In Vitro Antiproliferative Properties and Flavanone Profiles of Six Grapefruit (Citrus paradisi Macf.) Cultivars

Lorenzo Camarda* • Vita Di Stefano • Rosa Pitonzo • Domenico Schillaci

ABSTRACT

Recent studies suggest an inverse relationship between the intake of Citrus fruits and the incidence of cardiovascular diseases, stroke and different cancer types. Protective effects shown could be ascribed to the flavonoid content of Citrus fruits. A reversed-phase high-performance liquid chromatographic method was developed to determine the content of flavanone glycosides, which are the main flavonoids present in Citrus fruits. Fresh squeezed juices of six different grapefruit cultivars (Citrus paradisi Macf.) were analysed. In all grapefruit juices tested, we found total flavanone glycoside contents in the range from 33.93 to 44.97 mg/100 ml of juice; naringin was the main flavanone component (16.37-26.14 mg/100 ml of juice). To evaluate pharmacological activity and, in particular, inhibitory effects in vitro on proliferation and growth of cancer cells, the six freshly squeezed grapefruit juices were tested against K562 (human chronic myelogenous leukemia), NCI-H460 (human lung cancer) and MCF-7 (human breast adenocarcinoma) cell lines. Most of the tested grapefruit juices showed distinct antiproliferative activity against all three cancer cell lines; in particular ‘Ruby Red’ and ‘Foster’ grapefruit juices showed the best activity in inhibiting the growth of NCI-H460 and MCF-7 cell lines at 3% v/v (fresh juice volume diluted in cell culture medium).

Keywords: cancer cell lines, flavanone glycosides, HPLC, juice analyses

INTRODUCTION

Recent epidemiological studies, in vitro and in vivo, have shown that the intake of a protective effect against cancer and cardiovascular diseases (Borradaile et al. 1999; Tanaka et al. 2000, 2001; Tian et al. 2001; Poulou et al. 2005).

Citrus plants are a rich source of health-promoting flavonoids, especially flavanone glycosides, which show an important role in the prevention of oxidative stress-related chronic diseases and against some types of cancer (Robak and Gryglewski 1988; Francis et al. 1989; Chen et al. 1990; Kuo 1996; Yang et al. 2001; Yu et al. 2005). The content of flavanone glycosides in fruits can be influenced by various factors such as genotypic differences, climatic conditions, cultural practices and degree of ripeness (Albach et al. 1981; Rouzeft et al. 1987; Ross et al. 2000).

Grapefruit, widely cultivated in Sicily, was originally believed to be a spontaneous hybrid of pummelo (Citrus maxima). Botanist James MacFayden, in his Flora of Jamaica in 1837, was the first to assign grapefruit with a botanical name, Citrus paradisi Macf (Morton 1987). The varieties have different flesh colour of fruits due to the presence (pink/red) or absence (blond) of lycopenes.

Our aim was to investigate freshly squeezed juices of six different grapefruit cultivars: ‘Duncan’, ‘Imperial’, ‘Foster’, ‘Tompson’ with blond pulp, ‘Red Blush’ and ‘Ruby Red’ with pigmented pulp, to evaluate flavanone glycosides content.

In order to evaluate pharmacological activity and, in particular, inhibitory effects in vitro on proliferation and growth of cancer cells, the six freshly squeezed grapefruit juices were tested against K562 (human chronic myelogenous leukemia), NCI-H460 (human lung cancer) and MCF-7 (human breast adenocarcinoma) cell lines.

MATERIALS AND METHODS

Chemicals

Naringenin, hesperitin, eriocitrin (eriodictyol 7-rutinoside), neoeiocitrin (eriodictyol 7-neohesperidoside), narirutin (naringenin 7-rutinoside), naringin (naringenin-7-neohesperidoside), hesperidin (hesperetin-7-rutinoside), neohesperidin (hesperetin-7-neohesperidoside), isosakuranetin 7-rutinoside, and poncirin (isosakuranetin 7-neohesperidoside) were obtained from Apin Chemicals LTD UK; quercetin (quercetin 3-rhamnoside) was obtained from Sigma (Sigma-Aldrich Co., Germany). These served as standards. Methanol and water were HPLC grade (Lab-Scan, Ireland). All other chemicals used were analytical grade.

Fruit samples preparation

All samples of Citrus paradisi Macf. cvs. ‘Duncan’, ‘Imperial’, ‘Foster’, ‘Tompson’ (blond pulp) and cvs. ‘Red Blush’ and ‘Ruby Red’ (pigmented pulp) were collected from healthy mature trees in an experimental orchard, located near Palermo, at a commercial ripening stage (i.e. ripe enough to be eaten), in February 2006. All cultivars shared the same environmental, cultural and soil conditions, thus the differences of the chemical composition among cultivars are neither influenced by climatic factors or crop techniques.

Twelve fruits of each variety were randomly collected. Fruits were immediately hand-squeezed and juices were centrifuged at 7000 rpm for 20 min at 4°C. Clarified juices (20 ml) were diluted with water HPLC grade (1:1 v/v), filtered through a 0.45 μm hydrophilic cellulose acetate membrane syringe filter (Sartorius Stedim, Italy) and stored at -8°C. Sample juice (20 μl) was injected in triplicate into the HPLC system to determine the concentration of flavanones.

Preparation of stock solutions

Each flavanone stock solution (0.5 mg/ml) was prepared in metha-
nol (HPLC grade) and stored at 8°C. Daily, well-defined volume samples were drawn from each stock solution, joined and diluted in methanol to produce four working standard solutions at 0.005, 0.02 and 0.1 mg/ml for each flavanone. Standard solutions obtained were filtered through a 0.45 µm hydrophilic cellulose acetate filter (Sartorius Stedim, Italy), injected into the HPLC system and assayed in triplicate to generate the corresponding standard curves.

HPLC Analyses were performed on a liquid chromatograph Agilent 1100 Series coupled to a Chemstation HP. The instrumentation included a binary pump, model G1312A and a UV-variable wavelength detector, model G1314A.

To choose the best UV wavelength for all flavanones investigated, at the beginning of our study we looked for the maximum UV absorption wavelength for each flavanone standard. We found that 280 nm was the best wavelength that afforded good sensitivity and maximum signal/noise ratio for all flavanones. Separation was achieved using a reversed-phase Supelcosil LC-18 (Supelco, USA) analytical column (4.6 mm i.d. × 250 mm, 5 µm particle size), maintained at 25°C with a column temperature controller (Thermosphere TS-130, Phenomenex, Torrance, California).

HPLC gradient analyses were performed using the following mobile phase: water containing 0.1% formic acid (A) and methanol (B). A gradient program was used as follows: 0 min 10% B, 1-15 min 10-25% B linear, 16-50 min 95% B linear, 51-60 min 10% B for column equilibration. Each mobile phase was freshly prepared each day and degassed ultrasonically for 20 min before use. The flow rate was 0.7 ml/min and injected volume was 20 µl. The separation took about 60 min under the described conditions including column equilibration. HPLC conditions were optimized for separation of all investigated flavanone glycosides.

Flavanone identification was performed by comparing retention times of each flavanone standard solution (Table 1) with flavanones present in grapefruit juices tested.

**Data analysis**

Calibration curves were produced using linear regression of the peak area against concentration of each flavanone standard solution. All curves were linear over the concentration ranges considered for each flavanone (r²>0.99). Calculation of flavanone glycoside concentration (expressed in mg/100 ml) was carried out by an external standard method, using calibration curves.

<table>
<thead>
<tr>
<th>Rt (min.)</th>
<th>Flavanone glycosides</th>
</tr>
</thead>
<tbody>
<tr>
<td>29.06</td>
<td>neoeriocitrin</td>
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<tr>
<td>31.20</td>
<td>naringin</td>
</tr>
<tr>
<td>31.75</td>
<td>naringin</td>
</tr>
<tr>
<td>32.36</td>
<td>hesperidin</td>
</tr>
<tr>
<td>32.83</td>
<td>neohesperidin</td>
</tr>
<tr>
<td>35.82</td>
<td>didymin</td>
</tr>
<tr>
<td>36.16</td>
<td>poncirin</td>
</tr>
<tr>
<td>36.21</td>
<td>quercetin</td>
</tr>
</tbody>
</table>

In vitro cytotoxicity studies

Fruit juices of six different grapefruit cultivars were tested in vitro for antiproliferative activity against K562 (human chronic myelogenous leukemia), NCI-H460 (human lung cancer) and MCF-7 (human breast adenocarcinoma) cell lines. These cell lines were grown at 37°C under a humidified atmosphere containing 5% CO₂ in Roswell Park Memorial Institute-1640 medium (RPML, Sigma) or Minimum Essential medium (MEM, Sigma) in the case of NCI-H460 and MCF-7, supplemented with 10% fetal calf serum and antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml and amphotericin B 2.5 µg/ml). K562 cells were suspended at a density of 1 x 10⁶ cells/ml in growth medium, transferred to 24-well plate (1 ml per well), cultured with or without (in the case of control wells) a screening volume of 50 µl of fruit juices and incubated at 37°C for 48 h. Control wells were added with 50 µl of a citric acid solution (1% v/v), the pH of which is comparable to that of fresh juices (pH 5.5).

The number of viable cells was determined by counting in a hemacytometer after dye exclusion with trypan blue (Manfredini et al. 1992). Anti-proliferative activity against NCI-H460 and MCF-7 was determined by MTT (methyltetrazolium) assays (Car michael et al. 1987).

The experiment was carried out as follows: cells were suspended at a density of 4 x 10⁵ cells per ml for NCI-H460 or 2 x 10⁵ cells for MCF-7 per ml in MEM (Minimum Essential medium), supplemented with 10% fetal calf serum and antibiotics, transferred (100 µl per well) to a 96-well plate and incubated at 37°C for 4 days until cellular monolayer formation. After this time, the original medium was replaced with RPMI (Roswell Park Memorial Institute-1640 medium) without red phenol and with juice fruit screening volume of 5 µl (corresponding to 5% v/v) was added to each well and incubated for three days.

The optical density (OD) of each well was read by a microplate reader (ELX 800, Bio-Tek Instruments) at 570 nm with background subtraction at 630 nm.

The anti-proliferative effects of fruit juices were estimated in terms of percent growth inhibition by comparing the average of optical density of the growth control wells (with 5 µl of a citric acid solution 1%) respect of sample wells, by their reduction of the tetrazolium substrate MTT. We have calculated, by the following formula, the inhibition percentages for each concentration of juices:

\[
\text{OD growth control - OD sample} \times 100 \\
\text{OD growth control}
\]

**RESULTS AND DISCUSSION**

Table 2 shows the quantities (mg/100 ml of juice) of flavanone glycosides in filtered fresh squeezed juices of six different grapefruit cultivars 'Duncan', 'Imperial', 'Foster', 'Tompson' (blond pulp) and 'Red Blush' and 'Ruby Red' (pigmented pulp).

Naringin was the dominant flavanone glycoside in all grapefruit cultivars: 'Red Blush' (21.45 mg/100 ml), 'Duncan' (20.67 mg/100 ml), 'Tompson' (20.38 mg/100 ml), 'Foster' (19.84 mg/100 ml) and 'Imperial' (16.37 mg/100 ml). Significant amounts of naringin (26.14 mg/100 ml) and narinriut (10.67 mg/100 ml) were found in 'Ruby Red' and high amounts of neohesperidin were found in 'Imperial' (8.25 mg/100 ml); this is the only grapefruit cultivar that contained neoeriocitrin (3.04 mg/100 ml).

The total content of flavanone glycosides in all grapefruit juices tested ranged from 44.97 mg/100 ml in 'Ruby Red' variety to 33.93 mg/100 ml in 'Duncan'.

All the investigated juices exhibited evident antiproliferative activities against human chronic myelogenous leukemia K562, human breast adenocarcinoma MCF-7 and human lung cancer NCI-H460 (Table 3). 'Foster' and 'Ruby Red' exhibited the greatest significant inhibitory effect against all three cell lines at a screening concentration of 5% (v/v). As shown in Table 3, juice of 'Ruby Red' completely inhibited the proliferation of human lung cancer NCI-H460, and the proliferation of human breast adenocarcinoma MCF-7 by 98.3%.

Since juices of 'Foster' and 'Ruby Red' showed the highest growth inhibition percentages, we evaluated the
growth inhibition percentages at a lower screening concentration of 3% (v/v) (Table 4) with the aim of comparing the activity of juices.

Data obtained showed that juice of ‘Ruby Red’ had the best activity against all cell lines tested at both screening concentrations, i.e. 3 and 5% (v/v).

Various mechanisms have been identified about antitumor effects of different flavonanes which may act in different development stages of cancer by protecting DNA against oxidative damage, inactivating carcinogens, inhibiting the expression of the mutagenic genes and enzymes responsible for activating procarcinogenic substances, and activating the systems responsible for xenobiotic detoxification (Manthey and Guthrie 2002; Benavente-García and Castillo 2008). There are several studies about anti-inflammatory activity.

Table 2

<table>
<thead>
<tr>
<th>Grapefruit cultivar</th>
<th>neoeriocitin</th>
<th>narirutin</th>
<th>naringin</th>
<th>hesperidin</th>
<th>neohesperidin</th>
<th>didymin</th>
<th>poncirin</th>
<th>quercetin</th>
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<tbody>
<tr>
<td>‘Imperial’</td>
<td>3.04 ± 1.6</td>
<td>7.69 ± 0.9</td>
<td>16.37 ± 0.2</td>
<td>1.59 ± 2.3</td>
<td>8.25 ± 1.5</td>
<td>-</td>
<td>2.80 ± 1.1</td>
<td>-</td>
</tr>
<tr>
<td>‘Foster’</td>
<td>-</td>
<td>9.96 ± 1.5</td>
<td>19.84 ± 0.4</td>
<td>0.93 ± 0.5</td>
<td>0.15 ± 2.1</td>
<td>2.54 ± 1.8</td>
<td>3.90 ± 0.5</td>
<td>3.09 ± 0.3</td>
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<tr>
<td>‘Duncan’</td>
<td>-</td>
<td>7.93 ± 1.7</td>
<td>20.67 ± 1.6</td>
<td>1.41 ± 1.7</td>
<td>1.20 ± 1.2</td>
<td>1.80 ± 2.7</td>
<td>0.92 ± 2.3</td>
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<tr>
<td>‘Ruby Red’</td>
<td>-</td>
<td>10.67 ± 2.1</td>
<td>26.14 ± 0.9</td>
<td>1.63 ± 0.3</td>
<td>1.49 ± 0.8</td>
<td>1.54 ± 0.5</td>
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<tr>
<td>‘Red-Blush’</td>
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<td>6.70 ± 1.1</td>
<td>21.45 ± 0.6</td>
<td>0.55 ± 2.5</td>
<td>1.40 ± 0.2</td>
<td>0.82 ± 1.3</td>
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<tr>
<td>‘Tompson’</td>
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<td>7.05 ± 1.2</td>
<td>20.38 ± 1.1</td>
<td>1.08 ± 2.1</td>
<td>1.37 ± 1.3</td>
<td>1.09 ± 0.9</td>
<td>2.59 ± 0.6</td>
<td>1.22 ± 0.7</td>
</tr>
</tbody>
</table>

REFERENCES


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Robak J, Gryglewski RJ (1988) Flavonanes are scavengers of superoxide anions. Biochemical Pharmacology 37, 837-841


Table 3

<table>
<thead>
<tr>
<th>Grapefruit cv.</th>
<th>K-562</th>
<th>NCI-H460</th>
<th>MCF-7</th>
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<tbody>
<tr>
<td>‘Imperial’</td>
<td>ns</td>
<td>31.1</td>
<td>36.2</td>
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<tr>
<td>‘Foster’</td>
<td>67.5</td>
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<td>21.9</td>
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Table 4

<table>
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<tr>
<th>Grapefruit cv.</th>
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<td>‘Foster’</td>
<td>33.8</td>
<td>14.0</td>
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