present. The absorbance was then measured at a wavelength of 570 nm using a microplate reader (Multiskan Spectrum, Thermo Electron Corporation, Vantaa, Finland).

2.6. Statistical analysis of difference significance

Statistical analysis was performed using ANOVA Duncan's Multiple Range Test by SAS 8.0 (SAS Institute Inc., Cary, USA). A probabilistic value $p < 0.05$ was considered significant. Experimental results of contents of PMFs were expressed as means ± SD from the values of three replications of each sample.

3. Results and discussion

3.1. Isolation and identification of PMFs

Preparative isolation of the crude PMFs by HSCCC employed a two-phase solvent system composed of *n*-hexane–ethyl acetate–

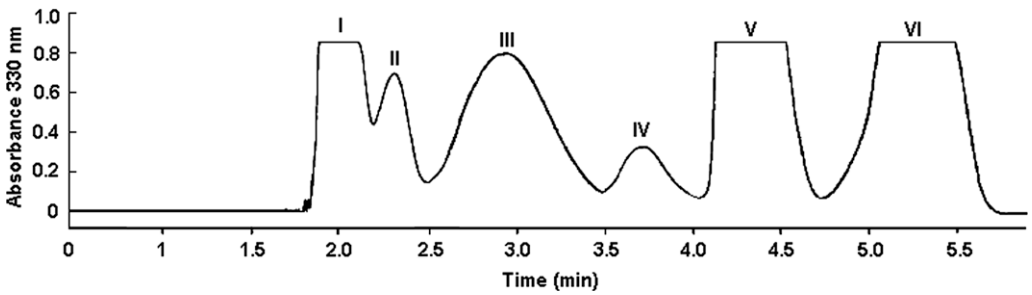

Fig. 1. HSCCC chromatogram of 5.0 g of the crude PMFs from ponkan peels. Two-phase solvent system: *n*-hexane–ethyl acetate–methanol–water (1:1:1:1:1.5, v/v); stationary phase: upper organic phase; elution mode in the coil system: head to tail; flow-rate: 5.0 mL/min; detection wavelength: λ = 330 nm; retention of stationary phase: 51%; I, II and IV: unknown impure components; III: tetramethyl-*o*-isoscutellarein; V: isosinensetin + sinensetin; VI: nobiletin.