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Isolation and syntheses of polymethoxyflavones and hydroxylated polymethoxyflavones as inhibitors of HL-60 cell lines

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Abstract—Fifteen polymethoxyflavones (PMFs) and hydroxylated PMFs were isolated from sweet orange (*Citrus sinensis*) peel extract and synthesized to investigate their biological activity. All obtained compounds were tested in HL-60 cancer cell proliferation and apoptosis induction assays. While some PMFs and hydroxylated PMFs had moderate anti-carcinogenic activities, 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone and 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone showed strong inhibitory activities against the proliferation and induced apoptosis of HL-60 cell lines.

1. Introduction

Polymethoxyflavones (PMFs) are found almost exclusively in *Citrus* genus, particularly in the peels of sweet orange (*Citrus sinensis* (L.) Osbeck) and mandarin (*Citrus reticulate* Blanco). They have been of particular interest due to their broad spectrum of biological activities, including anti-inflammatory,[1,2] anti-carcinogenic[3–5] and anti-atherogenic[6] properties. Chronic inflammation was closely associated with the increased risk of various human cancers.[7]

Many in vitro and in vivo studies indicate the protective effects of PMFs against the occurrence of cancer. For example, PMFs have been shown to selectively inhibit the growth of human HL-60 cell lines in vitro,[8] to reduce the invasion of tumors in animal models,[9] to induce the differentiation of myeloid leukemic cells, and to suppress proliferation while promoting apoptosis.[8,10] Studies of the biological activity of PMFs have focused on two of the most abundant PMFs in citrus peels: tangeretin and nobiletin. Anti-cancer activity of tangeretin has been well documented. Tangeretin exerts anti-cancer effects by multiple mechanisms.[11] It can inhibit cancer initiation stage by modulating hepatic enzymes, thus affecting xenobiotic activation and detoxification in liver. Our previous study showed that tangeretin inhibits tumor cell growth through inhibiting cyclin-dependent kinase 2 (Cdk2) and Cdk4 activities and elevating Cdk inhibitors p21 and p27 in human colorectal carcinoma cells.[12] Recent study also showed that tangeretin exerts its anti-tumor activity by repressing induced and constitutively expressed cyclooxygenase-2 (COX-2) in human lung cancer cells.[13] Moreover, there were many reports to show the anti-cancer activities of nobiletin. For example, it can inhibit the proliferation of human prostate cancer cells; inhibit the skin, breast, and colon carcinoma cell lines.[14] Recently, nobiletin was shown to have anti-proliferative and apoptotic effects on a gastric cancer cell line and have disruptive effect on cell-cycle progression.[15] Nobiletin showed the strongest anti-proliferative activity in a comparative evaluation of 42 flavonoids against six human cancer cell lines including lung, prostate, colon, melanoma, estrogen receptor positive and estrogen receptor negative breast cancer cells.[5] Recently nobiletin was demonstrated to be an inhibitor of both NO and O_2^- generation in human cells, and a powerful inhibitor of two distinct stages of skin

Abbreviations: PMF, polymethoxyflavones; heptaMF, heptamethoxyflavone; hexaMF, hexamethoxyflavone; pentaMF, pentamethoxyflavone.
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inflammation induced by double TPA application.[16] The bioactivity study of 3,5,6,7,8,3′,4′-heptamethoxyflavone (heptaMF, **III**, Fig. 1), another abundant PMF in the orange peel extract, was started a few years ago and it also showed anti-cancer property. Among seven PMFs and six hydroxylated PMFs screened for anti-tumor effects, 3,5,6,7,8,3′,4′-heptaMF showed the greatest inhibitory effects on mouse skin tumor promotion in an in vivo two-stage carcinogenesis test.[17]

We used anti-cancer screening of 15 PMFs and hydroxylated PMFs to elucidate structure–activity relationship (SAR) within this group of compounds. Five most abundant PMFs and two hydroxylated PMFs were isolated from sweet orange peel. Also, we synthesized seven hydroxylated PMF analogues of PMFs commonly found in sweet orange peel. Screening against cell proliferation and induction of apoptosis in HL-60 leukemia cells revealed that 5-hydroxy-6,7,8,3′,4′-pentamethoxy-

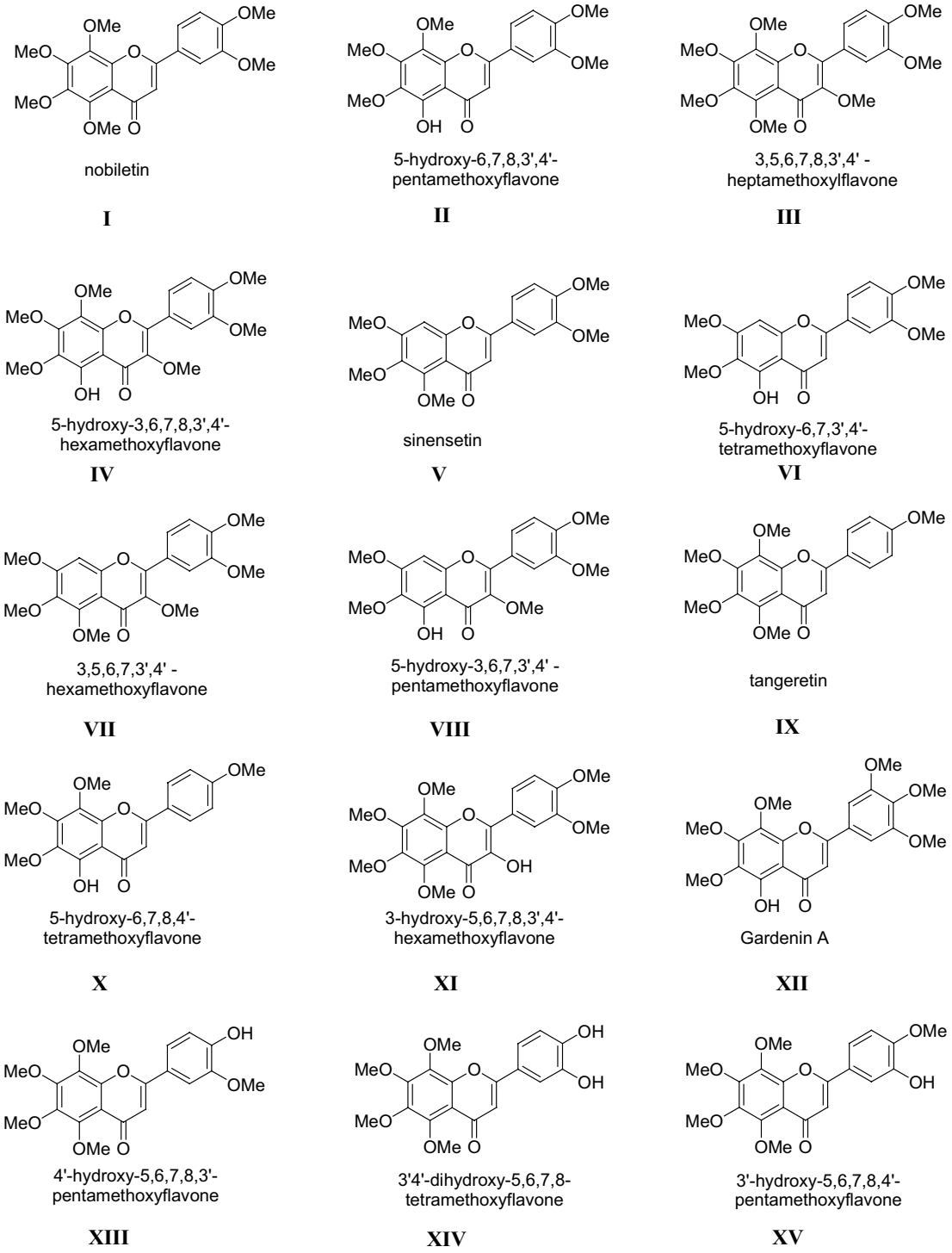

Figure 1. Structures of PMFs and hydroxylated PMFs.

flavone (5-demethylnobiletin, **II**, Fig. 1) and 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (5-demethylheptaMF, **IV**, Fig. 1) have the strongest activity, followed by 5-hydroxy-6,7,3′,4′-tetramethoxyflavone (5-demethylsinensetin, **VI** in Fig. 1). Overall anti-cancer activity of PMFs was significantly lower compared to hydroxylated PMFs. In this paper, we report the isolation, syntheses, and the activity of hydroxylated PMFs and PMFs against HL-60 cell lines.

2. Chemistry

2.1. Isolation

Five polymethoxyflavones and two hydroxylated polymethoxyflavones were isolated from sweet orange peel extract (cold pressed) and used in the biological activity screening against HL-60 cancer cells, namely, nobiletin (**I**), tangeretin (**IX**), sinensetin (**V**), heptaMF (**III**), 3,5,6,7,3′,4′-hexamethoxyflavone (hexaMF, **VII**), 5-demethylnobiletin (**II**), and 5-demethylheptaMF (**IV**). The separation and characterization of these compounds were similar to previously reported procedure.[18] In addition, seven hydroxylated PMFs either absent in orange peel extract or present in minute quantities were synthesized and used in screening:5-hydroxy-6,7,8,4′-tetramethoxyflavone (5-demethyltangeretin, **X**), 5-demethylsinensetin (**VI**), 3-hydroxy-5,6,7,8,3′,4′-hexamethoxyflavone (3-hydroxynobiletin, **XI**), 5-hydroxy-3,6,7,3′,4′-pentamethoxyflavone (5-demethylhexaMF, **VIII**), 3′-hydroxy-5,6,7,8,4′-pentamethoxyflavone (3′-demethylnobiletin, **XV**), 4′-hydroxy-5,6,7,8,3′-pentamethoxyflavone (4′-demethylnobiletin, **XIII**), and 3′,4′-dihydroxy-5,6,7,8-tetramethoxyflavone (3′,4′-didemethylnobiletin, **XIV**). They are listed in Figure 1 and synthesized according to synthetic routes described below. Gardenin A (**XII**) was purchased from a commercial source.

2.2. Syntheses

2.2.1. Synthesis of 3-hydroxynobiletin. Following previously published procedure,[19] 3-hydroxynobiletin (3-hydroxy-5,6,7,8,3′,4′-hexamethoxyflavone, **XI**) was synthesized in one-step by reacting nobiletin with dimethyldioxirane (DMD) in acetone at low temperature (Fig. 2). The oxidation reaction was regioselective with high yield in a short time period. It is worth mentioning that the yield of DMD preparation was low.

2.2.2. Syntheses of 5-hydroxylated PMFs. Polymethoxyflavones were used as a source material for synthesis of corresponding 5-hydroxylated PMFs. For instance, the synthesis of 5-demethylsinensetin (**VI**) started from sinensetin (**V**). Exposure of sinensetin to boron trichloride solution under low temperature within short period of time yielded the desired 5-demethylsinensetin (Fig. 3). This one-step reaction went through smoothly with moderate to high yield for different substrates.[19] Boron trichloride is a catalyst acting as a Lewis acid and it can be replaced with other Lewis acids such as boron tribromide. Recently, we found other acid like hydrochloric acid can be used in this one-step process with the optimization of reaction conditions.[20] Hence, this reaction can be generalized to prepare 5-hydroxylated PMFs with high efficiency.

2.2.3. Syntheses of other hydroxylated PMFs. B-ring demethylated PMFs, such as 3′-demethylnobiletin (**XV**), 4′-demethylnobiletin (**XIII**), and 3′,4′-didemethylnobiletin (**XIV**), were prepared in a multi-step synthesis by endorsing Mizuno's procedure[21] with some modification[22] (Fig. 4). In the five-step synthesis of 3′-demethylnobiletin (**XV**), the reaction started with ring fission of nobiletin, tangeretin and/or other A ring permethoxylated and 3-unsubstituted PMFs, which were isolated from OPE following the reported procedure.[18] Then the mixture or any one of these compounds

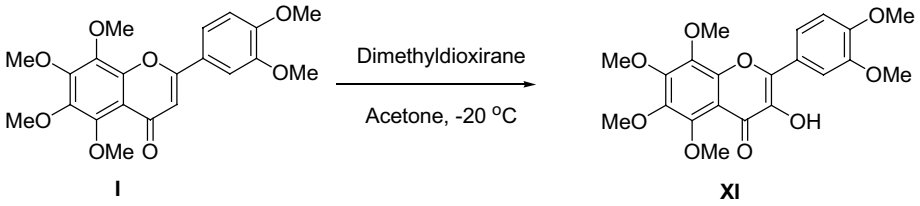

Figure 2. Synthetic scheme of 3-hydroxynobiletin (**XI**).

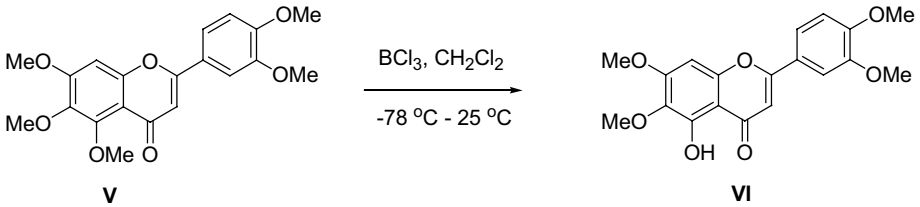

Figure 3. Synthetic example of 5-demethylated PMFs.

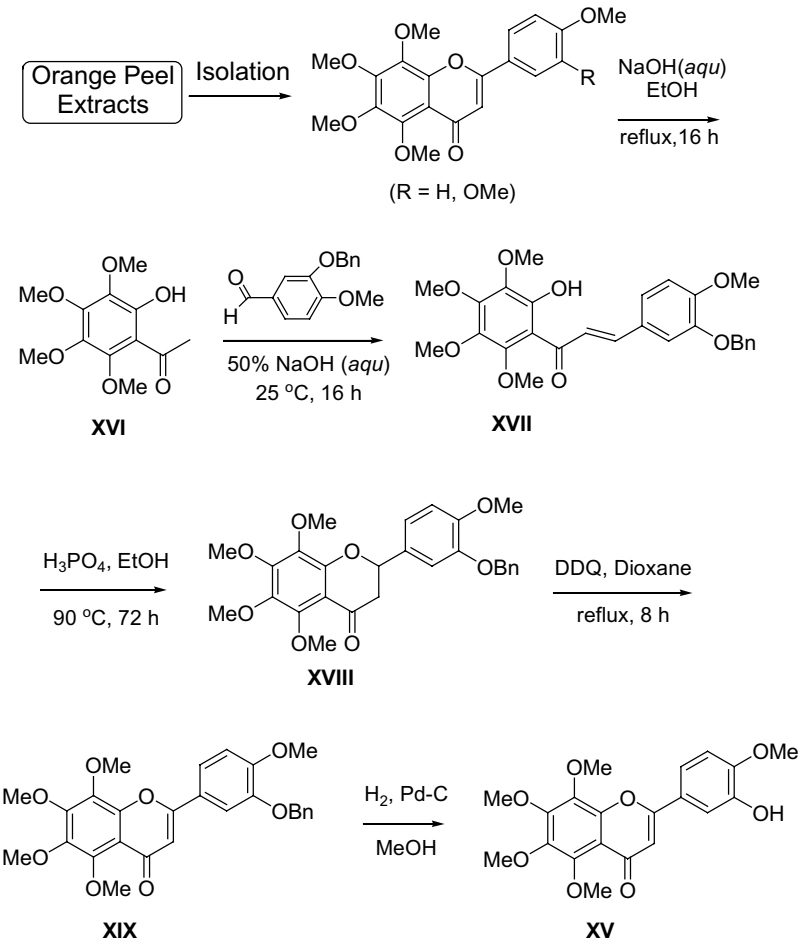

Figure 4. Synthesis of 3′-demethylated nobiletin (**XV**).

underwent basic hydrolysis and C ring fission product, 2′-hydroxy-3′,4′,5′,6′-tetramethoxyacetophenone (**XVI**), was obtained. Aldol condensation of the acetophenone (**XVI**) with 3-benzyloxy-4-methoxy-benzaldehyde yielded 3-benzyloxy-2′-hydroxy-4,3′,4′,5′,6′-pentamethoxylchalcone (**XVII**), which underwent cyclization under phosphoric acid and heating conditions to yield a corresponding flavanone (**XVIII**), Consequently, the flavanone was oxidized with DDQ to form 3′-benzylated nobiletin (**XIX**). Debenzylation reaction yielded 3′-demethylnobiletin and finished the synthesis.

It is worth mentioning that the starting material, 2′-hydroxy-3′,4′,5′,6′-tetramethoxyacetophenone (**XVI**), can also be obtained by methylating a commercial reagent, Gardenin A (**XII**), with iodomethane in the presence of anhydrous potassium carbonate and DMF. The reaction of this step was uncomplicated with quantitative

Table 1. Inhibition activity (IC_{50}) and apoptosis activity (AC_{50}) of PMFs and hydroxylated PMFs

Compound	Compound name	IC_{50} (μM)	AC_{50} (μM)
I	Nobiletin	41.50 ± 7.01	>100
II	5-Hydroxy-6,7,8,3′,4′-pentamethoxyflavone (5-demethylnobiletin)	2.07 ± 2.56	5.87 ± 0.13
III	3,5,6,7,8,3′,4′-Heptamethoxyflavone (HeptaMF)	13.31 ± 1.28	33.88 ± 0.01
IV	5-Hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (5-demethylheptaMF)	4.16 ± 2.33	5.90 ± 0.11
V	Sinensetin	>100	>100
VI	5-Hydroxy-6,7,3′,4′-tetramethoxyflavone (5-demethylsinensetin)	>100	>100
VII	3,5,6,7,3′,4′-Hexamethoxyflavone (HexaMF)	20.59 ± 1.01	92.10 ± 5.67
VIII	5-Hydroxy-3,6,7,3′,4′-pentamethoxyflavone (5-demethylhexaMF)	6.44 ± 2.50	7.26 ± 0.01
IX	Tangeretin	>100	>100
X	5-Hydroxy-6,7,8,4′-tetramethoxyflavone (5-demethyltangeretin)	>100	>100
XI	3-Hydroxy-5,6,7,8,3′,4′-hexamethoxyflavone (3-hydroxynobiletin)	>100	56.51 ± 2.39
XII	Gardenin A	>100	>100
XIII	4′-Hydroxy-5,6,7,8,3′-pentamethoxyflavone (4′-demethylnobiletin)	47.41 ± 3.64	87.10 ± 7.83
XIV	3′,4′-Dihydroxy-5,6,7,8-tetramethoxyflavone (3′,4′-didemethylnobiletin)	71.18 ± 1.93	>100
XV	3′-Hydroxy-5,6,7,8,4′-pentamethoxyflavone (3′-demethylnobiletin)	52.72 ± 0.22	94.62 ± 1.50

yield. This is a fast and efficient method of obtaining 2′-hydroxy-3′,4′,5′,6′-tetramethoxyacetophenone, especially when large quantity of material is not readily available through isolation of PMFs.

3. Inhibition of cell proliferation in PMF-treated HL-60 cell lines

The growth inhibitory activity of six PMFs and nine hydroxylated PMFs has been screened against HL-60 cell lines. The inhibition and apoptosis data are shown in Table 1. All compounds showed no cytotoxicity on normal human polymorphonuclear leukocyte (PMN) cells at concentration of 100 μM.

3.1. Inhibition activity

3.1.1. PMFs. We determined IC_{50} and AC_{50} for five PMFs with a number of methoxy groups ranging from 5 (pentaMF such as sinensetin and tangeretin) up to 7 (heptaMF). From the results presented in Table 1 it is evident that both the configuration and total number of methoxy groups substantially influence anti-cancer properties of PMFs. The increase of total number of methoxy side groups in PMFs is positively correlated with the overall anti-proliferative activity of compounds in HL-60 cell lines. There was no detectable activity against HL-60 cell lines treated with sinensetin (**V**) and tangeretin (**IX**), compounds containing only five methoxy groups. The compounds with six methoxy groups, 3,5,6,7, 3′,4′-hexaMF (**VII**, 20.6 μM) and nobiletin (**I**, 41.5 μM), showed moderate anti-proliferation activity. The highest level of anti-cancer activity among tested PMFs was measured in treatment with heptaMF (**III**, 13.3 μM), compound with the most methoxy groups. It is also indicative that heptaMF (**III**) was the only PMF with distinct proapoptotic activity (**III**, AC_{50} = 33.88 μM). Saturations of the A-ring with methoxy groups positively correlated with increased biological activity. IC_{50} rapidly decreased in a row of sinensetin < nobiletin < 3,5,6,7,8,3′,4′-heptaMF. Comparative analysis of anti-proliferation activity in HL-60 cells of sinensetin, 3,5,6,7,3′,4′-hexaMF, nobiletin and 3,5,6,7,8,3′,4′-heptaMF revealed that methoxylation in 3-position might boost biological activity more compared to methoxylation in position 8.

3.1.2. Hydroxylated PMFs. The SAR of hydroxylated PMFs did not completely followed that of PMFs. Among the 10 hydroxylated PMFs, 5-demethylated nobiletin (**II**) has the highest inhibition activity of 2.1 μM, followed by 5-demethylheptaMF (**IV**, 4.2 μM), 5-hydroxy-3,6,7,3′,4′-pentaMF (**VIII**, 6.4 μM), and B-ring demethylated PMFs ranging from 47 to 71 μM. The rest four hydroxylated PMFs, 5-demethyltangeretin (**X**), 5-demethylsinensetin (**VI**), 3-hydroxynobiletin (**XI**) and gardenin A (**XII**), showed no inhibition activity at 100 μM. For 5-hydroxylated PMF series, saturation of the A ring with methoxy groups significantly increased activity. However, it is unclear if this increase in activity was directly linked to the methoxy group at position 8. Also among the 5-demethylated PMFs, the methoxy group at 3′-position could boost the activity dramatically. For example, both 5-demethyltangeretin and 5-demethylsinensetin have no 3′-methoxy groups and are lacking activity at the highest tested concentration, whereas, both 5-demethylnobiletin (**II**, 2.1 μM) and 5-hydroxy-3,6,7,3′,4′-pentaMF (**VIII**, 6.4 μM), the 3′-monomethoxylated version of 5-demethyltangeretin and 5-demethylsinensetin, are strong inhibitors against HL-60 cell lines. In the absence of 8-methoxy groups, the 3-methoxy group plays a major role in boosting the biological activities, which can be evidenced by comparing 5-demethylsinensetin (**VI**) with completely no activity with 5-demethylhexaMF (**VIII**) with strong inhibition activity (IC_{50}, 6.44 μM) and apoptosis property (AC_{50}, 7.26 μM). Gardenin A was inactive, which is analyzed in next section.

3.1.3. PMFs and hydroxylated PMFs. In general, the anti-proliferative activity of hydroxylated PMFs was more evident compared to PMFs. Notably substitution of the methoxy groups with hydroxyls in 5-position was linked to significant increase in bioactivity. For instance, 5-demethylnobiletin (**II**) was more than 20-fold effective in suppressing HL-60 cell growth compared to nobiletin (**I**). Similar effect was observed in case of 5-demethylheptaMF (**IV**) and 5-demethylated 3,5,6, 7,3′,4′-hexaMF (**X**), which demonstrated more than threefold increase in the anti-proliferative activity compared to their corresponding PMFs. Only substitution of the methoxy group with hydroxyl in 5-position substantially increased anti-proliferative activity of PMFs. Substitution of the methoxy group with hydroxyl in 3′ or 4′ position of B ring did not substantially change bioactivity. Simultaneous demethylation in 3′ and 4′ positions might be linked to the decrease in activity. It is important to notice that the additional 3′-methoxy group renders gardenin A completely inactive. The hydroxyl at 3-position of 3-hydroxynobiletin also killed the activity.

3.2. Apoptosis

The apoptotic activity of PMFs was negligible with the exception of heptaMF (**III**) with a moderate AC_{50} value of 33.88 μM. Three hydroxylated PMFs showed strong proapoptotic activity: 5-demethylnobiletin (**II**, 5.87 μM), 5-demethylheptaMF (**IV**, 5.90 μM), and 5-demethylated 3,5,6,7,3′,4′-hexaMF (**VIII**, 7.26 μM). 3-hydroxynobiletin (**XI**) had demonstrated much lower activity (56.51 μM). Analysis of structure–function relationships in position of side groups and biological activity revealed that presence of 3′- and 8-methoxy group is critical for apoptotic activity of tested hydroxylated PMFs. Lack of either the 3′- or 8-methoxy group renders hydroxylated PMF inactive. This is evident in comparison of the inactive compounds 5-demethyltangeretin (**X**), 5-demethylsinensetin (**VI**) with analogues having aforementioned groups (see Table 1 for details). In the presence of 3′-methoxy group, the influence of 8-methoxy groups on the 5-demethylated PMFs drastically reduced, which can be observed from 5-demethylheptaMF (**IV**, 5.90 μM) and 5-hydroxy-3,6,7,3′,4′-pentaMF (**VIII**, 7.26 μM). There is no evident influence for the

3-methoxy group in 5-demethylnobiletin (**II**, 5.87 μM) and 5-demethylheptaMF (**IV**, 5.90 μM) in the presence of 8-methoxy group. However, in the absence of 8-methoxy group, the presence of 3-methoxy group is necessary for proapoptotic activity, which is evidenced by comparison of 5-demethylsinensetin (**VI**, >100 μM) and 5-demethylhexaMF (**VIII**, 7.26 μM). 3′-Methoxy group also plays a pivotal role for the apoptosis process to occur in the 5-demethylated PMFs, such as 5-demethylnobiletin (**II**, 5.87 μM) and 5-demethyltangeretin (**X**, >100 μM). It is noticeable that hydroxylated PMFs with the hydroxy groups on the B-ring had significantly lower apoptotic activity. An additional methoxy group at the 5′-position of 5-demethylheptaMF, that is, gardenin A (**XII**), totally eliminated the apoptotic property of the compound.

4. Conclusion

In summary, some PMFs like heptaMF showed strong inhibition activity, whereas nobiletin, 3,5,6,7,3′,4′-hexaMF, 3′- and 4′-demethylated nobiletin possess moderate inhibition properties against HL-60 cell lines. 5-Demethylated PMFs, such as 5-demethylnobiletin, 5-demethylheptaMF, and 5-demethylhexaMF, showed much stronger inhibition activity against HL-60 cells than their PMF counterpart. More importantly, these 5-hydroxylated PMFs have very strong apoptotic activity, whereas their corresponding PMFs lack apoptosis property. Therefore, the combination of the potent inhibition effect and strong apoptosis property makes the 5-demethylated PMFs very appealing in the exploration of their anti-cancer activities, such as anti-leukemia.

5. Experimental

5.1. Chemistry

5.1.1. Reagents. Sweet orange peel extract (OPE, from cold-pressed orange peel oil) was obtained from Florida Flavors Company (Lakeland, FL, USA). Solvents, including acetonitrile, acetone, dichloromethane, dioxane, ethanol, ethyl acetate, hexane, methanol, and water, were of HPLC grade and purchased from Fisher Scientific (Springfield, NJ, USA). Reagents, including ammonium hydroxide, boron trichloride, citric acid, formic acid, palladium on carbon, phosphoric acid, pyridine, sodium bicarbonate, sodium hydroxide, trifluoroacetic acid, and triethylamine, were of ACS reagent grade and purchased from Sigma–Aldrich Company (Allentown, PA, USA). 2,3-Dichloro-5,6-dicyanobenzoquinone was purchased from Alfa-Aesar-Lancaster (Pelham, NH, USA). Deuterated solvents including chloroform-*d*, DMSO-d_6, and methanol-d_4 were purchased from Norell Company (Landisville, NJ, USA). Gardenin A was purchased from Indofine Inc. (Hillsborough, NJ, USA).

5.1.2. General. NMR spectra were recorded on a Varian 300 and Varian 500 Spectrometer (Varian Inc., Palo Alto, CA). With TMS serving as an internal standard, ^{1}H NMR was recorded at 300 MHz and ^{13}C NMR at 75 MHz. LC–MS data were obtained from an HPLC-MS system being composed of an auto-sampler injector (Switzerland), an HP1090 system controller, with a variable UV wavelength (190–500 nm) detector, an evaporizing laser scattered deposition (ELSD) detector, and an ESI-MS detector from Micromass VG Platform **II** mass analyzer (Micromass, Beverly, MA). Octadecyl (C_{18}) derivatized silica gel (60 Å) reverse phase analytical HPLC column was purchased from Waters Corporation (Milford, MA, USA). ESI-MS conditions were as follows: acquisition mode, ESI-positive; mass scan range, 100–800 amu; scan rate, 0.4 s; cone voltage, 25 V; source temperature: 150 °C; probe temperature: 550 °C. Analytical HPLC conditions on HPLC-MS: column: Chromeabond WR C_{18}, 3 μm, 120 ; length and OD: 30 × 3.2 mm; injection volume, 15 μL; flow rate: 2 mL/min; run time: 3 min. Mobile phase consisted of acetonitrile and H_2O with 0.05% TFA, typical gradient of 10–90% acetonitrile and the gradient varied.

The isolation and purification were performed on liquid chromatography. An automated flash chromatography system (Model Foxy 200, sg100, ISCO Inc., Lincoln, NE) equipped with a pre-packed silica gel (particle size 35–60 μm) flash column (size 120 g) from Teledyne Isco Inc. was used. The mobile phase consisted of varying proportions of ethyl acetate and hexane with a flow rate of 80 mL/min at a monitoring wavelength of 254 nm. HPLC separation was conducted on a high performance liquid chromatograph (HPLC) equipped with a pump (Waters Delta Prep 4000 delivery pump, Milford, MA), UV–vis detector (Waters 486 tunable absorbance detector, Milford, MA), and an injector (Waters U6K injector, Milford, MA). A Regis Welk-O 1 (R,R) 450 g column (Regis Technologies, Inc., Morton Grove, IL) was used for the HPLC system. The mobile phase for the HPLC system was 35% absolute ethanol and 65% hexanes with a flow rate set at 90 mL/min. The eluent was detected with a UV wavelength at 326 nm.

5.1.3. Isolation of PMFs and hydroxylated PMFs. The general separation procedures from crude sweet orange peel extract were reported previously.[18] Ten grams of OPE was dissolved in 2 mL of dichloromethane and 2 mL of hexanes. The mixture was loaded onto an equilibrated silica gel flash column. The eluting solvents were ethyl acetate and hexanes. The fractions that had UV absorbance at 254 nm were collected and analyzed by LC-ESI-MS. The fractions were combined into six groups[18] according to their molecular weight. Each group was separated either on reverse phase HPLC or with chiral separation technology. The detailed separation procedure and characterization of each PMF and hydroxylated PMF were previously reported.[18] This procedure was used to isolate nobiletin, tangeretin, sinensetin, heptaMF, 3,5,6,7,8,3′,4′-hexaMF, 5-demethylnobiletin, and 5-demethylheptaMF.

5.1.4. Synthesis of 3-hydroxynobiletin (XI). The synthesis of 3-hydroxynobiletin was performed according to the published procedure[19] with slight modification. The process was initiated with the preparation of dimethyldiox-

irane (DMD). A mixture of acetone (30 mL), sodium bicarbonate (24 g), and water (30 mL) along with a magnetic stir bar was placed in a 250 mL three-necked, round-bottomed flask. One neck of the flask was equipped with an addition funnel containing 50 g (0.082 mol) of potassium monoperoxy sulfate (oxone), and second neck of the flask was connected through an air condenser to a receiving flask (50 mL) which was cooled by dry ice–acetone bath. While applying a slight vacuum (ca. 180 Torr, water aspirator) and vigorous stirring at 25 °C to the reaction mixture, oxone was slowly added in about 20 min. Light yellow colored DMD–acetone solution (25 mL) was collected in the receiving flask.

Nobiletin (50 mg) was dissolved in 2 mL of acetone and added 4 mL DMD solution at 0 °C. The reaction mixture was warmed up to ambient temperature and kept stirring for 30 min. The reaction mixture was concentrated in vacuo and 2 mL of methanol was added to the residue. Thus, formed solution was subjected to preparative HPLC purification and 3-hydroxynobiletin was obtained as light yellow solid (18 mg, 34% yield); MS (ESI) $m/z = 419$ [MH^+]; ^{1}H NMR (300 MHz, $CDCl_3$), δ 7.92 (dd, $J = 2$, 8.5 Hz, 1H), 7.90 (d, $J = 2$ Hz, 1H), 7.02 (d, $J = 8.5$ Hz, 1H), 4.16 (s, 3H), 4.04 (s, 3H), 3.98 (s, 3H), 3.96 (s, 3H); 3.94 (s, 3H), and compatible with literature value.[8]

5.1.5. General procedure of 5-demethylated PMF synthesis. *General method.* A PMF was dissolved in anhydrous THF and the solution was cooled to −78 °C with dry ice–acetone. After 10 min stirring, 1 M trichloroboron solution in THF (5–10 equiv) was added dropwise to above cooled solution. Upon completion of addition, the solution was stirred for 20–40 min at −78 °C. The reaction progress was monitored by TLC and LC/MS. Methanol was added slowly to quench the reaction at −78 °C, and then the cool bath was removed. After concentration, the residue was dissolved in a minimum amount of methylene chloride. Thus formed mixture was applied to silica gel flash chromatography and the corresponding 5-demethylated PMF was eluted with ethyl acetate and hexanes.

Alternative method.[20] A PMF was dissolved in 95% ethanol and to the solution was added 1 M hydrochloric acid. The resulted solution was heated to reflux for 16 h. The reaction progress was monitored by TLC and LC/MS. The reaction mixture was cooled and the ethanol was removed in vacuo. Ethyl acetate and water were added, and the organic layer was collected. After concentration, the residue was dissolved in a minimum amount of methylene chloride. Thus formed mixture was applied to silica gel flash chromatography and the corresponding 5-demethylated PMF was eluted with ethyl acetate and hexanes.

5.1.5.1. 5-DemethylhexaMF (VIII). 3,5,6,7,3′,4′-HexaMF (**VII**, 40.2 mg, 0.1 mmol) was dissolved in anhydrous THF (5 mL) and the solution was cooled to −78 °C with dry ice–acetone. After 10 min stirring, 1 mL of 1 M trichloroboron solution in THF (1 mmol) was added dropwise to above cooled solution. Upon completion of addition, the solution was stirred for 20 min at −78 °C. Analysis by LC/MS showed the completion of reaction. Methanol (1 mL) was added to the reaction mixture slowly at −78 °C then cool bath was removed. After removal of THF and excess methanol, the residue was dissolved in a minimum amount of methylene chloride. Thus, formed mixture was applied to silica gel flash chromatography and 5-demethylhexaMF (**VIII**) was eluted with ethyl acetate and hexanes as pale yellow solid (32 mg, 79% yield); MS (ESI) $m/z = 389$ [MH^+]; ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.40 (s, 1H), 7.72 (dd, $J = 2$, 9 Hz, 1H), 7.64 (d, 2 Hz, 1H), 7.18 (s, 1H), 7.15 (d, $J = 9$ Hz, 1H), 3.90 (s, 3H), 3.86 (s, 3H), 3.84 (s, 3H), 3.83 (s, 3H), 3.82 (s, 3H) and in agreement with known spectral data.[23]

5.1.5.2. 5-Demethyltangeretin (X). Same procedure was adapted as 5.1.5.1. White solid; 54% yield; MS (ESI) $m/z = 359$ [MH^+]; ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.75 (s, 1H), 8.05 (d, $J = 9$ Hz, 2H), 7.16 (d, 9 Hz, 2H), 6.95 (s, 1H), 4.03 (s, 3H), 3.92 (s, 3H), 3.87 (s, 3H), 3.82 (s, 3H), and identical with isolated counterpart.[18]

5.1.5.3. 5-Demethylsinensetin (VI).[8] Same procedure was adapted as 5.1.5.1. Light yellow solid, 61% yield, ESI-MS $m/z = 359$ [MH^+]; ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.75 (s, 1H), 7.53 (dd, $J = 2.1$, 8.4 Hz, 1H), 7.34 (d, 2.1 Hz, 1H), 6.98 (d, $J = 8.4$, 1H), 6.60 (s, 1H), 6.55 (s, 1H), 3.98(s, 3H), 3.96 (s, 3H), 3.92 (s, 3H), 3.86 (s, 3H).

5.1.6. Synthesis of 3′-demethylnobiletin (XV).[21,22] The following compounds were obtained as an intermediate step in synthesis of 3′-demethylnobiletin, 2′-hydroxy-3′,4′,5′,6′-tetramethoxyacetophenone (**XVI**), 3-benzyloxy-2′-hydroxy-3′,4,4′,5′,6′-pentamethoxylchalcone (**XVII**), 3′-benzyloxy-4′,5,6,7,8-pentamethoxyflavanone (**XVIII**), and 3′-benzyloxy-4′,5,6,7,8-pentamethoxyflavone (**XIX**).

5.1.6.1. 2′-Hydroxy-3′,4′,5′,6′-tetramethoxyacetophenone (XVI). A mixture of polymethoxylated flavones (1.4 g) that contains tangeretin and nobiletin was dissolved in ethanol (100 mL). While stirring, 50 mL of aqueous potassium hydroxide (50%) was added to above solution and the resulted solution was refluxed under nitrogen for 48 h. The reaction mixture was concentrated in vacuo to remove ethanol. Water (50 mL) and diethyl ether (100 mL) were added to the resulted viscous solution. The aqueous layer was collected and acidified with citric acid. Ethyl acetate (100× 3 mL) was added to extract the product. The combined organic layer was washed with water and brine, dried over sodium sulfate, and concentrated in vacuo. The yellow oil residue was loaded onto a silica gel flash column and the desired product was eluted with ethyl acetate and hexane to give 0.7 g of 2′-hydroxy-3′,4′,5′,6′-tetramethoxyacetophenone as a yellow oil: ESI-MS $m/z = 257$ [MH^+]; ^{1}H NMR (300 MHz, $CDCl_3$), δ 4.05 (s, 3H), 3.95 (s, 3H), 3.85 (s, 3H), 3.80 (s, 3H), 2.64 (s, 3H), compatible with known spectra.[21]

5.1.6.2. 3-Benzyloxy-2′-hydroxy-3′,4,4′,5′,6′-pentamethoxylchalcone (XVII). A solution of 3-benzyloxy-4-methoxy-benzaldehyde (218 mg, 0.90 mmol) and 2-hydroxy-3,4,5,6-tetramethoxyacetophenone (217 mg, 0.85 mmol) in 10 mL of 80% ethanol containing KOH (2 g) was stirred at room temperature overnight. The mixture was acidified with 20% HCl and then extracted with ethyl acetate. The extract was washed with water and brine, dried over $MgSO_4$, and concentrated in vacuo, and the residue was applied to silica gel flash chromatography to give 192 mg of 3-benzyloxy-2′-hydroxy-3′,4,4′,5′,6′-pentamethoxylchalcone as a light yellow solid; ESI-MS $m/z = 481$ [MH^+] and ^1H NMR was compatible with literature value.[21]

5.1.6.3. 3′-Benzyloxy-4′,5,6,7,8-pentamethoxyflavanone (XVIII). A solution of 3-benzyloxy-2′-hydroxy-3′,4,4′,5′,6′-pentamethoxylchalcone (192 mg) in ethanol (10 mL) containing 50 mg H_3PO_4 was heated to reflux for 16 h. Upon concentration, the resultant material was mixed with water and extracted twice with ethyl acetate (50× 2 mL). The combined organic layer was concentrated and purified by normal phase flash chromatography and 81 mg of 3′-benzyloxy-4′,5,6,7,8-pentamethoxyflavanone was obtained as a pale yellow solid; ESI-MS $m/z = 481$ [MH^+] and ^1H NMR was compatible with literature value.[21]

5.1.6.4. 3′-Benzyloxy-4′,5,6,7,8-pentamethoxyflavone (XIX). A solution of 3′-benzyloxy-4′,5,6,7,8-pentamethoxyflavanone (81 mg, 0.17 mmol) and 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ, 57 mg, 0.25 mmol) in dry dioxane (5 mL) was refluxed for 8 h. The reaction mixture was cooled, concentrated, and separated with silica gel chromatography to obtain 48 mg of 3′-benzyloxy-4′,5,6,7,8-pentamethoxyflavone as an off-white solid; ESI-MS $m/z = 479$ [MH^+].

5.1.6.5. 3′-Demethylnobiletin (XV). 3′-Benzyloxy-4′,5,6,7,8-pentamethoxyflavanone (48 mg) was dissolved in methanol (2 mL) and to this solution 10% of palladium on carbon (5 mg) was added. Hydrogen gas was introduced at 40 psi for 30 min while shaking to give 3′-hydroxy-4′,5,6,7,8-pentamethoxyflavone (3′-demethylnobiletin, 15 mg) as a white solid. ESI-MS $m/z = 389$ [MH^+]; ^1H NMR (300 MHz, $CDCl_3$): δ 7.50 (d, $J = 2$ Hz, 1H), 7.48 (dd, $J = 8.5$ and 2 Hz, 1H), 6.97 (d, $J = 8.5$ Hz, 1H), 6.60 (s, 1H), 5.78 (s, 1H), 4.10(s, 3H), 4.02 (s, 3H), 3.98 (s, 3H), 3.94 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 177.52, 161.32, 159.80, 149.69, 148.24, 147.92, 146.35, 144.40, 138.80, 124.42, 118.98, 115.50, 112.49, 111.04, 107.26, 62.434, 62.22, 61.98, 61.81; UV (acetonitrile and water): $\lambda_{max} = 250.5$ and 374.5 nm.[22]

5.1.7. 4′-Demethylnobiletin (XIII).[21,22] The same procedure as the previous synthesis of 3′-demethylnobiletin (**XV**) was followed using 4-benzyloxy-3-methoxy-benzaldehyde (218 mg, 0.90 mmol) and 2′-hydroxy-3′,4′,5′,6′-tetramethoxyacetophenone (217 mg, 0.85 mmol) as starting materials. 4-Benzyloxy-2′-hydroxy-3,3′,4′,5′,6′-pentamethoxylchalcone (181 mg) was obtained as a light yellow solid. ESI-MS $m/z = 481$ [MH^+]. From 4-benzyloxy-2′-hydroxy-3,3′,4′,5′,6′-pentamethoxylchalcone (181 mg, 0.38 mmol) and phosphoric acid (50 mg), 61 mg of 4′-benzyloxy-3′,5,6,7,8-pentamethoxyflavanone was obtained as a pale yellow solid. ESI-MS $m/z = 481$ [MH^+]. 4′-Benzyloxy-3′,5,6,7,8-pentamethoxyflavanone (61 mg, 0.13 mmol) was treated with DDQ (57 mg, 0.25 mmol) to obtain 4′-benzyloxy-3′,5,6,7,8-pentamethoxyflavone (38 mg). ESI-MS $m/z = 479$ [MH^+]. Upon hydrogenation of 4′-benzyloxy-3′,5,6,7,8-pentamethoxyflavone (38 mg), 4′-hydroxy-3′,5,6,7,8-pentamethoxyflavone (**XV**, 4′-demethylnobiletin, 12.5 mg) was obtained as a white solid. ESI-MS $m/z = 389$ [MH^+]; ^1H NMR (300 MHz, $CDCl_3$): δ 8.80 (br, 1H), 7.78 (s, 1H), 7.50 (d, $J = 8$ Hz, 1H), 7.0 (d, $J = 8$ Hz, 1H), 6.70 (s, 1H), 4.12(s, 3H), 3.98 (s, 3H), 3.94 (s, 3H), 3.92 (s, 6H); UV (acetonitrile and water): $\lambda_{max} = 250.5$ and 336.5 nm.[22]

5.1.8. 3′4′-Didemethylnobiletin (XIV). The same procedure as the previous synthesis of 3′-demethylnobiletin (**XV**) was followed using 3,4-dibenzyloxy-benzaldehyde (287 mg, 0.90 mmol) and 2′-hydroxy-3′,4′,5′,6′-tetramethoxyacetophenone (**XVIII**, 217 mg, 0.85 mmol) as starting materials. 3,4-Dibenzyloxy-2′-hydroxy-3′,4′,5′,6′-tetramethoxylchalcone (202 mg) was obtained as a light yellow solid; ESI-MS $m/z = 557$ [MH^+]. From 3,4-dibenzyloxy-2′-hydroxy-3′,4′,5′,6′-tetramethoxylchalcone (202 mg, 0.36 mmol) and phosphoric acid (50 mg), 89 mg (0.16 mmol) of 3′,4′-dibenzyloxy-5,6,7,8-tetramethoxyflavanone was obtained as a yellow solid, which was treated with DDQ (57 mg, 0.25 mmol) to obtain 3′,4′-dibenzyloxy-5,6,7,8-tetramethoxyflavone as an off-white solid (53 mg) as an off-white solid. ESI-MS $m/z = 555$ [MH^+]. Upon hydrogenation of 3′,4′-dibenzyloxy-5,6,7,8-tetramethoxyflavone, 3′,4′-dihydroxy-5,6,7,8-tetramethoxyflavone (3′,4′-didemethylnobiletin, **XVI**, 13 mg) was obtained as a white solid; ESI-MS $m/z = 375$ [MH^+]; UV (max in acetonitrile/water) 368.5 and 252.5 nm; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.70 (s, 1H), 7.50 (d, $J = 8.5$ Hz, 1H), 7.0 (d, $J = 8.5$ Hz, 1H), 6.74 (s, 1H), 4.12(s, 3H), 4.02 (s, 3H), 3.97 (s, 3H), 3.94 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz), δ 178.4, 163.1, 152.4, 148.9, 148.4, 148.2, 145.0, 144.9, 139.0, 123.2, 119.6, 115.7, 114.8, 113.5, 106.3, 62.4, 62.3, 61.3, 61.8.

5.2. Biological activity

5.2.1. Cell culture. Human leukemia HL-60 cell lines were obtained from American Type Culture Collection (Rockville, MD) and maintained in Roswell Park Memorial Institute 1640 medium (Life Technologies, Inc.) which was supplemented with 10% fetal bovine serum (Biological Industries, Israel), 2 mM glutamine (Gibco BRL) and 1% penicillin/streptomycin (10,000 units of penicillin/mL and 10 mg/mL streptomycin). The HL-60 cell lines was kept at 37 °C in humidified 5% CO_2 incubator.

5.2.2. Cell proliferation assay. HL-60 cells were placed into 12-well plates at a density of 2×10^5 per well. After overnight growth, cells were treated with different concentrations of the selected compounds for 24 h. Control cells are treated with DMSO to a final concentration of 0.05% (v/v). At the end of incubation, cells are harvested and counted in a hemocytometer.

5.2.3. Apoptotic ratio analysis. HL-60 cells (2×10^5) were cultured in 60-mm Petri dishes and incubated for 24 h. After treated with selected compounds for 24 h, the cells were then harvested, washed with PBS, resuspended in 200 μL PBS, and fixed in 800 μL of iced 100% ethanol at −20 °C. After being left to stand overnight, the cell pellets were collected by centrifugation, resuspended in 1 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/mL RNase,) and incubated at 37 °C for 30 min. Next, 1 mL of propidium iodide solution (50 μg/mL) was added, and the mixture was allowed to stand on ice for 30 min. Fluorescence emitted from the propidium iodide–DNA complex was quantitated after excitation of the fluorescent dye by FACScan cytometry (Becton–Dickinson, San Jose, CA).

5.2.4. Statistical analysis. A one-way ANOVA was used for statistical analysis of data. A Student's *t*-test was applied when multiple comparisons were performed.

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References and notes

1. Manthey, J. A.; Grohmann, K.; Guthrie, N. *Curr. Med. Chem.* **2001**, *8*, 135–153.
2. Middleton, E.; Kandaswami, C.; Theoharides, T. C. *Pharmacol. Rev.* **2000**, *52*, 673–751.
3. Murakami, A.; Ohigashi, H. In *Oxidative Stress and Disease*; Bao, Y., Fenwick, R., Eds.; Marcel Dekker: New York, 2004; Vol. 12, pp 187–211.
4. Lopez-Lazaro, M. *Curr. Med. Chem.* **2002**, 691.
5. Manthey, J. A.; Guthrie, N. *J. Agric. Food Chem.* **2002**, *50*, 5837–5843.
6. Whitman, S. C.; Kurowska, E. M.; Manthey, J. A.; Daugherty, A. *Atherosclerosis* **2005**, *178*, 25.
7. Ohshima, H.; Tatemichi, M.; Sawa, T. *Arch. Biochem. Biophys.* **2003**, *417*, 3–11.
8. Sugiyama, S.; Umehara, K.; Kuroyanagi, M.; Ueno, A.; Taki, T. *Chem. Pharm. Bull.* **1993**, *41*, 714–719.
9. Vermeulen, S.; Vanmarck, V.; Vanhoorde, L.; Vanroy, F.; Brack, M.; Mareel, M. *Pathol. Res. Pract.* **1996**, *192*, 694–707.
10. Hirano, T.; Abe, K.; Gotoh, M.; Oka, K. *Br. J. Cancer* **1995**, *72*, 11380–11388.
11. Kawaii, S.; Tomono, Y.; Katase, E.; Ogawa, K. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 896–899.
12. Pan, M. H.; Chen, W. J.; Lin-Shiau, S. Y.; Ho, C. T.; Lin, J. K. *Carcinogenesis* **2002**, *23*, 1677–1684.
13. Chen, K. H.; Weng, M. S.; Lin, J. K. *Biochem. Pharmacol.* **2007**, *73*, 215–227.
14. Kandaswami, C.; Perkins, E.; Soloniuk, D. S.; Drzewiecki, G.; Middleton, E. *Cancer Lett.* **1991**, *59*, 147–152.
15. Yoshimizu, N.; Otani, Y.; Saikawa, Y.; Kubota, T. *Aliment Pharmacol. Ther.* **2004**, *20*, 95–101.
16. Murakami, A.; Nakamura, Y.; Torikai, K.; Tanaka, T.; Koshiba, T.; Koshimizu, K.; Kuwahara, S.; Takahashi, Y.; Ogawa, K.; Yano, M.; Tokuda, H.; Nishino, H.; Mimaki, Y.; Sashida, Y.; Kitanaka, S.; Ohigashi, H. *Cancer Res.* **2000**, *60*, 5059–5066.
17. Iwase, Y.; Takemura, Y.; Ju-ichi, M.; Ito, C.; Furukawa, H.; Kawaii, S.; Yano, M.; Mou, X. Y.; Takayasu, J.; Tokuda, H.; Nishino, H. *Cancer Lett.* **2000**, *154*, 101–105.
18. Li, S.; Lo, C.-Y.; Ho, C.-T. *J. Agric. Food Chem.* **2006**, *54*, 4176–4185.
19. Chu, H. W.; Wu, H. T.; Lee, Y. J. *Tetrahedron* **2004**, *60*, 2647–2656.
20. Ho, C.-T.; Li, S.; Pan, M.-H.; Lo, C.-Y.; Dushenkov, S. U.S. Provisional Patent, 2006.
21. Mizuno, M.; Matoba, Y.; Tanaka, T.; Tachibana, H.; Iinuma, M.; Iwamasa, M. *J. Nat. Prod.* **1987**, *50*, 751–753.
22. Li, S.; Wang, Z.; Sang, S.; Huang, M.-T.; Ho, C.-T. *Mol. Nutr. Food Res.* **2006**, *50*, 291–299.
23. Banerji, A.; Chadha, M. S.; Malshet, V. G. *Phytochemistry* **1969**, *8*, 511–512.