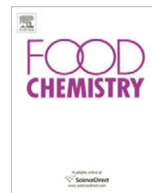
Food Chemistry 119 (2010) 567–572

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



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

Qizhen Du *, Hui Chen

Institute of Food and Biological Engineering, Zhejiang Gongshang University, 149 Jiaogong Road, Hangzhou, Zhejiang 310035, China

ARTICLE INFO

Article history:
Received 7 February 2009
Received in revised form 28 May 2009
Accepted 24 June 2009

Keywords:
Citrus reticulata Blanco cv. ponkan
Ponkan
Polymethoxyflavones
Composition
Antiproliferative activity
Cancer cells

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.

© 2009 Elsevier Ltd. All rights reserved.

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

* Corresponding author. Tel./fax: +86 571 88071024 8575.
E-mail address: qizhendu@163.com (Q. Du).

0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.06.059

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–

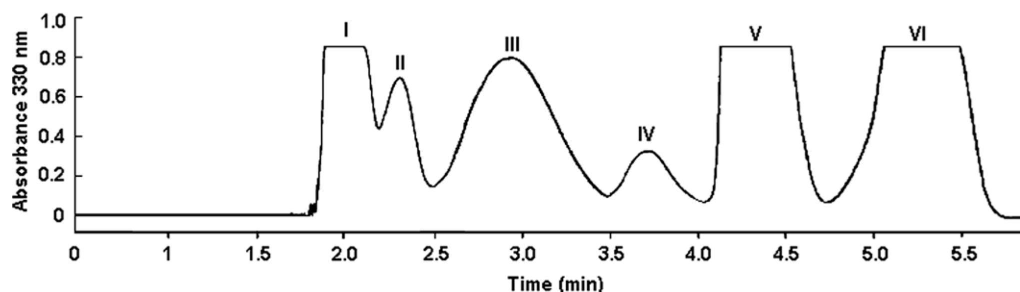


Fig. 1. HSCCC chromatogram of 5.0 g of the crude PMFs from ponkan peels. Two-phase solvent system: *n*-hexane–ethyl acetate–methanol–water (1:1:1:1:1.5, v/v); stationary phase: upper organic phase; elution mode in the coil system: head to tail; flow-rate: 5.0 mL/min; detection wavelength: λ = 330 nm;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

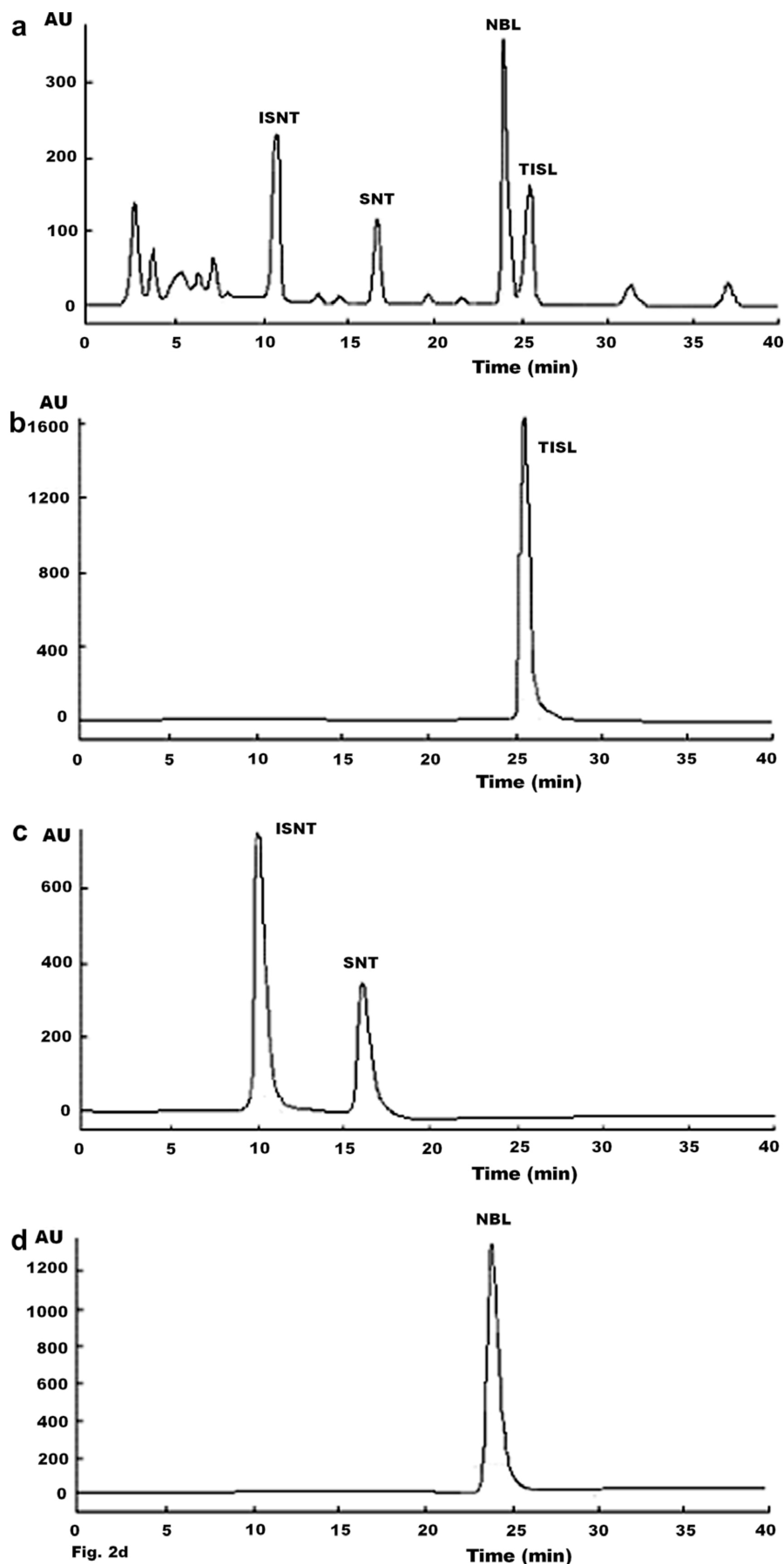


Fig. 2. HPLC analysis of the crude PMFs from ponkan peels and the components III, V and VI from HSCCC. Column: symmetry C-18, 5 µm, 250 × 4.6 mm; gradient profile of mobile phase: 0–20 min 65% H_2O; 20–40 min 65–50% H_2O; flow-rate: 1 ml/min; detection wavelength: λ = 330 nm; ISNT: isosinensetin; SNT: sinensetin; TISL: tetramethyl-*o*-isoscutellarein; NBL: nobiletin. Image (a): chromatogram of the crude PMFs; image (b): chromatogram of the component III; image (c): chromatogram of the component V; image (d): chromatogram of the component VI.

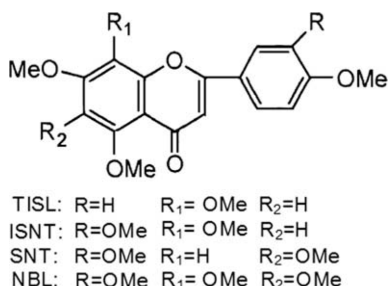


Fig. 3. The chemical structure of compounds **1**–**4** from ponkan peels. TISL: tetramethyl-*o*-isoscutellarein; ISNT: isosinensetin; SNT: sinensetin; NBL: nobiletin.

methanol–water (1:1:1:1:1.5, v/v) yielded fractions of six peaks which were combined to fractions I–VI, respectively (Fig. 1). Evaporation of the organic solvents under reduced pressure, and subsequent lyophilisation yielded three PMF components in which component III (compound **1**, 231 mg) and component VI (compound **2**, 413 mg) possessed purity more than 97% whilst component V (534 mg) was a mixture of two compounds analysed by HPLC (Fig. 2). Separation of 50 mg of component V by PHPLC resulting to 19.5 mg of compound **3** and 27.7 mg of compound **4**. Identification of the four compounds (Fig. 3) was carried out by physical properties, ESI-MS, ^{1}H, ^{13}C and DEPT 90/135 NMR spectra analysis as follows.

*Compound **1*** (TISL): Colorless needles; Mp 207–208 °C; ESI-MS: *m/z* 343 [M+H]$^+$, 365 [M+Na]$^+$; ^{1}H NMR δ: 7.14 (2H, (2H, d, *J* = 8.5 Hz, H-3′, 5′), 7.99 (2H, d, *J* = 8.5 Hz, H-2′, 6′), 6.68 (2H, s, H-3, 6), 3.88–4.00 (12H, m, 4 × OMe); ^{13}C NMR δ: 161.8 (C-2), 106.24 (C-3), 175.73 (C-4), 155.59 (C-5), 129.93 (C-6), 156.24 (C-7), 93.74 (C-8), 150.97 (C-9), 107.97 (C-10), 123.15 (C-1′), 127.52 (C-2′), 117.89 (C-3′), 159.50 (C-4′), 114.56 (C-5′), 127.52 (C-6′), 62.39, 56.31, 56.23, 55.41 (4 × OMe). Compound **1** was identified as 5,7,8,4′-tetramethoxyflavone (tetramethyl-*o*-isoscutellarein) from these spectral data and physical properties (Talatra, Swapan, & Mukhopadhyay, 1975).

*Compound **2*** (NBL): Colorless needles; Mp 137–138 °C; ESI-MS: *m/z* 403 [M+H]$^+$, 425 [M+Na]$^+$; ^{1}H NMR δ: 6.84 (1H, H-3), 7.528 (1H, H-2′), 7.140 (1H, d, *J* = 8.0 Hz, H-5′), 7.63 (1H, d, *J* = 8.0 Hz, H-6′), 3.77–4.03 (18H, 6 × OMe); ^{13}C NMR δ: 160.3 (C-2), 106.3 (C-3), 175.9 (C-4), 147.5 (C-5), 143.6 (C-6), 151.8 (C-7), 137.7 (C-8), 147.2 (C-9), 114.3 (C-10), 123.2 (C-1′), 108.9 (C-2′), 151.0 (C-3′), 151.8 (C-4′), 111.9 (C-5′), 119.4 (C-6′), 61.9, 61.8, 61.5, 61.4, 57.7, 55.7 (6 × OMe). Compound **2** was identified as 5,6,7,8,3′,4′-hexamethoxyflavone (nobiletin) from these spectral data and physical properties (Raman et al., 2005; Wang et al., 2005).

*Compound **3*** (ISNT): Colorless needles; Mp 145–146 °C; ESI-MS: *m/z* 373 [M+H]$^+$, 395 [M+Na]$^+$; ^{1}H NMR δ: 6.84 (1H, H-3), 6.68 (1H, H-6), 7.53 (1H, H-2′), 7.14 (1H, d, *J* = 8.0 Hz, H-5′), 7.63 (1H, d, *J* = 8.0 Hz, H-6′), 3.74–3.98 (15H, 5 × OMe); ^{13}C NMR δ: 159.5 (C-2), 106.5 (C-3), 175.9 (C-4), 155.6 (C-5), 129.8 (C-6), 156.3 (C-7), 93.6 (C-8), 151.0 (C-9), 107.9 (C-10), 123.2 (C-1′), 108.8 (C-2′), 148.9 (C-3′), 151.6 (C-4′), 111.8 (C-5′), 119.1 (C-6′), 60.9, 56.3, 56.2, 55.7, 55.6 (5 × OMe). Compound **3** was identified as 5,7,8,3′,4′-pentamethoxyflavone (isosinensetin) from these spectral data and physical properties (Talatra et al., 1975).

*Compound **4*** (SNT): Colorless needles; Mp 134–135 °C; ESI-MS: *m/z* 373 [M+H]$^+$, 395 [M+Na]$^+$; ^{1}H NMR δ: 6.96 (1H, H-3), 6.68 (1H, H-8), 7.54 (1H, H-2′), 7.12 (1H, d, *J* = 8.0 Hz, H-5′), 7.67(1H, d, *J* = 8.0 Hz, H-6′), 3.75–4.03 (15H, 5 × OMe); ^{13}C NMR δ: 160.3 (C-2), 106.3 (C-3), 175.9 (C-4), 147.5 (C-5), 143.5 (C-6), 151.8 (C-7), 137.7 (C-8), 147.2 (C-9), 114.3 (C-10), 123.2 (C-1′), 108.9 (C-2′), 151.0 (C-3′), 151.8 (C-4′), 111.9 (C-5′), 119.4 (C-6′), 61.9, 61.8, 61.5, 61.4, 57.7, 55.7 (5 × OMe). Compound **4** was identified as 5,6,7,3′,4′-pentamethoxyflavone (sinensetin) from these spectral data and physical properties (Rashid & Armstrong, 1992).

The above results demonstrate that HSCCC is a practical technique for the separation of TISL and NBL because these two compounds with a high purity can be attained by one-step HSCCC separation. Moreover, it is worthwhile to mention that the combination of HSCCC and PHPLC is a good way to achieve a excellent separation of the PMFs since we can see that SNT and ISNT were in one fraction in HSCCC, and TISL and NBL were very close in PHPLC, but high resolution between SNT and ISNT in HPLC, and between TISL and NBL in HSCCC was obtained. Really, HSCCC or PHPLC complements each other's shortcoming in some separations (Du, Jiang, & Wang, 2009) from a different separation mechanism.

3.2. Antiproliferative activity of polymethoxyflavones from ponkan

The polymethoxyflavones in *Citrus* are distinctive for their high antiproliferative activities against a number of human cancer cell lines (Sergeev, Li, Colby, Ho, & Dushenkov, 2006). Although the antiproliferative activities of SNT, TISL, ISNT and NBL against several cell lines have been assayed in some laboratories, no coinstantaneous antiproliferative assay of SNT, TISL, ISNT and NBL against the human ovarian cancer cell line HO8910, the human breast cancer cell line MCF-7, the human myeloid leukeamia cell line HL-60 or the human lung adenocarcinoma epithelial cell line A590 have been reported.

In the present study, we assayed the antiproliferative activities of the four polymethoxyflavones from ponkan against A590, MCF-7, HL-60 and HO8910 and assessed their IC50 values. The results are shown in Table 1. The order of sensitivity of the cell lines to the polymethoxyflavones was HO8910 > MCF-7 > HL-60 > A590. The IC_{50} values (mean) of the four polymethoxyflavones against HO8910 were 15.5 μM for TISL, 12.5 μM for SNT, 31.1 μM for SNT and 16.8 μM for NBL, which were about half of those in A590 cells. TISL, ISNT and NBL exhibited a stronger antiproliferative activity than SNT in the entire cell lines used. In particular, ISNT showed lower IC_{50} values in MCF-7 (15.1 μM) and HO8910 (12.5 μM), which suggests that higher ISNT content in food is beneficial for the prevention of female cancers. The IC_{50} for antiproliferation of MCF-7 cells showed a high correlation to the induction of cell death, apoptosis, and increased $[Ca^{2+}]_i$ (Sergeev et al., 2006). Therefore, the activity of ISNT against breast cancer warrants further investigation.

Table 1
Antiproliferative activities of polymethoxyflavones against cancer lines.

Polymethoxyflavone	Antiproliferative activities against cancer lines (IC50*)			
	A549 (μM)	HL-60 (μM)	MCF-7 (μM)	HO8910 (μM)
TISL	28.2 ± 2.1	20.1 ± 0.9	20.8 ± 1.2	15.5 ± 0.9
ISNT	23.6 ± 1.2	23.8 ± 1.5	15.1 ± 0.8	12.5 ± 0.4
SNT	62.4 ± 3.6	47.5 ± 2.9	40.9 ± 2.7	31.1 ± 1.7
NBL	31.9 ± 2.5	31.7 ± 1.8	28.2 ± 1.5	16.8 ± 1.2

* IC_{50}: the concentration that inhibits cell growth by 50% of the control experiment. All data represent a mean value of three replications.

Table 2
Polymethoxyflavones in ponkan peels from different cultivation regions.

Cultivation region	Polymethoxyflavone*			
	TISL (mg/g)	ISNT (mg/g)	SNT (mg/g)	NBL (mg/g)
Quzhou, Zhejiang	2.15 ± 0.23^{a}	3.52 ± 0.29^{a}	1.78 ± 0.13^{a}	8.61 ± 0.67^{a}
Lishui, Zhejiang	2.31 ± 0.17^{a}	3.31 ± 0.23^{ab}	1.73 ± 0.19^{a}	7.56 ± 0.36^{ab}
Yongchun, Fujian	2.24 ± 0.20^{a}	3.04 ± 0.26^{ab}	1.69 ± 0.11^{a}	6.92 ± 0.41^{bc}
Nanjing, Fujian	2.41 ± 0.32^{a}	2.89 ± 0.17^{bc}	1.62 ± 0.12^{a}	7.81 ± 0.56^{ab}
Nanfeng, Jiangxi	2.53 ± 0.29^{a}	3.14 ± 0.32^{ab}	1.76 ± 0.17^{a}	7.48 ± 0.33^{ab}

The superscripts sharing a letter in a column indicate that these samples are forming a group of not statistically different values.
* Contents of polymethoxyflavones were expressed as means ± SD from the values of three replications of each sample.

A comparison of ISNT and SNT showed that the methoxyl group at C-8 is essential for the antiproliferative activity of the flavones, which is consistent with the findings reported by Kawaii, Tomono, Katase, Ogawa, and Yano (1999), who assayed the antiproliferative activity of other polymethoxyflavones. Based on the comparison of ISNT and TISL, the IC_{50} values indicated that the reduced effects depended on the number of methoxyl groups in the C-ring.

3.3. Polymethoxyflavones in ponkan from different cultivation areas

Nogata, Ohta, Sumida, and Sekiya (2003) quantitatively analysed the *Citrus* flavonoids in dried edible parts of fruits, and found that the concentration of tangeretin (9.1 mg/100 g) in ponkan was highest and that of nobiletin (12.3 mg/100 g) was second highest amongst the 66 *Citrus* species in Japan. Similarly, Sugiyama, Umehara, Kuroyanagi, Ueno, and Taki (1993) reported that the concentration of sinensetin in the peel of ponkan in Japan was of 6.6 mg/100 g. However, no studies have reported the levels of polymethoxyflavones in ponkan peels obtained from the main cultivation regions in China.

In the present study, HPLC was used to measure the concentrations of four polymethoxyflavones in ponkan peels from five different regions (Quzhou, Lishui, Yongchun, Nanjing and Nanfeng) in three Chinese provinces. Statistical analysis of the data (Table 2) showed no difference ($p > 0.05$) in the peel content of TISL or SNT between regions, but there was a significant difference in ISNT levels between samples from Quzhou and Nanjing, and in NBL levels between samples from Quzhou and Yongchun. Of the four PMFs tested, the concentration of NBL was highest, which was consistent with the other species of *Citrus*. A feature of ponkan is its higher TISL and ISNT levels compared with the levels of PMFs in other *Citrus* species (Hirata et al., 2009; Kurowska & Manthey, 2004; Li et al., 2006; Li, Lambros, et al., 2007; Li, Pan, et al., 2007; Lin et al., 2003; Manthey et al., 1999; Miyazawa et al., 1999; Murakami et al., 2001; Raman et al., 2005; Wang et al., 2005, 2007).

Because polymethoxyflavones in ponkan peels from different cultivation regions displayed a relatively steady content and ISNT had lower IC_{50} values for MCF-7 and HO8910 cancer cell lines (Table 1), ponkan peels may offer excellent sources of functional polymethoxyflavones to help prevent female cancers.

4. Conclusions

Four compounds including isosinensetin, sinensetin, nobiletin and tetramethyl-*o*-scutellarein were identified as major polymethoxyflavones in the fruit (ponkan) peels of *Citrus reticulata Blanco cv. ponkan* by a combined separation using high-speed countercurrent chromatography and preparative high performance liquid chromatography and structure elucidation by ESI-MS and ^{1}H and ^{13}C NMR. ISNT had a lower IC_{50} value for MCF-7 and HO8910 cancer cell lines. Polymethoxyflavones in ponkan peels from different cultivation regions displayed a relatively steady content and a higher ISNT content. These findings suggest that ponkan peels are excellent sources of functional polymethoxyflavones that may help prevent female cancers, such as ovarian cancer and breast cancer.

Acknowledgments

This research was supported by a Foundation 2006C14040 from the Department of Science and Technology of Zhejiang Province, China. The authors are grateful Professor Gaoli Zheng in Zhejiang Academy of Medical Sciences for his support in the Assay of inhibiting proliferation of cancer cells.

References

Du, Q., Jiang, Z., & Wang, D. (2009). Excellent combination of counter-current chromatography and preparative high-performance liquid chromatography to separate galactolipids from pumpkin. *Journal of Chromatography A, 1216*, 4176–4180.
Green, C. O., Wheatley, A. O., Osagie, A. U., St. A. Morrison, E. Y., & Asemota, H. N. (2006). Determination of polymethoxyflavones in peels of selected Jamaican and Mexican citrus (*Citrus spp.*) cultivars by high-performance liquid chromatography. *Biomedical Chromatography, 21*(4), 8–54.
Hirata, T., Fujii, M., Akita, K., Yanaka, N., Ogawa, K., Kuroyanagi, M., et al. (2009). Identification and physiological evaluation of the components from Citrus fruits as potential drugs for anti-corpulence and anticancer. *Bioorganic and Medicinal Chemistry, 17*, 25–28.
Kawaii, S., Tomono, Y., Katase, E., Ogawa, K., & Yano, M. (1999). Antiproliferative activity of flavonoids on several cancer cell lines. *Bioscience, Biotechnology and Biochemistry, 63*, 896–899.
Kurowska, E. M., & Manthey, J. A. (2004). Hypolipidemic effects and absorption of citrus polymethoxyflavones in hamsters with diet induced hypercholesterolemia. *Journal of Agricultural and Food Chemistry, 52*, 2879–2886.
Li, S., Lambros, T., Wang, Z., Goodnow, R., & Ho, C. T. (2007). Efficient and scalable method in isolation of polymethoxyflavones from orange peel extract by supercritical fluid chromatography. *Journal of Chromatography B, 846*, 291–297.
Li, S., Lo, C. Y., & Ho, C. T. (2006). Hydroxylated polymethoxyflavones and methylated flavonoids in sweet orange (*Citrus sinensis*) peel. *Journal of Agricultural and Food Chemistry, 54*, 4176–4185.
Li, S., Pan, M.-H., Lai, C.-S., Lo, C.-Y., Dushenkov, S., & Ho, C.-T. (2007). Isolation and syntheses of polymethoxyflavones and hydroxylated polymethoxyflavones as inhibitors of HL-60 cell lines. *Bioorganic and Medicinal Chemistry, 15*, 3381–3389.
Li, S., Pan, M.-H., Lo, C.-Y., Tan, D., Wang, Y., Shahidi, F., et al. (2009a). Chemistry and health effects of polymethoxyflavones and hydroxylated polymethoxyflavones. *Journal of Functional Foods, 1*, 2–12.
Li, S., Pan, M.-H., Lo, C.-Y., Tan, D., Wang, Y., Shahidi, F., et al. (2009b). Citrus flavonoids: Molecular structure, biological activity and nutritional properties: A review. *Food Chemistry, 104*, 466–479.
Lin, N., Sato, T., Takayama, Y., Mimaki, Y., Sashida, Y., Yano, M., et al. (2003). Novel anti-inflammatory actions of nobiletin, a citrus polymethoxyflavonoid, on human synovial fibroblasts and mouse macrophages. *Biochemical Pharmacology, 65*, 2065–2071.
Manthey, J. A., Grohmann, K., Montanari, A., Ash, K., & Manthey, C. L. (1999). Polymethoxyflavones derived from citrus suppress tumor necrosis factor-α expression by human monocytes. *Journal of Natural Products, 62*, 441–444.
Miyazawa, M., Okuno, Y., Fukuyama, M., Nakamura, S., & Kosaka, H. (1999). Antimutagenic activity of polymethoxyflavonoids from *Citrus aurantium*. *Journal of Agricultural and Food Chemistry, 47*, 5239–5244.
Mizuno, M., Iinuma, M., Ohara, M., Tanaka, T., & Iwamasa, M. (1991). Chemotaxonomy of the genus citrus based on polymethoxyflavones. *Chemical and Pharmaceutical Bulletin, 39*, 945–949.
Mouly, P., Gaydou, E. M., & Auffray, A. (1998). Simultaneous separation of flavanone glycosides and polymethoxyflavones in citrus juices using liquid chromatography. *Journal of Chromatography A, 800*, 171–179.
Murakami, A., Kuwahara, S., Takahashi, Y., Ito, C., Furukawa, H., Ju-Tchi, M., et al. (2001). In vitro absorption and metabolism of nobiletin, a chemopreventive polymethoxyflavonoid in citrus fruits. *Bioscience, Biotechnology and Biochemistry, 65*, 194–197.
Nogata, Y., Ohta, H., Sumida, T., & Sekiya, K. (2003). Effect of extraction method on the concentrations of selected bioactive compounds in mandarin juice. *Journal of Agricultural and Food Chemistry, 51*, 7346–7351.
Raman, G., Jayaprakasha, G. K., Cho, M., Brodbelt, J., & Patil, B. S. (2005). Rapid adsorptive separation of citrus polymethoxyflavones in non-aqueous conditions. *Separation and Purification Technology, 45*, 147–152.
Rashid, A., & Armstrong, A. J. (1992). Alkaloids, flavonols and coumarins from *Drummondita hassellii* and *C. Calida*. *Phytochemistry, 31*, 1265–1269.
Sergeev, I. N., Li, S., Colby, J., Ho, C.-T., & Dushenkov, S. (2006). Polymethoxyflavones induce Ca^{2+}-mediated apoptosis in breast cancer cells. *Life Sciences, 80*, 245–253.

Sugiyama, S., Umehara, K., Kuroyanagi, M., Ueno, A., & Taki, T. (1993). Studies on the differentiation inducers of myeloid leukemic cells from citrus species. *Chemical and Pharmaceutical Bulletin, 41*, 714–719.

Talatra, S. K., Swapan, S. K., & Mukhopadhyay, A. (1975). Methoxylated flavones of *Fortunella japonica*. *Phytochemistry, 14*, 309–310.

Walle, T. (2007). Methoxylated flavones, a superior cancer chemopreventive flavonoid subclass. *Seminars in Cancer Biology, 17*, 354–362.

Wang, X., Li, F., Zhang, H., Geng, Y., Yuan, J., & Jiang, T. (2005). Preparative isolation and purification of polymethoxyflavones from Tangerine peel using high-speed counter-current chromatography. *Journal of Chromatography A, 1090*, 188–192.

Wang, D., Wang, J., Huang, X., Tu, Y., & Ni, K. (2007). Identification of polymethoxyflavones from green tangerine peel (*Pericarpium Citri Reticulatae Viride*) by chromatographic and spectroscopic techniques. *Journal of Pharmaceutical and Biomedical Analysis, 44*, 63–69.

Zhang, D. Y., Wu, J., Ye, F., Xue, L., Jiang, S., Yi, J., et al. (2003). Inhibition of cancer cell proliferation and prostaglandin E2 synthesis by *Scutellaria Baicalensis*. *Cancer Research, 63*, 4037–4043.