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Relationship Between the Structure of Methoxylated and Hydroxylated Flavones and Their Antiproliferative Activity in HL60 Cells

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Abstract. *As part of our continuing investigation on flavonoid derivatives as potential anticancer substances, a series of methoxylated and hydroxylated flavones was synthesized, and their cytotoxic and anti-proliferative activity was evaluated in leukemic HL60 cells. Their structure-activity relationship was also investigated. The correlation between the methoxylation/hydroxylation pattern and antiproliferative activity revealed the importance of the 5,4'- and 3',4'-dihydroxyl moieties in flavone nucleus.*

Many of the health-promoting effects of fruits, vegetables, legumes and beverages such as wine, coffee and tea, are attributed to flavonoids, which are naturally occurring polyphenolic compounds (1-4). They occupy an important part of human diet, and are reported to have a broad spectrum of biological activities, such as antibacterial and anti-inflammatory activities. They also participate in cellular mechanisms related to cancer. Although the positive biological actions of flavonoids have been assigned to their antioxidant properties (5), there is an emerging view that flavonoids and their metabolites do not act only as hydrogen-donating antioxidants, but could also modulate signaling pathways in cells (6). Flavonoids might be used not only for cancer prevention (7), but also as anticancer agents (8, 9).

Polymethoxyflavones (PMFs) are flavones substituted with two or more methoxyl groups. PMFs are found almost exclusively in the *Citrus* genus particularly in the peel of king orange (*C. nobilis*), sweet orange (*C. sinensis*), and mandarin orange (*C. reticulata*). They are coming to the center of interest due to their documented wide spectrum of biological activity including anti-inflammatory, anti-carcinogenic, anti-atherogenic, neurotropic and memory-enhancing properties (10-14). PMFs have also been reported to inhibit the growth of HL60 cells *in vitro* and to suppress proliferation, while promoting apoptosis (15). The potent differentiation-inducing activity toward HL60 leukemic cells was found from in the juice of *Citrus nobilis*, and the active components were isolated and identified as four PMFs (16). Their biological properties lead to horticultural breeding attempt to produce new citrus cultivar containing high amounts of PMFs (17, 18).

Our investigation on the structure-activity relationship of PMFs showed that there was a complicated correlation between the number/position of methoxyl group and their antiproliferative activity. Roughly speaking, an increase in the number of methoxyl groups on the A-ring enhanced the activity of PMFs, whereas the increase of B-ring methoxyl groups reduced the activity (19). In order to determine how the methoxyl/hydroxyl groups contribute to the antiproliferative activity of flavone, relatively simple methoxylated flavones and their corresponding hydroxylated flavones were synthesized and their antiproliferative activity was estimated.

Materials and Methods

General procedures. Chemicals and solvents from commercial sources were used without further purification unless specified. Reactions were carried out under argon, and monitored by thin-layer chromatography on silica gel (mesh size 60, F254) and with visualizedation under UV light. Standard and flash column chromatography procedures were not optimized. Nuclear magnetic resonance (NMR) spectra were recorded on a 400-MHz JEOL ECP-400 spectrometer (JEOL, Tokyo, Japan), and chemical shifts values are expressed in ppm (δ) relative to the residual ^{1}H signal of the solvents. Unless otherwise specified, compounds were dissolved in dimethylsufoxide-d_6. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) mass spectrometry were performed on Thermo Exactive (Thermo Fisher Scientific K.K, Tokyo, Japan) and Hitachi M8000 instruments (Hitachi, Tokyo, Japan), respectively.

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Key Words: Methoxyflavone, hydroxyflavone, antiproliferative activity, HL60 cells, structure and activity relationship.

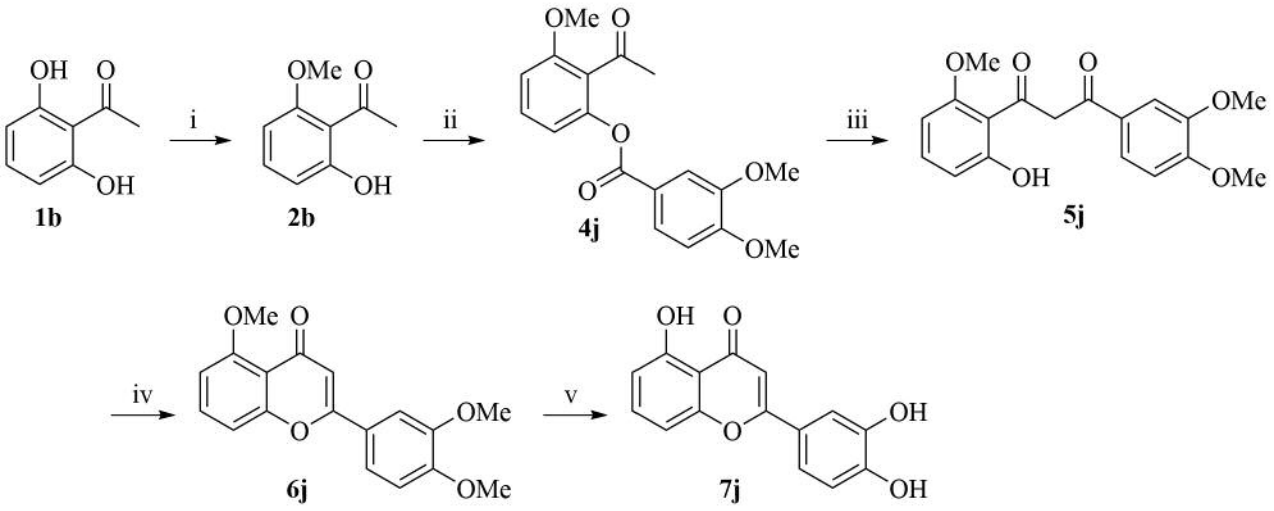


Figure 1. *Synthesis of 5,3',4'-trimethoxyflavone (**6j**) and 5,3',4'-trihydroxyflavone (**7j**). i) Me_2SO_4, K_2CO_3, acetone, rt, 6h; ii) DCC, DMAP, 3,4-dimethoxybenzoic acid (**3f**); iii) KOH, pyridine, 100˚C, 10 min; iv) 20% H_2SO_4/acetic acid, 100˚C, 10 min; v) 1.0 M BBr_3/CH_2Cl_2, rt, 12 h.*

*Synthesis of 5,3',4'-trimethoxyflavone (**6j**) and 5,3',4'-trihydroxyflavone (**7j**) (Figure 1).* General procedure for synthesis of methoxyflavones (Figure 2, **6a-6k**) and hydroxyflavones (Figure 2, **7a-7k**). To a suspension of 2',6'-dihydroxyacetophenone (1b, 6.53 g, 42.9 mmol) and K_2CO_3 (8.89 g, 64.4 mmol) in acetone (50 ml), dimethylsulfate (4.07 ml, 42.9 mmol) was dropwisely added. After the reaction mixture was refluxed overnight, the organic solvent was removed under reduced pressure. The residue was dissolved in dichloromethane (40 ml) and the organic solution was washed with deionized water. The concentrated residue of the organic phase was chromatographed over silica gel (hexane/ethyl acetate; 85:15) to afford 2'-hydroxy-6'-methoxyacetphenone (**2b**, 3.35 g, 47%).

To a suspension of dicyclohexylcarbodiimide (1.00 g, 4.85 mmol) and *N,N*-dimethyl-4-aminopyridine (107 mg, 0.876 mmol) in dry dichloromethane (12 ml), **2b** (718 mg, 4.32 mmol) and then 3,4-dimethoxybenzoic acid (**3f**, 977 mg, 5.37 mmol) were added. The reaction mixture was stirred at room temperature for 20 hours. The mixture was filtered to remove dicyclohexylurea as white precipitate. The solvent of the filtrate was removed under reduced pressure, and the residue was chromatographed over silica gel [hexane/dichloromethane; 2:8] to afford 6'-methoxy-2'-(3,4-dimethoxybenzoyloxy) acetophenone (**4j**) as a white solid. For **4j**: ^{1}H-NMR (CDCl3) δ 2.49 (s, 3H), 3.84 (s, 3H), 3.86 (s, 3H), 3.87 (s, 3H), 6.87 (d, 1H, *J*=8.8 Hz), 7.19 (dd, 1H, *J*=7.7, 1.5 Hz), 7.42 (d, 1H, *J*=1.5 Hz), 7.71 (dd, 1H, *J*=8.8, 2.2 Hz), 7.81 (d, 1H, *J*=7.7 Hz), 7.90 (d, 1H, *J*=2.2 Hz).

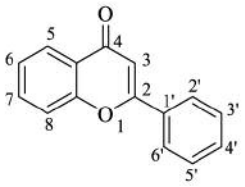
To a suspension of KOH (212 mg, 3.79 mmol) in dry pyridine (3 ml) **4j** (573 mg, 1.74 mmol) was added and the mixture was stirred at 100˚C for 10 min. After being cooled to room temperature, the reaction mixture was neutralized with acetic acid (approximately 3.0 ml). The mixture was added to ethanol (3.0 ml) and deionized water (3.0 ml). The resulting precipitate was filtered and washed with cold ethanol to give 1-(3,4-dimethoxyphenyl)-3-(2-hydroxy-4-methoxyphenyl) propane-1,3-dione (**5j**, 350 mg, yield from **4j**: 61%). For **5j**: ^{1}H-NMR ($CDCl_3$) δ 3.81 (s, 2H), 3.87 (s, 3H), 3.89 (d, 6H), 6.47 (m, 2H), 6.69 (s, 1H), 7.38 (d, 1H, J=8.8 Hz), 7.44 (d, 1H, *J*=2.5 Hz), 7.48 (d, 1H, *J*=1.5 Hz), 7.68 (d, 1H, *J*=8 Hz).

To a solution of containing **5j** (431 mg, 1.30 mmol) dissolved in acetic acid (6 ml) kept at 100˚C, 20% H_2SO_4/acetic acid (1 ml) was added. The mixture was stirred at 100˚C for 10 min. After cooling to room temperature, deionized water was added to the mixture. The resulting precipitate was filtered and washed with water, and was then chromatographed over silica gel (hexane/dichloromethane; 2:8) to afford 7,3'-dimethoxyflavone (**6j**, 379 mg, yield from **5j**: 93%).

To a solution containing **6j** (300 mg, 1.12 mmol) in dry dichloromethane kept at 0˚C, 1.0 M BBr_3/CH_2Cl_2 (3.9 ml, 3.9 mmol) was carefully added. After the mixture stirred at room temperature for 12 h, 7 ml of ethanol were added and the organic solvent was removed under reduced pressure. To the residue 11 ml of boiling 50% ethanol were added. After being cooled to room temperature, the resulting precipitate was filtered and washed with 50% ethanol. Recrystallization from ethanol afforded **7j** as yellow needles (211 mg, 74%).

By combination of 2'-hydroxyacetophenone derivatives, namely 2'-hydroxy- (**1a**), 2'-hydroxy-6'-methoxy- (**1b**), 2'-hydroxy-5'-methoxy- (**1c**), and 2'-hydroxy-4'-methoxy-acetophenones (**1d**), and benzoic acid (**2a**) and its derivatives, namely 2-methoxy- (**2b**), 3-methoxy- (**2c**), 4-methoxy- (**2d**), 3,4,5-trimethoxy- (**2e**), and 3,4-dimethoxybenzoic acids (**2f**), 11 methoxylated and 10 hydroxylated flavones were synthesized. Spectral data for **6j** and **7j** are shown in Table I along with data for other synthetic flavones.

Cell proliferation assay. HL60 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum. The level of cellular proliferation for HL60 cells grown in a 96-well microplate was measured by using Alamar Blue (Life Technologies Ltd., Tokyo, Japan). To each well, 100 μl of HL60 cell suspension (1.0×10^4 cells/100 μl) was were inoculated and 100 μl of medium containing serial dilutions of the samples to be assayed was were added. After three days of incubation, 20 μl of alamar blue was aseptically added to each well, and cells were further incubated for approximately 20 hours. Cellular proliferation (as a percentage that



Compound	Substituents							IC_{50} (μM)	Log *p*
	C5	C6	C7	C2'	C3'	C4'	C5'		
6a	OMe	H	H	H	H	H	H	48	2.95
6b	H	OMe	H	H	H	H	H	400<	2.95
6c	H	H	OMe	H	H	H	H	68	2.95
6d	H	H	H	OMe	H	H	H	101	2.95
6e	H	H	H	H	OMe	H	H	206	2.95
6f	H	H	H	H	H	OMe	H	77	2.95
6g	H	H	H	H	OMe	OMe	OMe	400<	2.69
6h	OMe	H	H	H	OMe	H	H	46	2.82
6i	OMe	H	H	H	H	OMe	H	36	2.82
6j	OMe	H	H	H	OMe	OMe	H	400<	2.69
6k	H	OMe	H	H	H	OMe	H	400<	2.82
8	H	H	H	H	OMe	OMe	H	400<	2.82
7a	OH	H	H	H	H	H	H	400<	2.68
7b	H	OH	H	H	H	H	H	89	2.68
7c	H	H	OH	H	H	H	H	400<	2.68
7d	H	H	H	OH	H	H	H	400<	2.68
7e	H	H	H	H	OH	H	H	400<	2.68
7f	H	H	H	H	H	OH	H	400<	2.68
7g	H	H	H	H	OH	OH	OH	196	1.9
7h	OH	H	H	H	OH	H	H	400<	2.29
7i	OH	H	H	H	H	OH	H	28	2.29
7j	OH	H	H	H	OH	OH	H	13	1.9
7k	H	OH	H	H	H	OH	H	n.t.	2.29
9	H	H	H	H	OH	OH	H	51	2.29
10	H	H	H	H	H	H	H	64	3.07

Figure 2. *Structures, IC_{50} (μM) and log P of methoxyflavones and hydroxyflavones. Calculation of log p was done by ChemDraw Professionl 15.1 (Perkin-Elmer Infomatics, Inc.).*

of the untreated control) was calculated with the following equation: Proliferation (%)

$$= \frac{([(A_{570}\text{-}A_{595}) \text{ of test agent dilution}]\text{-}[(A_{570}\text{-}A_{595}) \text{of blank}])}{([(A_{570}\text{-}A_{595}) \text{ of positive growth control}]\text{-}[(A_{570}\text{-}A_{595}) \text{ of blank}])}$$

where A_{570} and A_{595} are the absorbance at 570 nm and 595 nm, respectively.

Results and Discussion

Chemistry. In this study, 11 methylated flavones and 10 hydroxylated flavones were synthesized. The synthetic route to 5,3',4'-trihydroxyflavone (**7j**) through 5,3',4'-trimethoxyflavone (**6j**) is illustrated in Figure 1. 2',6'-Dihydroxyacetophenone (**1b**) was first treated with dimethylsulfate and K_2CO_3 in dry acetone to afford 2'-hydroxy-6'-methoxyactophenone (**2b**). In spite of the increased ratio of

Table I. *Analytical data of synthesized compounds*.

Compound	5-Methoxyflavone (**6a**)	6-Methoxyflavone (**6b**)	7-Methoxyflavone (**6c**)
weight (yield)	490 mg (42%)	1.05 g (72%)	2.58 g (71%)
^{1}H-NMR	4.00 (s, 3H), 6.75 (s, 1H), 6.82 (dd, 1H, J=8, 1 Hz), 7.13 (dd, 1H, J=8, 1 Hz), 7.51 (m, 3H),7.60 (t, 1H, J=8 Hz), 7.89 (m, 2H).	3.91 (s, 3H), 6.95 (s, 1H), 7.31 (dd, 1H, J=7.1, 3 Hz), 7.53 (m, 4H), 7.59 (d, 1H, J=3 Hz), 7.93 (m, 2H)	3.73 (s, 3H), 7.07 (dd, 1H, J=7.1, 3 Hz), 7.15 (s, 1H), 7.21 (d, 1H, J=7.1 Hz), 7.72 (m, 3H), 7.59 (d, 1H, J=3 Hz), 8.01 (m, 2H).
Compound	2'-Methoxyflavone (**6d**)	3'-Methoxyflavone (**6e**)	4'-Methoxyflavone (**6f**)
weight (yield)	2.20 g (91%)	1.21 g (75%)	176 mg (78%)
^{1}H-NMR	3.93 (s, 3H), 7.04 (d, 1H, J=8.4 Hz), 7.11 (dt, 1H, J=6.6, 1.1 Hz), 7.15 (s, 1H), 7.40 (dt, 1H, J=5.8, 1.1 Hz), 7.48 (dt, 1H, J=5.5, 1.8 Hz), 7.52 (dd, 1H, J=7.7, 0.7 Hz), 7.67 (dt, 1H, J=5.5, 1.8 Hz), 7.90 (dd, 1H, J=6.2, 1.8 Hz), 8.23 (dd, 1H, J=6.2, 1.8 Hz)	3.89 (s, 3H), 6.85 (s, 1H), 7.08 (dt, 1H, J=5.5, 2.6 Hz), 7.44 (m, 3H), 7.51 (dt, 1H, J=6.2, 0.7 Hz), 7.57 (d, 1H, J=8.4 Hz), 7.71 (dt, 1H, J=5.5, 1.8 Hz), 8.23 (dd, 1H, J=6.2, 1.8 Hz)	3.87 (s, 3H), 6.81 (s, 1H), 7.02 (dd, 1H, J=7.1, 1.5 Hz), 7.41 (dt, 1H, J=7.3, 1.0 Hz), 7.55 (d, 1H, J=8.4 Hz), 7.69 (dt, 1H, J=8.1, 1.5 Hz), 7.89 (d, 2H, J=7 Hz), 8.22 (d, 2H, J=7 Hz).
Compound	3',4',5'-Trimethoxyflavone (**6g**)	5,3'-Dimethoxyflavone (**6h**)	5,4'-Dimethoxyflavone (**6i**)
weight (yield)	1.64 g (85%)	659 mg (65%)	779 mg (69%)
^{1}H-NMR	3.93 (s, 3H), 3.96 (s, 6H), 6.78 (s, 1H), 7.14 (s, 2H), 7.43 (dt, 1H, J=6, 1.1 Hz), 7.58 (d, 1H, J=8 Hz), 7.71 (dt, 1H, J=6, 2 Hz), 8.23 (dd, 1H, J=6, 2 Hz)	3.90 (s, 3H), 3.92 (s, 3H), 6.70 (s, 1H), 6.98 (d, 1H, J=8.0 Hz), 7.14 (dd, 1H, J=5.9, 1.8 Hz), 7.24 (dd, 1H, J=7.4, 1.1 Hz), 7.48 (t, 1H, J=7.7 Hz), 7.56 (t, 1H, J=1.8 Hz), 7.61 (dt, 1H, J=5.1, 1.8 Hz), 7.67 (t, 1H, J=8.4 Hz).	3.87 (s, 3H), 3.99 (s, 3H), 6.67 (s, 1H), 6.81 (d, 1H, J=8.1 Hz), 6.99 (dd, 2H, J=4.8, 2.2 Hz), 7.11 (dd, 1H, J=7.7, 0.7 Hz), 7.55 (t, 1H, J=8.4 Hz), 7.84 (dd, 2H, J=4.8, 1.8 Hz).
Compound	5,3',4'-Trimethoxyflavone (**6j**)	6,4'-Dimethoxyflavone (**6k**)	5-Hydroxyflavone (**7a**)
weight (yield)	955 mg (71%).	674 mg (74%)	211 mg (74%)
^{1}H-NMR	3.96 (s, 3H), 3.97 (s, 3H), 4.00 (s, 3H), 6.80 (s, 1H), 6.83 (d, 1H, J=8.4 Hz), 6.97 (d, 1H, J=8.4 Hz), 7.14 (d, 1H, J=8.8 Hz), 7.35 (d, 1H, J=2.2 Hz), 7.55 (dd, 1H, J=6.6, 1.8 Hz), 7.59 (d, 1H, J=8.4 Hz).	3.86 (s, 3H), 3.87 (s, 3H), 6.95 (s, 1H), 7.12 (d, 2H, J=8.8 Hz), 7.41 (d, 1H, J=3.3 Hz), 7.43 (d, 1H, J=2.2 Hz), 7.75 (dd, 1H, J=4.4, 2.6 Hz), 8.07 (dd, 2H, J=5.1, 1.8 Hz).	6.79 (dt, 1H, J=8, 1.2 Hz), 6.92 (s, 1H), 7.16 (t, 1H, J=8.0 Hz), 7.63 (m, 3H), 7.68 (dt, 1H, J=8, 1.2 Hz), 8.11 (dd, 2H, J=6.2, 1.4 Hz), 12.6 (br.s, 1H)
Compound	6-Hydroxyflavone (**7b**)	7-Hydroxyflavone (**7c**)	2'-Hydroxyflavone (**7d**)
weight (yield)	352 mg (62%)	434 mg (38%)	863 mg (91%)
^{1}H-NMR	6.85 (s, 1H), 7.28 (dd, 1H, J=5.8, 2.9 Hz), 7.42 (d, 1H, J=2.9), 7.56 (m, 3H), 7.60 (d, 1H, J=8.8 Hz), 8.02 (dt, 2H, J=5.5, 2.5 Hz), 9.6 (br.s, 1H)	6.82 (s, 1H), 6.99 (dd, 1H, J=7.0, 1.1 Hz), 7.04 (d, 1H, J=1.1 Hz), 7.38 (m, 2H), 7.49 (m, 3H), 7.70 (d, 1H, J=7.0 Hz), 9.3 (br.s, 1H)	6.99 (dt, 1H, J=7.0, 1.1 Hz), 7.04 (d, 1H, J=1.1 Hz), 7.38 (m, 2H), 7.49 (dt, 1H, J=7.0, 1.1 Hz), 7.70 (d, 1H, J=8.4 Hz), 7.80 (dt, 1H, J=1.4 Hz), 7.96 (dd, 1H, J=6.2, 1.8 Hz), 8.14 (dd, 1H, J=6.2, 1.8 Hz), 9.4 (br.s, 1H)
Compound	3'-Hydroxyflavone (**7e**)	4'-Hydroxyflavone (**7f**)	3',4',5'-Trihydroxyflavone (**7g**)
weight (yield)	473 mg (77%)	3.90 g (83%)	530 mg (65%)
^{1}H-NMR	6.86 (s, 1H), 7.00 (dt, 1H, J=5.2, 1.4 Hz), 7.37 (t, 1H, J=8.1 Hz), 7.43 (t, 1H, J=1.8 Hz), 7.50 (m, 2H), 7.72 (dd, 1H, J=7.7, 0.7 Hz), 7.82 (dt, 1H, J=5.2, 1.8 Hz), 8.14 (dd, 1H, J=6.6, 1.1 Hz), 9.5 (br.s, 1H)	6.87 (d, 1H, J=4.4 Hz), 6.95 (m, 2H), 7.49 (dd, 1H, J=6.9, 0.7 Hz), 7.79 (m, 2H), 7.97 (m, 2H), 8.04 (dd, 1H, J=6.9, 0.8 Hz), 10.3 (br.s, 1H)	6.63 (s, 1H), 7.01 (m, 2H), 7.49 (dt, 1H, J=7.0, 1.1 Hz), 7.69 (d, 1H, J=8.1 Hz), 7.81 (dt, 1H, J=5.2, 1.8 Hz), 8.03 (dd, 1H, J=6.2, 1.5 Hz), 9.10 (br.s, 1H), 9.41 (br.s, 2H).

Table I. *Continued*

Table I. *Continued*

Compound	5,3'-Dihydroxyflavone (**7h**)	5,4'-Dihydroxyflavone (**7i**)	5,3',4'-Dihydroxyflavone (**7j**)
weight (yield)	149 mg (33%)	383 mg (71%)	549 mg (79%)
^{1}H-NMR	6.83 (d, 1H, J=8.0 Hz), 7.02 (s, 1H), 7.04 (d, 1H, J=1.4 Hz), 7.19 (d, 1H, J=8.4 Hz), 7.39 (t, 1H, J=8.0 Hz), 7.45 (t, 1H, J=1.8 Hz), 7.55 (d, 1H, J=8.8 Hz), 7.70 (t, 1H, J=8.4 Hz), 9.97 (br, 1H), 12.0 (br.s, 1H)	6.80 (d, 1H, J=8.4 Hz), 6.94 (d, 1H, J=4.0 Hz), 6.96 (s, 1H), 7.17 (d, 1H, J=8.4 Hz), 7.31 (d, 2H, J=8 Hz), 7.65 (t, 1H, J=8.4 Hz), 7.99 (d, 2H, J=8.8 Hz), 12.6 (br.s, 1H)	6.62 (dd, 1H, J=7.7, 0.8 Hz), 6.84 (s, 1H), 7.14 (dd, 1H, J=7.7, 0.8 Hz), 7.47 (m, 2H), 7.50 (d, 1H, J=2.2 Hz), 7.66 (t, 1H, J=8.4 Hz), 9.48 (br.s, 1H), 10.0 (br.s, 1H), 12.8 (br.s, 1H)

iodomethane added, 2',6'-dimethoxyacetophenone was not produced, fortunately because the nucleophilicity of either of two *ortho*-hydroxyl groups was weakened by the adjacent acetyl carbonyl group.

2b was then reacted with 3,4-dimethoxybenzoic acid (**3f**) to give the intermediate ester (**4j**) which was then subjected to the Baker-Venkataraman rearrangement in dry pyridine in the presence of KOH, to afford the 1,3-dione (**5j**). Acid-catalyzed cyclization of **5j** in acetic acid in the presence of H_2SO_4 yielded 5,3',4'-trimethoxyflavones (**6j**) in yields of 25-35%, which was then demethylated using BBr3 to give 5,3',4'-trihydroxyflavones (**7j**).

Antiproliferative activity. These synthesized compounds were tested for their antiproliferative activity *in vitro* against HL60 leukemic cells using serial dilution method in 96-well microplates and flavone (**10**) was used as the positive control. The obtained results are summarized in Figure 2. Among the compounds tested, 5,3',4'-trihydoxyflavone (**7j**, IC_{50}=13 μM) demonstrated the most potent activity, followed by 5,4'-dihydroxyflavone (**7i**, IC_{50}=28 μM). Furthermore the 5-methoxylated derivatives, namely 5,4'-dimethoxyflavone (**6i**, IC_{50}=36 μM), 5,3'-dimethoxyflavone (**6h**, IC_{50}=46 μM) and 5-methoxyflavone (**6a**, IC_{50}=48 μM) also demonstrated significant activity.

Structure and activity relationship. The introduction of hydroxyl groups both at C3' and C4' positions in flavone (**10**, IC_{50}=64 μM), slightly improved the activity (**9**, IC_{50}=51 μM). The role of 3',4'-dihydroxy moiety for in the activity was also supported by the observation that methylation of hydroxyl groups at C3' and C4' drastically reduced the activity (**8**, IC_{50}>400 μM). The results that the 3',4'-dihydroxy moiety of dihydroxyflavone independently showed the antiproliferative activity, gave us precise information about the role of the this structural moiety, although the importance of 3',4'-dihydroxy moiety in the polyhydroxyflavones had been reported (20, 21). Among the dihydroxyflavones tested, namely compounds **7h**, **7i**, **7k** and **9**, the most potent activity was shown by 5,4'-dihydroxyflavone (**7i**, IC_{50}=28 μM) which does not have 3',4'-dihydroxy moiety. Taking these observations into account, the 3',4'-dihydroxy and 5,4'-dihydroxy moieties were suggested to have a different mechanism of action. The activity enhancements found in **7j** (IC_{50}=13 μM) both from 4-hydroxylation of **9** (IC_{50}=51 μM) and from 3'-hydroxylation of **7i** (IC_{50}=28 μM) indicated the synergistic effects of these two dihydroxy moieties and thus strongly supported the above suggestion.

A noteworthy reduction of the antiproliferative activity was observed for compounds **6j** and **8** bearing 3',4'-dimethoxyl group. Moreover, the removal of one or two methoxyl groups from the B-ring of compound **6j** (IC_{50} >400 μM) caused an increased potency of compounds **6a** (IC_{50}=48 μM), **6h** (IC_{50}=46 μM) and **6i** (IC_{50}=36 μM). These findings indicating that the increase of methoxyl groups on the B-ring decrease activity are consistent with our previous study where the attachment of more than two methoxyl groups on the B-ring completely diminished the activity when the number of A-ring methoxyl groups was 0-3 (19).

The position of methoxyl group on A-ring had significant effects on the activity; 5-methoxyflavone (**6a**, IC_{50}=48 μM) and 7-methoxyflavone (**6c**, IC_{50}=68 μM) displayed the moderate activity whereas 6-methoxyflavone (**6b**, IC_{50}>400 μM) had no activity. Contrarily, the intact hydroxyl group on A-ring had an opposite effects on the activity profile; only 6-hydroxyflavone (**7b**, IC_{50}=89 μM) showed the activity among the A-ring monohydroxylated derivatives. These findings suggested the important role of the A-ring in the interaction with target molecules.

In summary the data presented here demonstrated that the mechanisms of action of the 3',4'-dihydroxy and 5,4'-dihydroxy moieties were different and thus synergistic, and that the increased number of B-ring methoxyl groups reduced the activity of PMFs towards HL60 cells.

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