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Antiproliferative Activities of Citrus Flavonoids against Six Human Cancer Cell Lines

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Citrus fruits contain high concentrations of several classes of phenols, including numerous hydroxycinnamates, flavonoid glycosides, and polymethoxylated flavones. The latter group of compounds occurs without glycosidic linkages and has been shown to inhibit the proliferation of a number of cancer cell lines. This antiproliferative property was further demonstrated against additional human cancer cell lines, and the antiproliferative actions of a series of synthetic methoxylated flavones were also studied. Similar to the naturally occurring compounds, the synthetic compounds exhibited strong antiproliferative activities. In many cases the IC_{50} values occurred below 10 μm. Other hydroxylated flavone and flavanone aglycons also exhibited antiproliferative activities against the cancer cell lines, with the flavones showing greater activities than the flavanones. Glycosylation of these compounds removed their activity. The strong antiproliferative activities of the polymethoxylated flavones suggest that they may have use as anticancer agents in humans.

KEYWORDS: Citrus; Rutaceae; flavonoid; polymethoxylated flavones; cancer; lung; prostate; breast; colon; melanoma

INTRODUCTION

The flavonoids in citrus occur in several groups, including the numerous flavanone and flavone glycosides and the highly methoxylated flavones, termed polymethoxylated flavones (*1*). The flavanones occur as *O*-glycosides, mainly as neohesperidosides or rutinosides, depending on the species of *Citrus* (*2*). The flavone glycosides in citrus occur as either *O*- or *C*-glycosides (**Figure 1**). Unlike these glycosidic compounds, the polymethoxylated flavones are considerably less polar and assume planar structures. These features of the polymethoxylated flavones influence their biological properties, including their permeabilities to biological membranes, metabolic fates, and binding properties. These properties, in turn, play critical roles in influencing the molecules' modes of actions, which often differ from those exhibited by the glycosidic flavonoids (*3*).

The main flavonoid glycoside in orange (*Citrus sinensis* L.), hesperidin, and its flavone analogue, diosmin, have exhibited anticarcinogenic activities in various in vivo studies (*4*−*6*). These actions likely depend on the molecules' antioxidant properties, as well as their abilities to modulate the activity levels of detoxifying hepatic enzymes. Typically, these molecules show little effect on the proliferation of cancer cells in vitro. Glycosidation likely blocks the entry of flavonoids into cells and may also sterically inhibit their binding to receptors involved in gene expression. In contrast, the polymethoxylated flavones have been shown in numerous in vitro studies to exert strong antiproliferative actions against cancer cells (*7*−*13*) and antigen-activated T-lymphocytes (*14*). In these studies, cellular differentiation by leukemic HL-60 cells co-occurs with the antiproliferative actions of the polymethoxylated flavones. Antimetastatic actions against human breast cancer cells have also been observed for tangeretin (*15*, and references cited therein), although antitumor activity for tangeretin was not observed in a follow-up in vivo study (*16*).

To further characterize the inhibitory effects of polymethoxylated flavones on cancer cell growth, we measured the antiproliferative properties of these compounds, as well as a group of synthetic polymethoxylated flavone analogues, against six common human cancer cell lines including lung, prostate, colon, melanoma, and estrogen receptor positive (ER+) and estrogen receptor negative (ER−) breast cancer. The synthetic derivatives were observed in this study to have antiproliferative activity levels similar to those obtained with the naturally occurring polymethoxlyated flavones, and in several instances, very high antiproliferative activities were observed. These findings suggest that the citrus polymethoxylated flavones may be effective in certain cases against cancer cell proliferation, hence creating potential valuable uses for these citrus compounds.

MATERIALS AND METHODS

Naturally Occurring Flavonoids. Naturally occurring polymethoxylated flavones were isolated from orange peel (*1*, and references cited therein). Apigenin, kaempferol, and quercetin were obtained from

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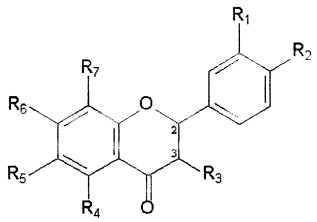

Compound	R1	R2	R3	R4	R5	R6	R7	C2-C3
1. 3,5,6,7,8,3',4'-heptamethoxyflavone	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	double
2. Tangeretin	H	OCH_3	H	OCH_3	OCH_3	OCH_3	OCH_3	double
3. Nobiletin	OCH_3	OCH_3	H	OCH_3	OCH_3	OCH_3	OCH_3	double
4. Sinensetin	OCH_3	OCH_3	H	OCH_3	OCH_3	OCH_3	H	double
5. Tetramethylscutellarein	H	OCH_3	H	OCH_3	OCH_3	OCH_3	H	double
6. 5-Desmethylnobiletin	OCH_3	OCH_3	H	H	OCH_3	OCH_3	OCH_3	double
7. Tetramethylisoscutellarein	H	OCH_3	H	OCH_3	H	OCH_3	OCH_3	double
8. 5-Desmethylsinensetin	OCH_3	OCH_3	H	OH	OCH_3	OCH_3	H	double
9. Quercetin 3,5,7,3',4'-pentamethylether	OCH_3	OCH_3	OCH_3	OCH_3	H	OCH_3	H	double
10. Quercetin 3,7,3',4'-tetramethylether	OCH_3	OCH_3	OCH_3	OH	H	OCH_3	H	double
11. Limocitrin 3,5,7,4'-tetramethylether	OCH_3	OCH_3	OCH_3	OCH_3	H	OCH_3	OCH_3	double
12. Quercetin 5,7,3',4'-tetramethylether	OCH_3	OCH_3	OH	OCH_3	H	OCH_3	H	double
13. Limocitrin 3,7,4'-trimethylether	OCH_3	OCH_3	OCH_3	OH	H	OCH_3	OCH_3	double
14. Quercetin 5,7,3',4'-tetramethylether-3-acetate	OCH_3	OCH_3	acetate	OCH_3	H	OCH_3	H	double
15. Limocitrin 3,7,4'-trimethylether-5-acetate	OCH_3	OCH_3	OCH_3	acetate	H	OCH_3	OCH_3	double
16. Quercetin 3,7,3',4'-tetramethylether-5-acetate	OCH_3	OCH_3	OCH_3	acetate	H	OCH_3	H	double
17. 5,8-dihydroxy-3,7,3',4'-tetramethoxyflavone	OCH_3	OCH_3	OCH_3	OH	H	OCH_3	OH	double
18. Rhamnetin	OH	OH	OH	OH	H	OCH_3	H	double
19. Kaempferol	H	OH	OH	OH	H	OH	H	double
20. Chrysoeriol	H	H	H	OH	H	OH	H	double
21. Apigenin	H	OH	H	OH	H	OH	H	double
22. Luteolin	OH	OH	H	OH	H	OH	H	double
23. Quercetin	OH	OH	OH	OH	H	OH	H	double
24. Eriodictyol	OH	OH	H	OH	H	OH	H	single
25. Hesperetin	OH	OCH_3	H	OH	H	OH	H	single
26. Naringenin	H	OH	H	OH	H	OH	H	single
27. Diosmin	OH	OCH_3	H	OH	H	*O*-rutinose	H	double
28. Naringin	H	OH	H	OH	H	*O*-neohesperidose	H	single
29. Isovitexin	H	OH	H	OH	*C*-glucose	OH	H	double
30. Neohesperidin	OH	OCH_3	H	OH	H	*O*-neohesperidose	H	single
31. Prunin	H	OH	H	OH	H	*O*-glucose	H	single
32. Quercetrin	OH	OH	OH	OH	H	*O*-glucose	H	double
33. Isosakuranetin Rutinoside	H	OCH_3	H	OH	H	*O*-rutinose	H	single
34. Rutin	OH	OH	O-rutinose	OH	H	OH	OH	double
35. Narirutin-4'-glucoside	H	*O*-glucose	H	OH	H	*O*-neohesperidose	H	single
38. Hesperidin	OH	OCH_3	H	OH	H	*O*-rutinose	H	single
39. Neoeriocitrin	OH	OH	H	OH	H	*O*-neohesperidose	H	single
40. Rhoifolin	H	OH	H	OH	H	*O*-neohesperidose	H	double

Figure 1. Structures of the flavonoids evaluated against the six human cancer cell lines.

Aldrich (St. Louis, MO). Rhamnetin was obtained from Mann Research Laboratories, Inc. (New York, NY), and quercetrin was obtained from California Corporation of Biochemical Research (Los Angeles, CA). All other flavonoids were obtained from a collection of purified compounds compiled by R. M. Horowitz (ARS, USDA, Pasadena, CA). Ultraviolet (UV) spectra of the flavonoids dissolved in ethanol were measured with a Shimadzu UV 2401-PC recording spectrophotometer.

Liquid Chromatography—Electrospray Ionization Mass Spectral Analysis. Mass spectral (MS) analysis of the flavonoids was carried out with a Waters ZQ single-quadrupole mass spectrometer equipped with a Waters 2695 HPLC pump and a Waters 996 detector (Waters Corp., Milford, MA). Photodiode array detection was monitored between 400 and 230 nm. Data handling was done with MassLynx software (Micromass, Division of Waters Corp., Beverly MA). The flavonoids were analyzed with the MS detector using a Whatman C18 5 μm analytical column (25 cm × 4.6 mm i.d.). Elution conditions included a three-solvent gradient composed initially of 2% formic acid/water/methanol (5:85:10, v/v/v), which was increased in a linear gradient to 5:62:22 (v/v/v) over 15 min. A final composition of 5:0:95 (v/v/v) was achieved with a linear gradient over 35 min using a flow rate of 0.75 mL min^{-1}. Postcolumn split to the photodiode array detector and ZQ detector was 10:1. MS parameters were as follows: ionization mode, ES+; scan range, 150−900 amu; scan rate, 1 scan/s; cone voltage, 20 eV.

Quercetin 3,5,7,3′,4′-Pentamethyl Ether (9). Quercetin (1.0 g) was added to a solution of 50 mL of acetone, 25 mL of water, and 2 mL of KOH (0.3 g mL^{-1}) in a 250 mL three-neck round-bottom flask. The reaction was heated to reflux. Dimethyl sulfate (DMS) (0.8 mL) was added, and the solution was allowed to turn yellow (∼15 min). KOH solution (1.0 mL) was added, producing a dark brown solution. Four sequential additions were made of DMS and KOH. Following the final addition of KOH, the solution was refluxed for 1 h and allowed to cool. Evaporation of the acetone produced a light yellow precipitate. Repeated washing of the yellow product with ice-cold acetone yielded pure **9**: UV 340, 263sh, 247 nm [lit. 339, 264sh, 249 nm (*17*)]; *m*/*e* 372; mp 150−152 °C [lit. 148−149 °C (*18*)].

Quercetin 3,7,3′,4′-Tetramethyl Ether (10). Quercetin (1.0 g) was added to a solution of 50 mL of acetone, 25 mL of water, and 2 mL of KOH (0.3 g mL^{-1}) in a 250 mL three-neck round-bottom flask. The reaction solution was heated to reflux. DMS (0.8 mL) was added, and the solution was refluxed for 15 min. KOH solution (1.0 mL) was added, producing a dark brown solution. An additional 0.8 mL of DMS was added, and the solution was again refluxed for 10 min. KOH (1.0 mL) was added, followed by 0.2 mL of DMS. A 1.0 mL aliquot of DMS was added; the solution was refluxed for 1 h and allowed to cool. Evaporation of the acetone produced a dark yellow precipitate. This precipitate, containing a mixture of **9** and **10**, was dissolved in dichloromethane and dried onto silica gel (10 g) (70−230 mesh, Sigma, St. Louis, MO). The sample-loaded silica gel was layered onto 20 g of new silica gel resin in a glass column (2.5 cm × 25 cm) and washed with ethyl acetate/hexane (1:3, v/v). The desired product, **10** (124 mg), eluted in the initial wash, while **9** remained bound to the silica gel. The product **10** had UV 353, 266sh, 253 nm [lit. 352, 269sh, 254 nm (*17*)]; *m*/*e* 358; and mp 156−157 °C [lit. 155 °C (*19*)].

Limocitrin 3,5,7,4′-Tetramethyl Ether (11). Oxidation of **10** was done by using a modified procedure of Rao and Seshadri *(20)*. Three hundred milligrams of **10** was added to 35 mL of KOH (0.04 g mL^{-1}) and 20 mL of pyridine in a 500 mL three-neck round-bottom flask. Dilute potassium persulfate solution (200 mL of 0.0165 g mL^{-1}) was added dropwise to the reaction solution maintained at 4 °C over 2 h. The solution was stirred and cooled in an ice bath overnight and yielded a dark olive green solution. The pH of the reaction solution was adjusted to pH 4 with HCl, then the solution was extracted with ethyl ether. Sodium sulfite (3.5 g) was added to the aqueous reaction solution, and this was further acidified with HCl to pH <2 and refluxed for 30 min. 5,8-Dihydroxy-3,7,3′,4′-tetramethoxyflavone (**17**) was subsequently extracted into and recovered from $CHCl_3$. The $CHCl_3$-recovered products were dried onto silica gel (2 g) (70−230 mesh) and then layered onto a dry bed of silica gel (39 cm × 2.5 cm). Hexane (200 mL) was passed through the column, followed by ethyl acetate. Compound **17** eluted from the column in the ethyl acetate as a single component: UV 338, 280, 257 nm [lit. 330, 280, 255 nm (*17*)]; *m*/*e* 374. Compound **17** (50 mg) was dissolved in 75 mL of dry acetone with 1 g of K_2CO_3. The solution was heated to reflux. Methyl iodide (1.0 mL) was added, and the reaction was refluxed for 90 min. The brown solution was removed from the remaining K_2CO_3. Crystals of **11** were recovered from acetone or ethanol. Product **11** had UV 351, 270, 252 nm [lit. 351, 273, 252 nm (*21*)]; *m*/*e* 402; and mp 170−172 °C [lit. 171−172 °C (*22*)].

Limocitrin 3,7,4′-Trimethyl Ether (13). Demethylation of the 5-position of **11** was run according to methods described by Tatum and Berry (*21*) with modifications. Glacial acetic acid (10 mL) and concentrated HCl (10 mL) were added to 100 mg of **11**. The reaction was heated for 2 h on a steam bath, then poured into 75 mL of ice water, and extracted with $CHCl_3$. Compound **13** had UV 360, 274, 256 nm [lit. 360, 272, 255 nm (*21*)]; *m*/*e* 388; mp 154−157 °C [lit. 156−157 °C (*21*)].

Limocitrin 3,7,4′-Trimethyl Ether-5-acetate (15). 13 (20 mg) was dissolved in 0.5 mL of pyridine. Acetic anhydride (0.5 mL) was added and left stirring in a 50 mL stoppered flask overnight at room temperature. The reaction solution was poured into 30 mL of water and allowed to stir for 1 h. Recovery of **15** was accomplished by extraction into ethyl ether and collected as off-white crystals. Compound **15** had UV 347, 249 nm [lit. 347, 250 nm (*17*)]; *m*/*e* 430; and mp 156−158 °C.

Quercetin 3,7,3′,4′-Tetramethyl Ether-5-acetate (16) and Quercetin 5,7,3′,4′-Tetramethyl Ether-3-acetate (14). Acetylations of **10** and quercetin 5,7,3′,4′-tetramethyl ether (**12**) were done in an identical manner as described above. Compound **12** had UV 360, 249 nm [lit. 360, 250 nm (*17*)]; mp 195−197 °C; and *m*/*e* 358. Product **14** had UV 332, 260, 247 nm [lit. 333 nm (*17*)] and *m*/*e* 400. Compound **16** had UV 340, 244 nm; *m*/*e* 400; and mp 162−165 °C [lit. 167−169 °C (*19*)].

Cell Culture. MDA-MB-435 ER− human breast cancer cells, MCF-7 ER+ human breast cancer cells, DU-145 androgen receptor-negative human prostate cancer cells, HT-29 human colon cancer cells, DMS-114 human lung cancer cells, and SK-MEL5 human melanoma cells were maintained in minimum essential medium-α modification (α-MEM) purchased from Gibco BRL (Burlington, ON) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL) and 100 mM penicillin/streptomycin (Gibco BRL). Once the cells reached 80−90% confluency, they were passaged by removing their growth medium and washed with citrate saline (4.49 g of $Na_3C_4H_3O{\cdot}8H_2O$, 10 g of KCl, pH 7.4). One millliliter of 5% trypsin (Difco, Becton Dickinson, Sparks, MD) was then used to loosen cells from the flask surface and was neutralized with 9 mL of α-MEM. Stock cultures were seeded at a density of 2 × 10^4 cells mL^{-1} and allowed to multiply for 48−72 h.

Incorporation of [^{3}H]Thymidine into DNA. Cells (MDA-MB-435, DU145, HT-29, DMS114, and SK-MEL5) were plated at 2 × 10^4 cells/well in 96-well, flat-bottom culture plates in a total volume of 200 μL of medium (Gibco, Burlington, ON, Canada) and incubated at 37 °C for 48 h with or without test compounds. [^{3}H]Thymidine (ICN, Irvine CA; 0.5 μCi/well) was added, and after 4 h, the cells were harvested onto a glass fiber filter using a semiautomatic 12-well cell harvester. Radioactivity on the filters was counted using Scintiverse (Fisher Scientific, Nepean, ON, Canada) in a liquid scintillation counter. MCF-7 cells were seeded at a density of 2 × 10^4 cells/well in 96-well, flat-bottom tissue culture plates and were incubated at 37 °C for 5 days. The test compounds were then added at concentrations of serial 1/10 dilutions. The plates were incubated at 37 °C for 2 days. [^{3}H]Thymidine (0.5 μCi) was added to each well to determine the number of dividing cells at each concentration. Four hours later, the medium and excess radioactive label were removed. Citrate saline with trypsin was added, the cells were harvested onto a glass fiber filter, and the radioactivity was counted. The percent of dividing cells was determined by comparing the number of counts per minute (cpm) for the treated cells to that for the control cells.

Viability of Cells. Viability of cells was measured by the MTT assay (*23*), which gives the concentration at which 50% of the cells remain viable after any drug treatment (LD_{50}). In this assay, MTT is converted to a blue formazan dye by mitochondrial enzymes in actively respiring but not necessarily proliferating cells. The intensity of the color formed

Table 1. Antiproliferative Activities (IC_{50} and IC_{90}) of Naturally Occurring and Synthetic Analogues of Polymethoxylated Flavones in Citrus against Six Human Cancer Cell Lines

compound	μM	lung	colon	breast ER−	breast ER+	prostate	melanoma
(**1**) 3,5,6,7,8,3′,4′-heptamethoxyflavone	IC_{50}	4.6	2.1	0.9	0.2	1.8	9.9
	IC_{90}	14.5	8.8	2.8	2.3	7.4	16.4
(**2**) tangeretin	IC_{50}	3.2	1.6	1.3	0.34	0.54	0.27
	IC_{90}	6.7	4.0	3.5	2.7	2.7	3.8
(**3**) nobiletin	IC_{50}	3.5	4.7	1.2	2.9	1.0	0.50
	IC_{90}	10.1	13.9	3.7	6.2	2.2	2.0
(**4**) sinensetin	IC_{50}	13.7	9.5	3.9	5.5	16.5	10.8
	IC_{90}	23.6	13.9	7.4	9.7	22.3	17.9
(**5**) tetra-*O*-methylscutellarein	IC_{50}	21.5	6.3	0.80	0.53	3.9	2.9
	IC_{90}	39.4	13.7	2.9	2.4	8.1	7.6
(**6**) 5-desmethylnobiletin	IC_{50}	38.8	8.5	0.77	0.23	2.8	3.6
	IC_{90}	143	171	2.6	0.77	162	92
(**7**) tetra-*O*-methylisoscutellarein	IC_{50}	18.1	6.6	ND[a]	ND	2.6	11.3
	IC_{90}	25.8	19.7	ND	ND	11.5	19.7
(**8**) 5-desmethylsinensetin	IC_{50}	0.11	5.0	0.06	0.03	2.2	1.1
	IC_{90}	1.7	9.5	1.7	1.1	7.8	4.7
(**9**) quercetin 3,5,7,3′,4′- pentamethyl ether	IC_{50}	2.2	33	19	18	6.0	12.1
	IC_{90}	5.5	110	59	57	74	45
(**10**) quercetin 3,7,3′,4′- tetramethyl ether	IC_{50}	6.9	0.84	26	14	16	8.1
	IC_{90}	21	47	54	41	56	42
(**11**) limocitrin 3,5,7,4′-tetramethyl ether	IC_{50}	17	45	2.0	0.50	14	6.2
	IC_{90}	47	107	7.2	4.0	97	42
(**12**) quercetin 5,7,3′,4′-tetramethyl ether (S)[b]	IC_{50}	3.1	15.6	27	14.2	5.6	3.9
	IC_{90}	5.9	28	70	64	21	5.6
(**13**) limocitrin 3,7,4′-trimethyl ether (S)	IC_{50}	6.4	54	8.0	1.3	11	5.4
	IC_{90}	44	115	21	8.2	69	41
(**14**) quercetin 5,7,3′,4′-tetramethyl ether-3-acetate (S)	IC_{50}	21	14	8.5	3.0	20	14
	IC_{90}	27	22	20	15	27	21
(**15**) limocitrin 3,7,4′-trimethyl ether-5-acetate (S)	IC_{50}	2.3	18	2.1	0.11	2.6	5.3
	IC_{90}	17	69	8.0	1.6	23	20
(**16**) quercetin 3,7,3′,4′-tetramethyl ether-5-acetate (S)	IC_{50}	15	8.8	ND	ND	7.0	3.0
	IC_{90}	18	45	ND	ND	50	6.3
(**17**) 5,8-dihydroxy-3,7,3′,4′-tetramethoxyflavone (S)	IC_{50}	18	21	ND	ND	41	14
	IC_{90}	59	85	ND	ND	152	48

[a] ND, not determine. [b] Compounds designated with (S) are synthetic analogues.

can be correlated to untreated controls to obtain the LD_{50} value by reading the absorbance at 590 nm. Cancer cells (8×10^4 well^{-1}) were seeded with various concentrations of the test compounds in a 96-well plate in a total volume of 200 μL of medium. Sterile MTT (25 mL of 5 mg mL^{-1} in PBS) was added to each well, and the plates were covered in foil and incubated at 37 °C. After 3 h, 100 μL of extraction buffer consisting of 20% SDS dissolved in a dimethylformamide/water (1:1, v/v) solution at pH 4.0 was added. Blue color formation was measured at 590 nm with a Dynatech MRX micorplate reader (Dynatech Laboratories). The percentage of cells surviving was determined by comparing the absorbance of the solutions recovered from the treated cells with that of the control cells.

RESULTS

Inhibition of cancer cell growth by the flavonoids in this study was measured by the decreases that occurred in the uptake of [^{3}H]thymidine by treated cells. Many of the flavonoids in this study exhibited antiproliferative activities toward the human lung DMS-114, colon HT-29, ER+ MCF-7 and ER− MDA-MB-435 breast, prostate DU-145, and melanoma SK-MEL5 cancer cell lines. The polymethoxylated flavones (**Table 1**), including the naturally occurring compounds **1**−**11** and a number of synthetic analogues, **12**−**17**, exhibited the highest activities against the six cancer cell lines. Notably high activities were observed for 5-desmethylsinensetin, **8**, a minor compound in orange peel, with average IC_{50} and IC_{90} values of 1.4 and 4.4 μM, respectively, against the six cancer cell lines. In contrast, the average IC_{50} and IC_{90} values for the entire set of naturally occurring compounds, **1**−**11**, against the six cancer cell lines were 7.6 ± 9.4 and 29.2 ± 39.4 μM, respectively. Individually, the average IC_{50} values of compounds **1**−**11** toward the human lung, colon, melanoma, prostate, breast ER− and ER+ cell lines were 11.7 ± 11.0, 11.2 ± 13.6, 6.1 ± 4.4, 6.1 ± 5.9, 6.2 ± 8.5, and 4.2 ± 6.2 μM, respectively. The human lung and colon cancer cell lines showed the least sensitivity toward **1**−**11**.

The synthetic derivatives **12**−**17** exhibited low IC_{50} levels toward the six human cancer cell lines, similar to compounds **1**−**11**. In a number of instances the IC_{50} values for the synthetic compounds occurred below 10 μM, and in a few instances the IC_{90} values also occurred below 10 μM. Among the synthetic derivatives, the most potent antiproliferative compound, limocitrin 3,7,4′-trimethyl ether-5-acetate (**15**), had IC_{50} values of 5.3, 2.6, 0.11, and 2.1 μM against the melanoma, prostate, and ER+ and ER− breast cancer cell lines, respectively. For the ER+ breast cancer cell line, **15** exhibited an IC_{90} of 1.6 μM, an indication of potent activity against this cancer cell line.

Toxicity measurements were taken for a number of the naturally occurring polymethoxylated flavones and synthetic analogues (**Table 2**). These activities were analyzed by monitoring cell viability of treated and untreated cells by their reduction of the tetrazolium substrate, MTT. The prostate cancer cell line, DU-145, was the least susceptible to the cytotoxic effects of the polymethoxylated flavones. Among the compounds analyzed, **12**, quercetin 5,7,3′,4′-tetramethyl ether, exhibited the highest toxicity, exhibiting average LC_{50} and LC_{90} values of 7.0 ± 0.6 and 61.7 ± 31.5 μM, respectively (omitting the anomalously high values for the prostate cancer cell line). For this compound the IC_{50} (**Table 1**) and LC_{50} values toward the individual cancer cell lines occurred at very similar values,

Table 2. Toxicity ($LC_{50,90}$) of Polymethoxylated Flavones toward Six Human Cancer Cell Lines

compound	μM	lung	colon	prostate	melanoma
(**1**) 3,5,6,7,8,3′,4′-heptamethoxyflavone	LC_{50}	48	41	126	34
	LC_{90}	>200	124	>200	142
(**9**) quercetin 3,5,7,3′,4′-pentamethyl ether	LC_{50}	79	47	110	47
	LC_{90}	>200	165	>200	191
(**10**) quercetin 3,7,3′,4′- tetramethyl ether	LC_{50}	47	41	>153	56
	LC_{90}	>200	>200	>200	>200
(**11**) limocitrin 3,5,7,4′-tetramethyl ether	LC_{50}	50	47	111	47
	LC_{90}	>200	133	>200	190
(**12**) quercetin 5,7,3′,4′-tetramethyl ether	LC_{50}	6.4	7.8	117	6.9
	LC_{90}	44	35	>200	106
(**13**) limocitrin 3,7,4′-trimethyl ether	LC_{50}	85	39	>100	39
	LC_{90}	>200	97	180	>200
(**15**) limocitrin 3,7,4′-trimethyl ether-5-acetate	LC_{50}	24	22	104	23
	LC_{90}	171	72	>200	123
(**16**) quercetin 3,7,3′,4′-tetramethyl ether-5-acetate	LC_{50}	18	13	112	2.5
	LC_{90}	>200	87	>200	170
(**17**) 5,8-dihydroxy 3,7,3′,4′-tetramethoxyflavone	LC_{50}	>200	48	>200	>200
	LC_{90}	>200	189	>200	>200

Table 3. Antiproliferative Activities (IC_{50} and IC_{90}) of Hydroxylated Flavone Aglycons against Five Human Cancer Cell Lines

compound	μM	lung	colon	breast ER+	prostate	melanoma
(**18**) rhamnetin	IC_{50}	38	76	85	22	25
	IC_{90}	174	>200	>200	158	>200
(**19**) kaempferol	IC_{50}	115	42	>200	95	>200
	IC_{90}	>200	>200	>200	>200	>200
(**20**) chrysoeriol	IC_{50}	17	20	7	30	23
	IC_{90}	36	46	20	69	59
(**21**) apigenin	IC_{50}	41	29	22	37	41
	IC_{90}	100	85	85	93	125
(**22**) luteolin	IC_{50}	3.1	10.5	21	32	32
	IC_{90}	12	28	46	70	77
(**23**) quercetin	IC_{50}	59	40	73	86	>200
	IC_{90}	158	115	168	181	>200

Table 4. Antiproliferative Activities ($IC_{50,90}$) of Naturally Occurring Hydroxylated Flavanone Aglycons against Five Human Cancer Cell Lines

compound	μM	lung	colon	breast ER+	prostate	melanoma
(**24**) eriodictyol	IC_{50}	87	62	56	42	>200
	IC_{90}	>200	180	156	180	>200
(**25**) hesperetin	IC_{50}	181	149	181	181	>200
	IC_{90}	>200	>200	>200	>200	>200
(**26**) naringenin	IC_{50}	102	154	84	150	77
	IC_{90}	>200	>200	180	>200	158

suggesting that the antiproliferative activity of **12** is linked to its cytotoxicity.

Antiproliferative activities were also measured for a number of hydroxylated flavone (**Table 3**) and flavanone (**Table 4**) aglycons. These compounds occur in citrus almost exclusively as either *C*-glycosides or *O*-glycosides containing either rutinose (6-*O*-α-L-rhamnopyranosyl-α-D-glucopyranose) or neohesperidose (2-*O*-α-L-rhamnopyranosyl-α-D-glucopyranose) groups, typically at either the 7- or 3-positions (*1*). Of these compounds, chrysoeriol (**20**), apigenin (**21**), and luteolin (**22**) exhibited the strongest antiproliferative activities. In a few cases the activity levels of these compounds were comparable to those of the polymethoxylated flavones. The flavanone eriodictyol (**24**) exhibited lower IC_{50} values than the other flavanones, hesperetin (**25**) and naringenin (**26**) (**Table 4**), but showed weaker activities than those measured for its flavone analogue, luteolin (**Table 3**). The glycosidic flavonoids in citrus (**Table 5**) were inactive as antiproliferative agents.

Table 5. Antiproliferative Activities (IC_{50}) of Flavanone and Flavone Glycosides against Five Human Cancer Cell Lines

compound	lung	colon	breast ER+	prostate	melanoma
(**27**) diosmin	>200	>200	>200	>200	>200
(**28**) naringin	>200	>200	>200	>200	>200
(**29**) isovitexin	>200	>200	17	>200	>200
(**30**) neohesperidin	>200	>200	>200	>200	>200
(**31**) prunin	>200	>200	>200	>200	>200
(**32**) quercetrin	>200	>200	>200	>200	>200
(**33**) isosakuranetin rutinoside	>200	>200	>200	>200	>200
(**34**) rutin	>200	>200	>200	>200	>200
(**35**) hesperetin trisaccharide	>200	>200	>200	>200	>200
(**36**) narirutin 4′-glucoside	>200	>200	>200	>200	>200
(**37**) hesperetin 7-glucoside	42	>200	46	86	61
(**38**) hesperidin	106	77	51	101	>200
(**39**) neoeriocitrin	70	>200	>200	>200	>200
(**40**) rhoifolin	>200	>200	>200	>200	>200
(**41**) neodiosmin	>200	>200	>200	>200	>200
(**42**) margaretin	>200	>200	>200	>200	>200

DISCUSSION

The potential health-promoting properties of citrus flavonoids have been researched for decades. Early studies by Szent-Györgyi and co-workers (*24*) demonstrated that a citrus bioflavonoid complex suppressed capillary fragility and permeability in animal trials. This complex, termed citrin, was shown to contain hesperidin and eriocitrin (*25*). Subsequent work demonstrated potent vitamin C-sparing properties of these citrus flavonoids (*26*, and references cited therein), and this strong synergistic effect between flavonoids and vitamin C is now well documented (*27*). Consistent with these early findings, many of the beneficial actions of citrus flavonoids in animals have ultimately been shown to be attributable to their protection of the microvascular endothelium during oxidative stress, particularly in cases involving cancer and inflammation. Much of the anti-inflammatory activities of citrus flavonoids arise from the antioxidant properties of these compounds as well as their abilities to block key steps in arachidonic acid metabolism (for

a review, see ref *3*). Recently the polymethoxylated flavones have also been shown to influence inflammation via a different mechanism (*28*), leading to inhibited cytokine production. Yet, in nearly all of these cases, the endpoints of citrus flavonoid functions are closely tied to the health of the microvascular endothelium. In this manner, then, it is not surprising that the citrus flavonoids might also mediate the occurrence of cancers in humans.

Interest in the anticancer properties of citrus flavonoids focuses primarily on the anticarcinogenic properties of the flavone and flavanone glycosides and on the antitumor properties of the polymethoxylated flavones. Differences in these modes of actions can be anticipated on the basis of the differences in chemical structures between the flavonoid glycosides and the polymethoxylated flavones. Against the six human cancer cell lines included in this present study, negligible antiproliferative activities are observed for the citrus flavonoid glycosides (**Table 5**), and these results are consistent with previous studies (*11*, *12*). Yet, the glycosidic compounds, hesperidin and diosmin, have previously exhibited potent anticarcinogenic properties in vivo (*4*−*6*). These compounds appear able to inhibit cancer initiation, possibly through the induction of hepatic detoxifying enzymes as well as through their antioxidant properties.

The absence of antiproliferative activities for the flavonoid glycosides is linked to a large extent to the presence of the glycosidic substituents, an association supported by the activities exhibited by the aglycon species in **Tables 3** and **4**. The antiproliferative activities of the hydroxylated flavone and flavanone aglycons are consistent with recent studies that have shown antiproliferative activities of flavones against a number of other human cancer cell lines. Apigenin has been shown to inhibit the proliferation of various human cancer cell lines, including OCM-1 melanoma (*29*), MCF-7 and MDA-MB-468 breast (*30*), SW480, HT-29, and Caco-2 colon carcinoma (*31*), prostate (*32*), thyroid (*33*), and HL-60 cancer cell lines (*34*). For a number of these cell lines antiproliferative activities were also observed for quercetin, luteolin, and/or kaempferol. The mechanisms of action of a number of these flavonoids in mediating tumor growth have been investigated in vitro as well as in vivo. Treatment of the colon carcinoma cell lines with apigenin reduced the accumulation of the p34 (cdc2) and cyclin B1 proteins, hence inhibiting the p34 (cd2) kinase (*31*), a critical enzyme in the G2/M transition phase of cell proliferation. Similar arrest at the G2/M cell cycle phase along with the suppression of a number of kinases involved in cellular signal transduction was observed for several of the other apigenin-treated cancer cell lines. In an animal trial, Caltagirone et al. (*35*) recently demonstrated that intraperitoneal administration of apigenin into syngenetic mice at the same time of injection of B16-BL6 melanoma cells resulted in significant dose-dependent delay of tumor growth. Significant decreases were observed in the number of cancer cells metastasized to the lungs. Similar results were observed with quercetin.

The flavanone aglycons, naringenin, hesperetin, and eriodictyol (**Table 4**), also exhibited antiproliferative activities, although these activities were significantly weaker than those of the flavones (**Table 3**). The *o*-catechol moiety in the B ring of the flavanone eriodictyol and its flavone analogue, luteolin, was previously shown to be important in its antiproliferative activity against several human cancer cell lines (human lung carcinoma A549, B16 melanoma 4A5, human T-cell leukemia CCRF-HSB-2, lymph node TGBC11TKB) (*12*). Methyl substitution at the 4′-position in hesperetin resulted in lower activity levels, and no activity was detected for naringenin. In our studies, antiproliferative activities were observed for both hesperetin and naringenin against the six human cancer cell lines, although these activities were significantly lower than those observed for eriodictyol.

The polymethoxylated flavones in citrus are distinctive for their high antiproliferative activities against a number of human cancer cell lines. In addition to the six human cancer cell lines listed in **Table 1**, antiproliferative activity has also been shown with these compounds against squamous cell carcinoma HBT43 (*7*), human lung carcinoma A549, T-cell leukemia (CCRF-HSB-2), gastric cancer, lymph node metastated (TGBC11TKB), A549 and B16 melanoma (*12*), and leukemia HL-60 (*8*, *11*). These findings indicate that citrus polymethoxylated flavones exhibit antiproliferative activity against a wide spectrum of human cancer cell lines. The potent antiproliferative activities of many of these compounds are further illustrated by their low IC_{90} values against the human colon, lung, melanoma, prostate, and ER+ and ER− breast cancer cell lines (**Table 1**), where many of the IC_{90} values occur below 10 μM.

A number of polymethoxylated flavone analogues were also analyzed in this study. These compounds exhibited antiproliferative activity levels comparable to those of the naturally occurring compounds. This is interesting in light of the fact that a number of these analogues contained acetate groups at the 5-position of the flavone A-ring. The absence of consistent differences in activity was observed with the 5-desmethyl analogues of nobiletin (**3** vs **6**) and quercetin 3,5,7,3′,4′-pentamethyl ether (**9** vs **10**) (**Table 1**). The fact that there are only small differences in the activity levels of **11** and **17** further suggests that the substitution on the flavone A-ring has little influence on antiproliferative activity in most cases. A notable exception to this finding was the significantly higher activities observed for 5-desmethysinensetin compared to sinensetin. In a number of instances the compounds in this study showed strong toxicity toward several of the human cancer cell lines. The lowest LC_{50} values were exhibited by **12** and **16** against the lung, colon, and melanoma cell lines.

The strong antiproliferative activities of the polymethoxylated flavones suggest their potential as anticancer agents. In addition to their antiproliferative actions, citrus polymethoxylated flavones have also been shown to exert in vitro multidrug reversing effects in adriamycin-resistant human myelogenous leukemia cells (*36*). Indications of anti-invasive actions for nobiletin and tangeretin have been shown by their down-regulation of matrix metalloproteinases in brain tumor cells in vitro (*37*). Their usefulness as such agents awaits verification by in vivo testing, although a recent study by Bracke et al. (*16*) contradicts the use of high doses of nobiletin and tangeretin against mammary cancers. It is likely that multiple mechanisms will be found for the several classes of citrus flavonoids, thus holding promise as potentially useful anticancer agents.

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