Original paper

Cellular Antioxidant Activity and Pharmacokinetic Study of Polymethoxylated Flavonoids in Extract of *Citrus reticulata* ‘Chachi’ Peel

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Received October 23, 2013; Accepted January 9, 2014

A rat pheochromocytoma (PC12) cell line was used to study the effects of several polymethoxylated flavonoids (PMFs) and extract of *Citrus reticulata* ‘chachi’ peel (ECRP) on hydrogen peroxide (H$_2$O$_2$)-induced apoptosis. In PC12 cells, H$_2$O$_2$-induced apoptosis was accompanied by rising malondialdehyde (MDA) level, as well as reducing activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). However, treating PC12 cells with PMFs and ECRP blocked H$_2$O$_2$-induced apoptosis by decreasing the MDA level and augmenting the activities of SOD and GSH-Px. Moreover, liquid chromatography coupled with UV detection was developed to simultaneously determine nobiletin and tangeretin in rat plasma after oral administration of ECRP, using quercetin as the internal standard. Plasma samples extracted by liquid-liquid extraction were separated on a Phenomenex Luna C$_{18}$ column and detected by a UV detector. The calibration curves were linear over the range of 5.5 – 2757 ng mL$^{-1}$ for nobiletin ($r^2 > 0.999$) and 6.3 – 2847 ng mL$^{-1}$ for tangeretin ($r^2 > 0.999$), respectively. The lower limits of quantification were 0.2 ng mL$^{-1}$ for nobiletin and tangeretin. The intra- and inter-day precisions were less than 15% and the accuracies ranged from 3.4% to 12.2%. The recoveries of nobiletin and tangeretin were 91.1% – 95.4% and 93.7 – 96.2% respectively. Nobiletin and tangeretin remained stable at low temperatures (−20°C and 4°C) and after freeze–thaw cycles. The validated method was successfully applied to investigate the pharmacokinetics of the main PMFs of ECRP extract after oral administration to rat.

Keywords: polymethoxylated flavonoid, *citrus reticulata* ‘chachi’ peel, H$_2$O$_2$, PC12 cell, pharmacokinetics, determination

Introduction

Oxidative stress is a risk factor in the occurrence and progression of various diseases including Alzheimer’s disease (AD), atherosclerosis and ischemia (Wadsworth, 2008). Fortunately, since plants contain a wide variety of free radical scavenging molecules such as flavonoids, anthocyanins, carotenoids, dietary glutathione and vitamins, they have received growing attention as chemopreventive agents against oxidative damage (Fabiana et al., 2008). Especially, natural flavonoids, which are promising antioxidant products, can easily penetrate the blood-brain barrier (Seung et al., 2012; Gilgun-Sherki et al., 2001; Manthey et al., 2001) due to lipophilic chemical structures. Recently, the antioxidant effects of citrus flavonoids have been spotlighted. Flavonoids are the major antioxidant bioactive constituents of *Citrus reticulata* ‘chachi’ peel (ECRP), and polymethoxylated flavonoids (PMFs) are peculiar to Rutaceae citrus. Besides inhibiting the key enzymes in mitochondrial respiration and protecting against coronary heart disease, these flavonoids also have anti-spasmodytic, anti-inflammatory, antioxidative, vascular, estrogenic, cytotoxic, antitumor, and antimicrobial activities (Wang et al., 2007; Benavente-Garcia et al., 2008).

In our previous studies, the extraction process of ECRP was optimized by using response surface methodology with antioxidant activity as the indicator (Mo et al., 2012). A series of *in vitro* antioxidant models suggested that the extract of ECRP possessed

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excellent antioxidant activity and scavenging activity against free radicals. In addition, D-galactose-induced aging mice model experiments showed that ECRP not only reduced the malondialdehyde (MDA) levels but also improved the activities of SOD and GSH-Px in blood, liver and brain. Furthermore, the components of ECRP in plasma were mainly identified as PMFs based on the principle of serum medicinal chemistry (Li et al., 2012). Then the PMFs were isolated and purified by silica gel column chromatography and the structures were identified by nuclear magnetic resonance spectroscopy and UV absorption spectroscopy.

Taken together, ECRP exhibited significant antioxidant effects in vitro and in vivo, and PMFs were mainly absorbed in blood. However, the intracellular antioxidant activities of these flavanones have not been tested. Therefore, it is necessary to investigate whether and how PMFs work in vivo by cellular antioxidant activity assay. Although the protective effects of citrus flavanones on hydrogen peroxide-induced cytotoxicity have been reported (Lu et al., 2010; Hwang et al., 2008), the antioxidant effects of other PMFs of ECRP, except for nobiletin and tangeretin, have never been studied. Thus, this study aimed to first evaluate the protective effects of PMFs of ECRP on PC12 cells against H$_2$O$_2$-induced cytotoxicity. Moreover, pharmacokinetics should be studied to determine how much PMFs can be absorbed in blood to exert effects, and to unravel the mechanism of compatibility and action of ECRP, especially because oral bioavailability significantly limits the efficacy of the PMFs. It is of great importance to obtain the detailed pharmacokinetics and to find out the relationship between oral dosage and antioxidant effect of ECRP PMFs. Although Manthey et al. (2011) and Saigusa et al. (2011) have developed simultaneous determination methods to measure the contents of PMFs in sera of animals, they just focused on the pharmacokinetics of purified compounds. In contrast, the pharmacokinetics and bioavailability of PMFs in plant extract have never been reported hitherto. Thus, this study also evaluated the pharmacokinetics of nobiletin and tangeretin that have the highest contents of PMFs.

1 A Cellular Antioxidant Activity Assay

Materials and Methods

**Materials** Nobiletin, tangeretin, hesperidin, 5-hydroxy-6,7,8,3',4'-5 methoxy flavones, 5,7,8,3',4'-4 methoxy flavones, 5,6,7,3',4'-5 methoxy flavones, 3,5,6,7,8,3',4'-7 methoxy flavones and ECRP were prepared by our group. 3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), Dulbecco’s Modified Eagle’s Medium (DMEM) and H$_2$O$_2$ were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum was purchased from HyClone (Logan, UT). The medium was changed every other day. Before treatment, the cells were seeded at an appropriate density on 96-well plates and cultured for 24 h.

Then the cells were divided into an experimental group, a control group and an injured group, and those in the experimental group were pretreated for 24 h with various concentrations of samples. Then, for the experiment group and the injured group, the culture medium was refreshed without adding samples, and the cells were exposed to a certain concentration of H$_2$O$_2$ for another 4 h. For the control group, identical culture medium was added for continuous culture. The MDA levels and total activities of SOD and GSH-Px in all groups were measured.

**Determination of optimum effect concentration** To evaluate the activities of PMFs, ECRP and hesperidin at different concentrations without inducing cytotoxicity in normal cells, and to determine the concentrations of PMFs and ECRP that exert optimum effects, the samples treated with H$_2$O$_2$ were tested at 3.13 – 50.00 µg mL$^{-1}$. As mentioned above, PC12 cells were seeded in 96-well plates at a density of 1.0 × 10$^4$ cells/100 µL in 96-well plates for 24 h. After being divided into an experimental group and a control group. Likewise, a blank group was set by adding culture medium only. In the experimental group, the culture medium was refreshed by adding different concentrations of H$_2$O$_2$, and that in the control group was refreshed without adding H$_2$O$_2$. All groups were cultured continuously for 4 h, and the cells’ viability was measured by MTT reduction assay.

Preparation of ECRP extract ECRP was authenticated by Dr. Li Shuyuan who works in Medicinal Plants and Identification Science of TCM from Guangdong Pharmaceutical University.

The crude drug (100 g) was extracted by refluxing with 42% ethanol (1:17, v/v) at 62°C for 3.1 h and then filtered. The solution was concentrated under reduced pressure to give an extract of ECRP (yield: 36.57%), and stored at 4°C until use.

The contents in ECRP were determined to be 34.16, 13.30 and 10.68 mg/g for hesperidin, nobiletin, and tangeretin, respectively.

**Cell culture and treatment** Rat pheochromocytoma PC12 cells were purchased from the Shanghai Cell Bank. Briefly, PC12 cells were cultured in DMEM supplemented with heat-inactivated 10% fetal bovine serum in a water-saturated 5% CO$_2$ atmosphere at 37°C. All cells were cultured in collagen-coated culture dishes or flasks. The medium was changed every other day. Before treatment, the cells were seeded at an appropriate density on 96-well plates and cultured for 24 h.

To evaluate the activities of PMFs, ECRP and hesperidin at different concentrations without inducing cytotoxicity in normal cells, and to determine the concentrations of PMFs and ECRP that exert optimum effects, the samples treated with H$_2$O$_2$ were tested at 3.13 – 50.00 µg mL$^{-1}$. As mentioned above, PC12 cells were seeded in 96-well plates at a density of 1.0 × 10$^4$ cells/100 µL in 96-well plates for 24 h. After being divided into an experimental group and a control group. Likewise, a blank group was set by adding culture medium only. In the experimental group, the culture medium was refreshed by adding different concentrations of H$_2$O$_2$, and that in the control group was refreshed without adding H$_2$O$_2$. All groups were cultured continuously for 4 h, and the cells’ viability was measured by MTT reduction assay.
Measurement of Cell Viability  The viability of PC12 cells was determined by MTT reduction assay. MTT, a tetrazolium salt, is cleaved to formazan by succinate dehydrogenase, an active enzyme of the mitochondrial respiratory chain in live cells. After incubation and treatment mentioned above, cells in all groups were treated with the MTT solution at a final concentration of 5 mg mL\(^{-1}\) for 4 h. The dark blue formazan crystals formed in intact cells were solubilized with dimethylsulfoxide (DMSO), and their optical density was measured at 570 nm using a microplate reader.

Measurement of MDA, total SOD, and GSH-Px  At the end of the treatment, cells were digested with trypsin and collected by centrifuge at 1000 \(\times\) g for 3 min, and cell pellets were washed 3 times with PBS and then centrifuged again. The supernatant was discarded, and 400 \(\mu\)L of cold PBS (4°C) was added to suspend the cell pellets. Then the homogenate was prepared by ultrasonication at 4°C. MDA level and total activities of SOD and GSH-Px in the supernatant were measured spectrophotometrically according to the manufacturer’s instructions, respectively.

Statistical analysis  All experiments were performed in triplicate. Data were presented as mean ± SD. The Duncan test and one-way ANOVA were used for multiple comparisons using SPSS 12.0 software (SPSS, Chicago, USA). P < 0.05 was used to indicate that the difference between groups was statistically significant.

2 Pharmacokinetics Study

Materials and Methods

Materials and reagents  Nobiletin (>97% purity) and tangeretin (>96% purity) were prepared in our group. Quercetin (99.3% purity), which was used as the internal standard, was purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and acetonitrile (HPLC-grade) were purchased from VWR International Company (Darmstadt, Germany). Acetic acid and ethyl acetate (analytical grade) were purchased from Guangzhou Chemical Reagent No. 1 Factory (Guangzhou, China). Water was Watson’s spring water.

Instrumentation and operational conditions  The HPLC system used was from Agilent 1200 series LC system (Agilent, USA), consisting of a G1311A quaternary pump, a G1316A thermostatted column compartment, a G1322A degasser, a G1328B manual injector (with a 20 \(\mu\)L loop) and a G1314B UV detector. Data were collected and integrated by using Agilent ChemStation for LC (Agilent, USA). Chromatographic separation was conducted at 25°C on a Luna C\(_18\) column (250 mm × 4.6 mm, i.d. 5 \(\mu\)m, Phenomenex, USA) and a C\(_18\) guard column (5 \(\mu\)m, Phenomenex, USA). The mobile phase consisted of acetonitrile (A) and 0.2% acetic acid-water (B) using a gradient elution of 8 → 25% A at 0 → 10.0 min; 25 → 30% A at 10.0 → 20.0 min; 30 → 50% A at 20.0 → 30.0 min; 50% A at 30.0 → 40.0 min. The flow rate was 1.0 mL/min, and the injection volume was 20 \(\mu\)L. The mobile phase was filtered through a Millipore 0.22 \(\mu\)m filter prior to use. The optimum UV wavelength for detecting nobiletin and tangeretin was set at 330 nm.

Preparation of standard solutions  Stock solutions of nobiletin and tangeretin were prepared at 2 mg mL\(^{-1}\) and IS (quercetin) was prepared at 1 mg mL\(^{-1}\) in methanol after the correction for purity. All the stock solutions were stored at 4°C, brought to room temperature and further diluted to make working solutions. Intermediate solutions were prepared by mixing aliquots of respective stock solutions with methanol to prepare the working solutions containing 5000, 2000, 1000, 500.0, 200.0, 50.00, 20.00 and 10.00 ng mL\(^{-1}\) nobiletin and tangeretin, respectively. The stock solution of IS was further diluted with methanol to give the working solution containing 5000 ng mL\(^{-1}\) quercetin.

Animals, drug administration and plasma collection  Six Sprague–Dawley male rats (body weight, 250 ± 10 g) were supplied by the Animal Center of Traditional Chinese Medicine University Of Guangzhou (SCXK 2000A037). The experimental protocol was approved by the University Ethics Committee for the use of experimental animals and conformed to the Guide for Care and Use of Laboratory Animals. The rats were maintained in air-conditioned animal quarters under the following conditions: temperature 22 ± 2°C, relative humidity 55 ± 10%, free access to water, and feeding with laboratory rodent chow (Guangzhou, China). The animals were acclimatized to the facilities for 14 days and then fasted with free access to water for 12 h prior to the experiment.

To calculate the administration dose, the contents of nobiletin and tangeretin in ECRP were first quantitatively analyzed. The conditions for HPLC analysis were modified based on a previously published method (Li et al., 2012). The contents of nobiletin and tangeretin in ECRP were determined to be 13.30 and 10.68 mg/g, respectively.

Nobiletin and tangeretin were orally administered to rats at the dosages of 260.6 and 213.6 mg kg\(^{-1}\) respectively for 20 mL kg\(^{-1}\) ECRP dissolved by 0.5% sodium carboxymethyl cellulose(CMC-Na). Blood samples (0.5 mL) were collected at 0.33, 0.66, 1, 1.5, 2, 2.5, 4, 6, 8, 12 and 24 h after a single dose. The rats had free access to water during the experiment. The blood samples were immediately heparinized and centrifuged at 4000 \(\times\) g for 15 min, and the supernatant was harvested into 0.2 mL aliquots and stored in 1.5 mL polypropylene tubes at −20°C prior to analysis. All pharmacokinetic parameters were processed by non-compartmental analysis using DAS 2.0 pharmacokinetic program.

Sample preparations  All the standards and the spiked plasma were similarly treated. For quantitative analysis, an aliquot (0.2 mL) of rat plasma was pipetted into 1.5 mL plastics centrifuge tubes with addition of 1 mL of ethyl acetate, which was spiked with 50 \(\mu\)L (5 \(\mu\)g mL\(^{-1}\)) of IS (quercetin). The samples were vortex-mixed for 3 min and centrifuged at 12000 \(\times\) g for 10 min. The upper layer was transferred into another 1.5 mL plastics centrifuge
tube and the extraction was repeated again. Two pieces of upper layer were combined and blow-dried by nitrogen in a 45 °C water bath. The residual was re-dissolved by adding 100 µL of methanol and then centrifuged at 12000 × g for 5 min. Aliquots (20 µL) of the supernatant were injected into the LC-UV system.

**Method validation** The specificity of the method was assessed by comparing the lowest concentration in the calibration curves with that of blank rat plasma that had undergone the same pretreatment and analysis.

Calibration curves were plotted by spiking blank plasma with proper volumes of one of the working solutions mentioned above to produce the final standard curve points equivalent to 2500, 1000, 500, 250.0, 100.0, 25.0, 10.00 and 5.000 ng mL⁻¹ nobiletin and tangeretin, respectively. The subsequent assay procedures were the same as those described above. Calibration curves were generated by using the ratios of the analyte peak area to the IS peak area vs. concentration and were fitted to the equation \( y = bx + a \) by weighted least-squares linear regression, where \( x \) corresponds to the peak area ratio and \( y \) refers to the concentration added to plasma.

Quality control (QC) samples were prepared by spiking blank plasma with proper volumes of one of the working solutions mentioned above to produce the final concentrations equivalent to 5.000 ng mL⁻¹ (low level), 250.0 ng mL⁻¹ (moderate level) and 2500 ng mL⁻¹ (high level) nobiletin and tangeretin, respectively. The following procedures were the same as those described above. The limit of detection (LOD) and the limit of quantification (LOQ) were determined as the concentrations with signal-to-noise ratios of 3 and 10, respectively.

The precision was evaluated by assaying five replicates of QC samples at low, moderate and high concentrations (5.000, 250.0 and 2500 ng mL⁻¹) on the same day for five consecutive days. The accuracy was determined by analyzing five sets of spiked plasma samples of nobiletin and tangeretin at each QC level on the same day. Precision was measured by intra- and inter-day relative standard deviations (RSDs) and accuracy was described as relative error (RE).

The extraction recoveries were determined at three QC levels by comparing the peak area obtained from plasma sample spiked before extraction with from plasma samples spiked those after extraction. This procedure was repeated (\( n = 5 \)) at each QC level (5.000, 250.0 and 2500 ng mL⁻¹).

The stabilities of nobiletin and tangeretin were assessed by determining QC plasma samples kept at low temperature (−20 °C) for 5 days. The freeze-thaw stability was tested by analyzing QC plasma samples undergoing three freeze (−20 °C) and thaw (room temperature) cycles on consecutive days. This procedure was repeated (\( n = 5 \)) at each QC level (5.000, 250.0 and 2500 ng mL⁻¹) as well.

**Results and Discussion**

### 1 Cellular Antioxidant Activity Assay

**Determination of injury concentration of \( \text{H}_2\text{O}_2 \)** The viability of PC12 cells after treatment with 100 µM \( \text{H}_2\text{O}_2 \) for 4 h, which was measured by MTT reduction assay, decreased to 85% of controls (Fig. 1). In other words, PC12 cells were only mildly damaged. Thus, 100 µM \( \text{H}_2\text{O}_2 \) was used to produce oxidative stress to PC12 cells in the cellular antioxidant activity assay.
Table 1. Effects of different drugs on activities of SOD, MDA, GSH-Px in H₂O₂-treated PC12 cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MDA (nmol mg⁻¹ prot)</th>
<th>SOD (U mg⁻¹ protein)</th>
<th>GSH-Px (U mg⁻¹ prot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>7.03 ± 1.26</td>
<td>105.53 ± 6.85</td>
<td>295.14 ± 12.46</td>
</tr>
<tr>
<td>H₂O₂-injured group</td>
<td>22.59 ± 1.89</td>
<td>80.35 ± 3.62</td>
<td>241.16 ± 11.63</td>
</tr>
<tr>
<td>ECRP</td>
<td>12.26 ± 1.47</td>
<td>91.07 ± 5.93</td>
<td>265.12 ± 16.94</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>21.18 ± 1.12</td>
<td>87.54 ± 7.63</td>
<td>252.61 ± 13.55</td>
</tr>
<tr>
<td>Nobiletin</td>
<td>13.84 ± 1.53</td>
<td>93.68 ± 6.17</td>
<td>270.38 ± 11.14</td>
</tr>
<tr>
<td>Tangeretin</td>
<td>12.98 ± 1.22</td>
<td>90.3 ± 2.88</td>
<td>263.56 ± 18.32</td>
</tr>
<tr>
<td>PMF 3</td>
<td>21.71 ± 1.37</td>
<td>84.38 ± 5.45</td>
<td>244.17 ± 12.96</td>
</tr>
<tr>
<td>PMF 4</td>
<td>21.03 ± 1.97</td>
<td>83.24 ± 4.12</td>
<td>234.8 ± 16.55</td>
</tr>
<tr>
<td>PMF 5</td>
<td>7.91 ± 1.14</td>
<td>96.28 ± 3.77</td>
<td>280.96 ± 14.16</td>
</tr>
<tr>
<td>PMF 6</td>
<td>7.86 ± 0.96</td>
<td>96.41 ± 2.87</td>
<td>281.02 ± 15.93</td>
</tr>
</tbody>
</table>

PC12 cells were pretreated with ECRP or PMFs for 24 h and then exposed to 100 μM H₂O₂ for additional 4 h. PMF3, 5-hydroxy-6,7,8,3′,4′-5 methoxy flavones; PMF4, 5,7,8,4′-4 methoxy flavones; PMF5, 5,6,7,3′,4′-5 methoxy flavones; PMF6, 3,5,6,7,8,3′,4′-7 methoxy flavones. Data were represented as means ± SD (n = 5). (▲) (P < 0.01), compared to that of the control group; (▲▲) (P < 0.05), compared to that of the injured group; (▲▲▲) (P > 0.05), compared to that of the injured group.

 Determination of Optimum concentrations of PMFs and ECRP
As shown in Fig. 2, the viabilities of PC12 cells rise to various extents after pretreatment with PMFs and ECRP, inferring that they could protect PC12 cells. But with increasing drug concentration, the viabilities of PC12 cells remained unchanged or declined slightly. Thus, the optimum pretreatment concentrations of all samples were as follows: ECRP, 5,6,7,3′,4′-5 methoxy flavones, 6.25 μmol L⁻¹; hesperidin, nobiletin, tangeretin and 3,5,6,7,8,3′,4′-7 methoxy flavones, 12.5 μmol L⁻¹; 5-hydroxy-6,7,8,3′,4′-5 methoxy flavones, 25.0 μmol L⁻¹.

PMFs and ECRP Protected PC12 Cells against H₂O₂-Induced Cytotoxicity
The effects of PMFs and ECRP on MDA level reduction, lipid peroxidation, activities of SOD and GSH-Px were evaluated as described above. H₂O₂ alone increased the MDA level and decreased the activities of SOD and GSH-Px significantly compared with those of the control groups (P < 0.01) (Table 1). In contrast, except for PMF 3 and 4, pre-incubation with other PMFs, ECRP and hesperidin observably reduced the MDA levels and improved the activities of SOD and GSH-Px compared with those of the injured group (P < 0.05). Hence, H₂O₂-induced cytotoxicity in PC12 cells was suppressed by several PMFs and ECRP.

2 Pharmacokinetics Study
Method validation
Representative chromatograms obtained from blank plasma, blank plasma spiked with the analytes and IS, and plasma sample after oral administration are shown in Fig. 3. No endogenous interference appeared at around the retention times of the analytes and IS in drug-free specimens. The retention times of IS, nobiletin and tangeretin (Fig. 3, panel 3) were 26.2 (peak 1), 35.6 (peak 2) and 39.1 min (peak 3), respectively. This method exhibited an excellent linear response over the selected concentration range of 6.500 – 2500 ng mL⁻¹ for nobiletin and tangeretin by weighted (1/x) least-squares linear regression analysis. The mean standard curve was typically described by the equation: y = 0.0012x + 0.0202, r = 0.999 for nobiletin and y = 0.0012x + 0.0202, r = 0.999 for tangeretin. The LODs of both nobiletin and tangeretin were 0.2 ng/mL and the LOQs were 0.5 ng mL⁻¹.

Intra-day and inter-day precisions and accuracies of the method for nobiletin and tangeretin are presented in Table 2. The intra-day and inter-day precision deviations were all within 15% of the RSD at each QC level, and the accuracy deviations were all within (100 ± 15)% of the actual values at each QC level. Therefore, this
The extraction efficiencies measured for nobiletin and tangeretin in rat plasma were consistent, precise and reproducible. The mean absolute extraction recoveries at each QC level (6.500, 250.0 and 2500 ng mL\(^{-1}\)) were 95.35 ± 3.25, 90.59 ± 2.49 and 91.11 ± 6.33% for nobiletin (n = 5), and 96.22 ± 2.99, 94.12 ± 1.68 and 93.68 ± 2.35% for tangeretin (n = 5), respectively.

The low-temperature (−20 °C and 4 °C) and freeze–thaw stabilities of nobiletin and tangeretin (Table 3) showed that they were reliably stable under the tested conditions.

**Pharmacokinetic study** The developed assay was sensitive adequately to measure nobiletin and tangeretin in rat plasma samples obtained following oral administration of ECRP. Since the two compounds were eliminated from the body almost linearly, the plasma concentration-time profiles (Fig. 4 and Fig. 5) were analyzed by non-compartmental methods based on the statistical

### Table 2. Precisions and accuracies of the method for determining nobiletin and tangeretin in rat plasma

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration added (ng mL(^{-1}))</th>
<th>Intra-batch (n=5)</th>
<th>Inter-batch (n = 5)</th>
<th>Accuracy (mean ± SD, ng mL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration found (mean ± SD, ng mL(^{-1}))</td>
<td>RSD (%)</td>
<td>Concentration found (mean ± SD, ng mL(^{-1}))</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>Nobiletin</td>
<td>2758</td>
<td>2738 ± 95.20</td>
<td>3.48</td>
<td>2868 ± 206.8</td>
</tr>
<tr>
<td></td>
<td>275.8</td>
<td>291.3 ± 17.48</td>
<td>6.00</td>
<td>374.4 ± 15.85</td>
</tr>
<tr>
<td></td>
<td>5.520</td>
<td>5.740 ± 0.41</td>
<td>7.07</td>
<td>5.86 ± 0.56</td>
</tr>
<tr>
<td>Tangeretin</td>
<td>3145</td>
<td>2944 ± 160.3</td>
<td>5.44</td>
<td>3082 ± 234.15</td>
</tr>
<tr>
<td></td>
<td>314.5</td>
<td>329.0 ± 18.02</td>
<td>5.47</td>
<td>371.0 ± 23.43</td>
</tr>
<tr>
<td></td>
<td>6.290</td>
<td>4.480 ± 0.21</td>
<td>4.76</td>
<td>5.34 ± 0.27</td>
</tr>
</tbody>
</table>

RSD: Relative Standard Deviation; RE: Relative Error.

### Table 3. Stabilities of nobiletin and tangeretin in rat plasma under tested conditions (n = 5)

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration added (ng mL(^{-1}))</th>
<th>−20 °C, 5 days</th>
<th>4 °C, 5 days</th>
<th>Freeze-thaw cyclese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nobiletin</td>
<td>250.0</td>
<td>113.8 ± 8.50</td>
<td>105.4 ± 1.96</td>
<td>101.5 ± 2.88</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>98.86 ± 5.75</td>
<td>101.1 ± 4.79</td>
<td>101.4 ± 2.20</td>
</tr>
<tr>
<td>Tangeretin</td>
<td>2500.0</td>
<td>114.8 ± 9.14</td>
<td>105.9 ± 1.42</td>
<td>102.3 ± 2.92</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>106.3 ± 1.19</td>
<td>102.9 ± 5.26</td>
<td>106.4 ± 3.03</td>
</tr>
</tbody>
</table>

Fig. 4. Plasma concentration–time profiles for nobiletin in rat plasma after oral administration of ECRP. The data were presented as mean ± SD.
moment theory. The estimated pharmacokinetic parameters presented in Table 4 demonstrated that both nobiletin and tangeretin were rapidly absorbed and then slowly decreased. The main pharmacokinetic parameters in our study were different from those reported in a previous study, probably owing to the difference between monomer and herbal medicine, as the latter has interactions between components.

### Discussion

Oxidative damage in brain occurs in most neurodegenerative diseases, such as AD, Parkinson’s disease, and Huntington’s disease (Ankacrona et al., 1995). PC12 cell line, which was initially derived from a rat adrenal medullary pheochromocytoma, displays phenotypic properties of both adrenal chromaffin cells and sympathetic neurons. For having typical characteristics of nerve cells in morphology and function, PC12 cells have been used widely as the in vitro model for neuronal injury and oxidative stress because of the homogeneous cell population (Greene and Tischler, 1976). Of the chemicals capable of inducing apoptosis of PC12 cells, including H$_2$O$_2$, linoleic acid hydroperoxide, tert-butyl hydroperoxide, 2,2’-azobis(2-amidinopropane) dihydrochloride, dopamine and amyloid β-peptide (Aβ), H$_2$O$_2$ is a representative ROS (Sasaki et al., 2001; Chen et al., 2003; Heo et al., 2004). Although H$_2$O$_2$ is not reactive per se, it forms highly reactive and deleterious hydroxyl radicals by the Fenton reaction in the presence of transition metal ions. Meanwhile, H$_2$O$_2$ partially mediates the toxicity of Aβ, the major pathological hallmark for AD (Behl et al., 1994). Therefore, H$_2$O$_2$ has been adopted extensively to induce the apoptosis of various cells (Park et al., 2003; Ju et al., 2004).

In this study, the protective effects of hesperidin and PMFs of ECRP on H$_2$O$_2$-induced cytotoxicity in PC12 cells were investigated by several biochemical assays. The data showed that most PMFs and ECRP were neuroprotective against H$_2$O$_2$-induced cytotoxicity in PC12 cells by diverse mechanisms and can at least be attributed to their antioxidant properties such as reduction of MDA levels and improvement of SOD and GSH-Px activities. Unlike the widely studied nobiletin and tangeretin, the intracellular antioxidant activities of PMF3-6 have never been reported before. However, it is worth noting that hesperidin, PMF3 and PMF4 exhibited weaker antioxidant effects in this study compared with other PMFs did. Because of the aglycone thereof, hesperidin’s polar is so high that it can hardly penetrate the cell membrane. The relationship between structure and bioactivity of PMFs has been investigated (Kwaii et al., 2012; Vermeer et al., 2010). Increasing methoxyl groups on the B-ring moiety lowers the activities of PMFs by A-ring methoxylation. When the number of A-ring methoxyl groups is 0 – 3, attachment of more than two methoxyl groups on the B-ring moiety would completely diminish the activity. Although each of the PMFs with four methoxyl groups on
the A-ring moiety is significantly active, the activity is continuously reduced by increasing B-ring methoxyl groups. On the contrary, increasing A-ring methoxyl groups enhances the activity. In the meantime, the position of methoxyl group on the B-ring moiety evidently affects the activity. The activity is decreased when a methoxyl group is attached to the 4′-position. In contrast, many 3′-methoxylated PMFs are remarkably active. In short, the different antioxidant effects of PMFs in this study can be ascribed to the increase of the A-ring or the B-ring methoxyl groups.

Su et al. (2012) have investigated the effects of nobiletin and its metabolite 3′,4′-dihydroxy-5,6,7,8-tetramethoxyflavone (DTF) on radical-scavenging, GSH level, glutamate-cysteine ligase gene expression, heme oxygenase-1 (HO-1) gene expression, NF-κB activation and other signaling pathways. Only DTF, but not nobiletin, suppressed the intracellular ROS accumulation in H2O2-treated cells, and induced heme oxygenase-1 gene expression by suppressing the NF-κB activation. The action mechanisms of PMF derivatives based on the findings are being studied in our group.

To determine the contents of PMFs absorbed in blood, and to evaluate the bioavailability of PMFs in plant extract, the pharmacokinetics of nobiletin and tangeretin, which had maximum antioxidative components, were also studied. Compared with those of purified nobiletin and tangeretin in rat serum, the pharmacokinetic parameters in our study were different. The peak-reaching time of tangeretin in serum after administration of purified tangeretin was close to that of ECRP administration, while the administration of purified nobiletin rendered the peak serum level much earlier than that of ECRP did. The results may be attributed to the difference between pure compound and plant extract, as the latter was subject to interaction between constituents. In addition, the method was the first LC–UV quantitative assay of nobiletin and tangeretin in rat plasma samples following oral administration of ECRP. Being specific, sensitive, accurate and reproducible, the method was successfully applied to the pharmacokinetic studies of PMFs in biological samples. Given the valuable findings herein, the tissue distributions of nobiletin and tangeretin, especially their presence in brain, should be further studied.

References


