

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

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

Università degli Studi di Palermo, Facoltà di Farmacia, Dipartimento di Chimica e Tecnologie Farmaceutiche, via Archirafi n°32, 90123 Palermo, Italy

Corresponding author: * camarnat@unipa.it

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 *Citrus* has a protective effect against cancer and cardiovascular diseases (Borradaille *et al.* 1999; Tanaka *et al.* 2000, 2001; Tian *et al.* 2001; Poulose *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; Rouseff *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 lycopene.

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), neoeriocitrin (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; quercetrin (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-

Table 1 Flavanone glycosides standard retention times (Rt).

Rt (min.)	Flavanone glycosides
29.06	neeriocitrin
31.20	narirutin
31.75	naringin
32.36	hesperidin
32.83	neohesperidin
35.82	didymin
36.16	poncirin
36.21	quercetrin

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 ($r^2 > 0.99$). Calculation of flavanone glycosides concentration (expressed in mg/100 ml) was carried out by an external standard method, using calibration curves.

Linear simple regression model ($y = mx + q$) by the least-squares method.

neeriocitrin	$y = 0.0002x + 0.0103$	$r^2 = 0.9987$
narirutin	$y = 0.0002x - 0.0029$	$r^2 = 0.9990$
naringin	$y = 0.0002x + 0.0135$	$r^2 = 0.9998$
hesperidin	$y = 0.0002x + 0.0120$	$r^2 = 0.9997$
neohesperidin	$y = 0.0002x - 0.0208$	$r^2 = 0.9993$
didymin	$y = 0.0003x - 0.0138$	$r^2 = 0.9996$
poncirin	$y = 0.0002x - 0.0131$	$r^2 = 0.9998$
quercetrin	$y = 0.0004x - 0.0172$	$r^2 = 0.9999$

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 (RPMI, 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 1×10^5 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% w/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 (Carmichael *et al.* 1987).

The experiment was carried out as follows: cells were suspended at a density of 4×10^5 cells per ml for NCI-H460 or 2×10^5 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:

$$\frac{\text{OD growth control} - \text{OD sample}}{\text{OD growth control}} \times 100$$

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 narirutin (10.67 mg/100 ml) were found in 'Ruby Red'.

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

Table 2 Flavanones content expressed in mg/100 ml of juice (mean of three samples replicated two times) \pm % SD in filtered fresh squeezed juices of six different grapefruit cvs. 'Duncan', 'Imperial', 'Foster', 'Tompson' (blond pulp) and cvs. 'Red Blush' and 'Ruby Red' (pigmented pulp).

Grapefruit cultivar	neoroicitrin	narirutin	naringin	hesperidin	neohesperidin	didymin	poncirin	quercetrin
'Imperial'	3.04 \pm 1.6	7.69 \pm 0.9	16.37 \pm 0.2	1.59 \pm 2.3	8.25 \pm 1.5	-	2.80 \pm 1.1	-
'Foster'	-	9.96 \pm 1.5	19.84 \pm 0.4	0.93 \pm 0.5	0.15 \pm 2.1	2.54 \pm 1.8	3.90 \pm 0.5	3.09 \pm 0.3
'Duncan'	-	7.93 \pm 1.7	20.67 \pm 1.6	1.41 \pm 1.7	1.20 \pm 1.2	-	1.80 \pm 2.7	0.92 \pm 2.3
'Ruby Red'	-	10.67 \pm 2.1	26.14 \pm 0.9	1.63 \pm 0.8	1.49 \pm 0.8	1.54 \pm 0.5	3.50 \pm 1.1	-
'Red-Blush'	-	6.70 \pm 1.1	21.45 \pm 0.6	0.55 \pm 2.5	1.40 \pm 0.2	0.82 \pm 1.3	2.47 \pm 1.1	1.55 \pm 1.0
'Tompson'	-	7.05 \pm 1.2	20.38 \pm 1.1	1.08 \pm 2.1	1.37 \pm 1.3	1.09 \pm 0.9	2.59 \pm 0.6	1.22 \pm 0.7

Table 3 Growth inhibition percentages evaluated at screening concentration of 5% (v/v). Values are the mean of three independent determinations; coefficient of variation was less than 15%; ns = not significant i.e. below 10% inhibition.

Grapefruit cv.	K-562	NCI-H460	MCF-7
'Imperial'	ns	31.1	36.2
'Foster'	67.5	80.8	66.2
'Duncan'	47.7	34.1	30.7
'Ruby Red'	92.0	100	98.3
'Red Blush'	41.5	33.0	33.3
'Tompson'	36.9	22.1	21.9

Table 4 Growth inhibition percentages evaluated at screening concentration of 3% (v/v). Values are the mean of five independent determinations; coefficient of variation was less than 15%.

Grapefruit cv.	K-562	NCI-H460	MCF-7
'Ruby Red'	90.7	100	96.8
'Foster'	33.8	14.0	20.1

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 antitumoral effects of different flavanones 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 *in vitro* about anticancer activity of unconjugated flavanones such as the aglycone naringenin (Hakimuddin *et al.* 2004; Harmon and Patel 2004) but only few studies about flavanone glycosides such as naringin (Jeon *et al.* 2001; Kanno *et al.* 2003, 2004).

Our data show the best antiproliferative activity for 'Ruby Red' grapefruit juices that present high levels of flavanone glycosides (44.97 mg/100 ml) and in particular naringin (26.14 mg/100 ml). It is clear that the activity cannot be attributed to one single flavanone, but to the synergistic effects of several constituents.

Further experiments could be useful to relate 'Ruby Red' grapefruit intake with cancer incidence, as well as biomarker studies on biological pathways, such as biotransformation of carcinogens, DNA damage, cell proliferation, apoptosis and inflammation.

REFERENCES

- Albach RF, Redman GH, Cruse RR (1981) Annual and seasonal changes in naringin concentration of Ruby Red grapefruit juice. *Journal of Agricultural and Food Chemistry* **29**, 808-811
- Benavente-García O, Castillo J (2008) Update on uses and properties of *Citrus* flavonoids: new findings in anticancer, cardiovascular, and anti-inflammatory activity. *Journal of Agricultural and Food Chemistry* **56**, 6185-6205
- Borradaile NM, Carroll KK, Kurowska EM (1999) Regulation of hep2 cell apolipoprotein b metabolism by the *Citrus* flavanones hesperetin and naringenin. *Lipids* **34**, 591-659
- Carmichael J, DeGraff WG, Gazdar AP, Minna JD, Mitchel JB (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Research* **47**, 936-942
- Chen YT, Zheng RL, Jia ZJ, Ju Y (1990) Flavonoids as superoxide scavengers and antioxidants. *Free Radical Biology and Medicine* **9**, 19-21
- Francis AR, Shetty TK, Bhattacharya RK (1989) Modulating effect of plant flavonoids on the mutagenicity of N-methyl-N-nitro-N-nitrosoguanidine. *Carcinogenesis* **10**, 1953-1955
- Hakimuddin F, Paliyath G, Meckling K (2004) Selective cytotoxicity of a red grape wine flavonoid fraction against MCF-7 cells. *Breast Cancer Research and Treatment* **85**, 65-79
- Harmon AW, Patel YM (2004) Naringenin inhibits glucose uptake in MCF-7 breast cancer cells: a mechanism for impaired cellular proliferation. *Breast Cancer Research and Treatment* **85**, 103-110
- Jeon SM, Bok SH, Jang MK, Lee MK, Nam KT, Park YB, Rhee SJ, Choi MS (2001) Antioxidative activity of naringin and lovastatin in high cholesterol-fed rabbits. *Life Sciences* **69**, 2855-2866
- Kanno S, Shouji A, Asou K, Ishikawa M (2003) Effects of naringin on hydrogen peroxide-induced cytotoxicity and apoptosis in P388 cells. *Journal of Pharmacological Sciences* **92**, 166-170
- Kanno S, Shouji A, Hirata R, Asou K, Ishikawa M (2004) Effects of naringin on cytosine arabinoside (Ara-C)-induced cytotoxicity and apoptosis in P388 cells. *Life Sciences* **75**, 353-365
- Kuo SM (1996) Antiproliferative potency of structurally distinct dietary flavonoids on human colon cancer cells. *Cancer Letters* **110**, 41-48
- Manfredini S, Bazzanini R, Baraldi PG, Guarneri M, Simoni D, Marongiu ME, Pani A La Colla P, Tramontano E (1992) Pyrazole-related nucleosides. Synthesis and antiviral/antitumor activity of some substituted pyrazole and pyrazolo[4,3-d]-1,2,3-triazin-4-one nucleosides. *Journal of Medicinal Chemistry* **35**, 917-924
- Manthey JA, Guthrie N (2002) Antiproliferative activities of *Citrus* flavonoids against six human cancer cell lines. *Journal of Agricultural and Food Chemistry* **50**, 5837-5843
- Morton JF (Ed) (1987) Grapefruit. In: *Fruits of Warm Climates*, Miami, FL, pp 152-158
- Poulose SM, Harris ED, Patil BS (2005) Citrus limonoids induce apoptosis in human neuroblastoma cells and have radical scavenging activity. *Journal of Nutrition* **135**, 870-877
- Robak J, Gryglewski RJ (1988) Flavonoids are scavengers of superoxide anions. *Biochemical Pharmacology* **37**, 837-841
- Ross SA, Ziska DS, Zhao K, ElSohly MA (2000) Variance of common flavonoids by brand of grapefruit juice. *Fitoterapia* **71**, 154-161
- Rousseff RL, Martin SF, Youtsey CO (1987) Quantitative survey of narirutin, naringin, hesperidin and neohesperidin in citrus. *Journal of Agricultural and Food Chemistry* **35**, 1027-1030
- Tanaka T, Kohno H, Murakami M, Shimada R, Kagami S, Sumida T, Azuma Y, Ogawa H (2000) Suppression of azoxymethane-induced colon carcinogenesis in male F344 rats by mandarin juices rich in beta-cryptoxanthin and hesperidin. *International Journal of Cancer* **88**, 146-150
- Tanaka T, Maeda M, Kohno H, Murakami M, Kagami S, Miyake M, Wada K (2001) Inhibition of azoxymethane-induced colon carcinogenesis in male F344 rats by the *Citrus* limonoids obacunone and limonin. *Carcinogenesis* **22**, 193-198
- Tian Q, Miller ED, Ahmad H, Tang L, Patil BS (2001) Differential inhibition of human cancer cells proliferation by Citrus limonoids. *Nutrition and Cancer* **40**, 180-184
- Yang CS, Landau JM, Huang MT, Newmark HL (2001) Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annual Review of Nutrition* **21**, 381-406
- Yu J, Wang L, Walzem RL, Miller EG, Pike LM, Patil BS (2005) Antioxidant activity of Citrus limonoids, flavonoids and coumarins. *Journal of Agricultural and Food Chemistry* **53**, 2009-2014