Antiproliferative Constituents in Plants 10. Flavones from the Leaves of *Lantana montevidensis* Briq. and Consideration of Structure–Activity Relationship[1)]

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The flavonoid fraction from the leaves of *Lantana montevidensis* Briq. (Verbenaceae) showed antiproliferative activity against human gastric adenocarcinoma (MK-1, GI_{50}: 12 μg/ml), human uterus carcinoma (HeLa, 5 μg/ml), and murine melanoma (B16F10, 5 μg/ml) cells *in vitro*. Bioactivity-guided chemical investigation of the fraction has resulted in the isolation of apigenin (10) and ten 5,6,7-oxygenated flavones: cirsilineol (1), eupatorin (2), 5,4′-dihydroxy-6,7,3′,5′-tetramethoxyflavone (3), 5,6-dihydroxy-7,3′,4′-trimethoxyflavone (4), 5,6,4′-trihydroxy-7,3′,5′-trimethoxyflavone (5), 5,6,3′-trihydroxy-7,4′-dimethoxyflavone (6), 5,3′,4′-trihydroxy-6,7,5′-trimethoxyflavone (7), cirsiliol (8), hispidulin (9), and eupafolin (11). Antiproliferative activity of the isolated flavones, some other related flavones (luteolin, baicalein, 6-hydroxyluteolin, pectolinarigenin, jaceosidin, desmethoxycentaureidin, eupatilin, and chrysin) from other plant materials, and synthetic 6- and 7-methoxyflavones was evaluated, and the structure–activity relationships were examined.

Key words *Lantana montevidensis*; flavones; antiproliferative activity; structure–activity relationship; 5,6,4′-trihydroxy-7,3′,5′-trimethoxyflavone; 5,6,3′-trihydroxy-7,4′-dimethoxyflavone

Lantana camara L. is indigenous to tropical America, and its leaves have been used in folk medicine for the treatment of bronchitis and stomach disorders and as a sudorific, and are also put into baths to treat rheumatism in South America.[2)] However, this species has been known to be toxic to livestock, which develop hepatotoxicity and photosensitization on ingestion of the leaves.[3)] There have been many reports on the biological action of this plant and the toxic constituents. Scores of triterpene derivatives have been isolated,[3—6)] and some of them have been reported to exhibit inhibitory activity against Epstein–Barr virus early antigen activation in Raji cells induced by 12-*O*-tetradecanoyl-phorbol-13-acetate.[7)]

L. montevidensis Briq. is native to Brazil (Montevideo), and the tea and infusion of the dried leaves have been used in folk medicine for the same purposes as *L. camara*.[2)] However, to our knowledge there is only one report on the essential oil from the leaves[2)] and there is no report on the other constituents.

Among several Verbenaceae plants in which we investigated the antiproliferative activity of the MeOH extracts, *L. camara* and *L. montevidensis* exhibited higher activity against tumor cell growth. The MeOH extract of the leaves of *L. montevidensis* showed 50% growth inhibition (GI_{50}) at the concentrations of 8 μg/ml for human gastric adenocarcinoma (MK-1), 3 μg/ml for human uterus carcinoma (HeLa), and 4 μg/ml for murine melanoma (B16F10), which were more potent than the inhibition by the MeOH extract of the leaves of *L. camara* (12.5 μg/ml for MK-1 and 25 μg/ml for HeLa and B16F10). The inhibitory activity was found to be localized in the nonglycoside fraction that contains several flavonoids. The nonflavonoid fraction exhibited higher activity, however, the flavonoid fraction also showed significant activity. We therefore first investigated the active constituents in the flavonoid fraction. This paper deals with the isolation and identification of the flavone constituents in the leaves of *L. montevidensis* and their antiproliferative activity.

MATERIALS AND METHODS

Materials, Reagents, and Instruments The leaves of *L. montevidensis* were collected in Munakata City, Fukuoka, Japan, in July 2000 and air-dried. 6-Hydroxyluteolin, desmethoxycentaureidin, and jaceosidin were isolated from the herb of *Lippia canescens* Kunth. (Verbenaceae),[1)] and eupatilin was isolated from the herb of *Artemisia ludoviciana* Nutt. subsp. *mexicana* (Willd. *ex* Sprengel) (Compositae).[8)] Pectolinarigenin was obtained by acid hydrolysis of linariin isolated from the herb of *Trifolium dubium* Sibth. (Leguminosae) and identification was performed by comparison of the NMR data with those reported.[9)] Chrysin, luteolin, baicalein, and 6- and 7-methoxyflavones were purchased from Aldrich Chemical Company (Milwaukee, WI, U.S.A.). Reagents for culture of the tumor cells and for the bioassay were described in a previous paper.[10)] The instruments used in this study were a Shimadzu Double Beam Spectrometer UV-200S (UV spectra), JEOL JNM A-500 Spectrometer (^{1}H-NMR and ^{13}C-NMR spectra), JEOL JMS HX-110 Mass Spectrometer (MS spectra), and Yanaco Micro Melting Point Apparatus (mp).

Cells MK-1 cells were provided by Professor M. Katano, Faculty of Medicine, Kyushu University. HeLa and B16F10 cells were supplied by the Cell Resource Center for the Biomedical Research Institute of Development, Aging and Cancer, Tohoku University.

Measurement of Antiproliferative Activity against Tumor Cell Lines Cellular growth was determined using the MTT assay on 96-well microplates as described in the previous paper.[10)]

Extraction and Fractionation The dried leaves (50 g) were extracted three times with MeOH (300 ml) under reflux for 1 h. The MeOH extract (15 g) was suspended in 60% MeOH and passed through a column of styrene polymer, Diaion HP 20 (200 ml), and the column was successively washed with 60% MeOH, MeOH, and then AcOEt. The 60%

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Table 1. GI_{50} Values (μg/ml) of the MeOH Extract and Fractions

	HeLa	MK-1	B16F10
MeOH extract	3	7	4
Fr. I	>100	>100	70
Fr. II	33	15	6
Fr. III	<3	<3	<3
Fr. IIIa	<3	<3	<3
Fr. IIIb	5	12	5
Fr. IV	5	10	6

Values are means of two determinations.

MeOH eluate was concentrated to remove MeOH, and the aqueous solution was passed through a new column of Diaion HP 20 (100 ml) and washed with H_2O and 60% MeOH. The GI_{50} values of the H_2O eluate (Fr. I, 4.52 g), 60% MeOH eluate (Fr. II, 5.72 g), MeOH eluate (Fr. III, 3.22 g), and AcOEt eluate (Fr. IV, 1.52 g) are shown in Table 1. Fr. III (3.08 g) was further chromatographed on Sephadex LH 20 (200 ml, solvent MeOH) and separated into the fast-eluting nonflavonoid fraction (Fr. IIIa, 2.6 g) and later-eluting flavonoid fraction (Fr. IIIb, 0.45 g). The GI_{50} values of Fr. IIIa and Fr. IIIb are also presented in Table 1.

Fraction IIIb (4.5 g from 900 g of dried leaves) was repeatedly chromatographed on silica gel using 2% MeOH–$CHCl_3$ as an eluant and separated into 4 fractions (Fr. IIIb-1—4). Each fraction was further chromatographed on silica gel using hexane–AcOEt (1 : 2) to give 11 flavones: from Fr. IIIb-1, compounds **1** (205 mg), **2** (29 mg), **3** (67 mg), and **4** (9 mg); from Fr. IIIb-2, compounds **5** (95 mg) and **6** (33 mg); from Fr. IIIb-3, compounds **7** (61 mg), **8** (492 mg), **9** (24 mg), and **10** (148 mg); and from Fr. IIIb-4, compound **11** (1.5 g).

Identification of Known Flavones The structures of the isolated flavones were determined by MS and NMR spectral analyses, although the NMR spectra of some reported flavones were measured in $CDCl_3$ or CD_3OD and the NMR data could not be directly compared for identification with our data measured in DMSO-d_6. Identification was performed as shown in Table 2 by NMR spectral analysis referring to the reported data of pectolinarigenin, salvigenin, luteolin, and tricetin all measured in DMSO-d_6.[9] All except for **5** and **6** are known compounds. The ^{1}H- and ^{13}C-NMR data are shown in Tables 3 and 4.

Structures of Compounds 5 and 6 Compound **5** was obtained as a yellow powder from MeOH, mp 278—282 °C, UV λ_{max} (MeOH) (log ε): sh (3.79), 285 (3.73), 345 (3.99). The molecular formula $C_{18}H_{16}O_8$ was deduced from high-resolution FAB-MS (required for $[M+H]^+$, 361.0923; observed, 361.0929). The ^{1}H-NMR spectrum depicted signals of three methoxyl groups (δ 3.91, 6H and 3.94, 3H) and singlet signals at δ 6.96 (1H), 6.97 (1H), 7.35 (2H), and 12.65 (1H). The same chemical shifts of the two methoxyl groups (δ 3.91) and two phenyl protons (δ 7.35) indicated that the substitution pattern at ring B must be symmetrical, *viz.* 3′,5′-di-OCH_3-4′-OH. The signal at δ 12.65 was assigned to the proton of C_5-OH, and the signals at δ 6.96 and 6.97 were assigned to C_3-H and the proton in ring A. The ^{1}H-NMR signals at δ 6.96 and 6.97 showed correlating signals to the ^{13}C-NMR signals at δ 103.2 and 91.3 in the ^{13}C–^{1}H COSY spectrum. The former was assigned to C_3 and the latter to C_8, referring to the reported NMR data.[9] Assignment of the corresponding proton signals, C_3-H and C_8-H, could not be achieved because the chemical shifts were too close to be distinguished. The remaining methoxyl group therefore must be C_6-OCH_3 or C_7-OCH_3. The ^{13}C-NMR chemical shifts of the carbons at ring A are similar to those reported for negletein (5,6,4′-trihydroxy-7-methoxyflavone)[11] and slightly different from those of pectolinarigenin (5,7-dihydroxy-6,4′-dimethoxyflavone).[11] To confirm the position of the methoxyl group, the heteronuclear multiple bond connectivity (HMBC) spectrum was recorded. The C_5-OH proton showed correlation with the carbon signals at δ 105.1, 129.9, and 146.2, which were reasonably assigned to C_{10}, C_6, and C_5, respectively. The methoxyl proton signal at δ 3.94 showed correlation with the carbon signal at δ 154.4 and not with the signal at δ 129.9, indicating the methoxyl group is linked to C_7. From this spectral evidence, the structure of **5** was determined to be 5,6,4′-trihydroxy-7,3′,5′-trimethoxyflavone.

Compound **6** was obtained as a yellow powder from MeOH, mp 256—258 °C, UV λ_{max} (MeOH) (log ε): 245 (4.03), 283 (4.14), 340 (4.26). The molecular formula $C_{17}H_{14}O_7$ was deduced from high-resolution FAB-MS (required for $[M+H]^+$, 331.0818; observed, 331.0820). The ^{1}H-NMR spectrum depicted signals of two methoxyl groups (δ 3.88 and 3.93), singlet signals at δ 6.75 (1H), 6.88 (1H), and 6.97 (1H), doublets at δ 7.10 (1H, J=9 Hz) and 7.46 (1H, J=2 Hz), a double-doublet at δ 7.56 (1H, J=2, 9 Hz), and an OH signal at δ 12.60 which was assigned to C_5-OH. Comparison of the NMR spectral data of **6** to those of **5** indicated that the structure of **6** might be 5,6,3′ or 4′-trihydroxy-7,4′ or 3′-dimethoxyflavone. The position of the methoxyl group in ring B was determined to be $C_{4'}$ from the HMBC spectral analysis. The methoxyl proton signal at δ 3.88 was correlated with the carbon signal at δ 151.0, and to which the proton signals at δ 7.46 ($C_{2'}$-H) and 7.56 ($C_{6'}$-H) showed correlation. The proton signal at δ 7.10 ($C_{5'}$-H) showed intense cross peaks with the carbon signals at δ 123.2 ($C_{1'}$) and 146.8 ($C_{3'}$).

From the spectral evidence, the structure of **6** was determined to be 5,6,3′-trihydroxy-7,4′-dimethoxyflavone.

RESULTS AND DISCUSSION

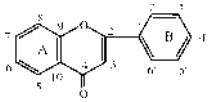
From the flavonoid fraction of the MeOH extract of the leaves of *L. montevidensis*, 11 flavones (**1**—**11**) were isolated: apigenin (**10**), two 5,7-di-OH-6-OCH_3-flavones (**9**, **11**), three 5,6-di-OH-7-OCH_3-flavones (**4**—**6**), and five 5-OH-6,7-di-OCH_3-flavones (**1**—**3**, **7**, **8**). The antiproliferative activity of the isolated flavones, eight related flavones from other plant origins, and two synthetic flavones was evaluated and the results are presented in Table 2. The compounds are arranged in groups according to the substituents at ring A, so that differences in activity may be discerned between groups having different substituents, and within groups having different substituents.

Among flavones from this plant, **7**, cirsiliol (**8**), apigenin (**10**), and eupafolin (**11**) showed higher activity against the three tumor cell lines followed by eupatorin (**2**), **4**, **5**, and **6**. Cirsilineol (**1**) and **3** showed the least activity, although cirsilineol (**1**) has selectively high activity against MK-1 cells. Considering the yield of each flavone, eupafolin (**11**) and cir-

Table 2. GI_{50} Values (μM) of Flavones

	Substituents at ring B			Tumor cell line		
	3′	4′	5′	HeLa	MK-1	B16F10
6-Methoxyflavone	H	H	H	8	398	398
7-Methoxyflavone	H	H	H	87	119	119
5,7-Di-OH						
Chrysin	H	H	H	8	63	51
Apigenin (**10**)	H	OH	H	15	22	26
Luteolin	OH	OH	H	10	31	21
5,6,7-Tri-OH						
Baicalein	H	H	H	30	26	11
6-Hydroxyluteolin	OH	OH	H	30	26	13
5,7-Di-OH-6-OCH_3						
Hispidulin (**9**)	H	OH	H	17	83	67
Pectolinarigenin	H	OCH_3	H	10	115	64
Eupafolin (**11**)	OH	OH	H	6	29	16
Desmethoxylcentaureidin	OH	OCH_3	H	9	24	64
Jaceosidin	OCH_3	OH	H	33	27	27
Eupatilin	OCH_3	OCH_3	H	35	55	58
5,6-Di-OH-7-OCH_3						
6	OH	OCH_3	H	55	55	18
4	OCH_3	OCH_3	H	73	73	29
5	OCH_3	OH	OCH_3	44	33	39
5-OH-6,7-di-OCH_3						
Cirsiliol (**8**)	OH	OH	H	21	18	9
Eupatorin (**2**)	OH	OCH_3	H	15	58	44
Cirsilineol (**1**)	OCH_3	OH	H	203	17	73
7	OH	OH	OCH_3	14	22	14
3	OCH_3	OH	OCH_3	>267	>267	241

Values are means of four determinations.



siliol (**8**) are considered to be responsible for Fr. IIIb activity.

Eupafolin (**11**) has been isolated from many plants belonging to Compositae, Verbenaceae, and Umbelliferae,[12] and its cytotoxicity against KB cells was first reported in 1969 by Kupchan *et al.*[13] Cirsiliol (**8**) has also been isolated from some Compositae plants,[12] and it has been known as an arachidonate 5-lipoxygenase inhibitor.[14] The cytotoxicity or biological properties of some flavones isolated from this plant have previously been reported.[15,16] Several researchers who reported on the cytotoxicity or biological activities of flavonoids have commented on their structure–activity relationships. However, confining the materials to the flavones, what can be deduced from those comments is that the presence of hydroxyl groups at C_5 and C_7 in ring A, and at $C_{3'}$ and $C_{4'}$ in ring B, appears to be important for enhanced activity, but the influence of the other substituents at other positions is not clear.

The potency of activity of the flavones might be influenced by the structures of (or substituents at) rings A and B. Chrysin (5,7-di-OH-flavone), apigenin (4′-OH-chrysin), acacetin (4′-OCH_3-chrysin), and luteolin (3′,4′-di-OH-chrysin), which all have 5,7-di-OH groups at ring A, showed different potency of inhibition on the avian myeloblastosis virus reverse transcriptase, and luteolin, 6-OH-luteolin, and pedalitinin, which all have 3′,4′-di-OH at ring B and different susbstituents at ring A also showed different potency[17] suggesting that the substitution patterns of both rings A and B influence the activity.

We estimated the antiproliferative activity of 21 flavones and tried to determine the effect of the susbstitution patterns on the activity, although the number of flavones examined might not be sufficient to clarify the structure–activity relationships. Viewed generally, the data show that the growth-inhibitory activity of one flavone against the three tumor cell lines is not always the same, indicating differences in the sensitivity of tumor cells to flavones.

The influence of ring A substituents on the activity against HeLa cells was examined by comparison of the activities of flavones with the same ring B structures. When the activities of the flavones with the 3′,4′-dihydroxy groups at ring B were compared, the order of the potency was eupafolin (**11**)>luteolin>cirsiliol (**8**)>6-hydroxyluteolin, and therefore the order of contribution of the A ring substitution pattern to the potency would be 5,7-di-OH-6-OCH_3>5,7-di-OH>5-OH-6,7-di-OCH_3>5,6,7-tri-OH. The position of 5,6-di-OH-7-OCH_3 could not be determined because we do not have the corresponding 3′,4′-di-OH flavone. However, considering that the 3′,4′-di-OH-flavone and 3′-OH-4′-OCH_3-flavone showed almost the same activity and that the activity of **6** is weaker than that of 6-hydroxyluteolin, 5,6-di-OH-7-OCH_3 could be safely put after 5,6,7-tri-OH.

Introduction of the hydroxyl group at C_6 of the 5,7-di-OH-flavones decreases the activity, as seen in the cases of chrysin *vs.* baicalein and luteolin *vs.* 6-OH-luteolin. Methylation of the 6-OH of the 5,6,7-tri-OH recovers the activity, and methylation of 7-OH lowers the activity compared with that of 5,6,7-tri-OH-flavone. However, in the case of **5** *vs.* **3**, the order of contribution of the two ring A substitutions are reversed (5,6-di-OH-7-OCH_3≫5-OH-6,7-di-OCH_3). Therefore, roughly speaking, the order of contribution of the ring A substitution patterns to activity would be 5,7-di-OH-6-OCH_3>5,7-di-OH>5-OH-6,7-di-OCH_3>5,6,7-tri-OH>5,6-di-OH-7-OCH_3, although there is one unexplainable exception.

The influences of the substituents at ring B were examined based on the data of six 5,7-di-OH-6-OCH_3-flavones. The order would be 3′,4′-di-OH≒3′-OH-4′-OCH_3>4′-OCH_3≒4′-OH>3′-OCH_3-4′-OH≒3′,4′-di-OCH_3. Methylation of 4′-OH appears not affect activity, although methylation of 3′-OH or introduction of 3′-OCH_3 decreases the activity.

In five 5-OH-6,7-di-OCH_3-flavones, the effect of 3′,4′-di-OH, 3′-OH-4′-OCH_3, and 3′,4′-di-OH-5′-OCH_3 is the greatest and that of 3′-OCH_3-4′-OH, and 3′,5′-di-OCH_3-4′-OH is very low. Judging from the above data, 4′-OH, 3′,4′-di-OH, 3′,4′-di-OH-5′-OCH_3, and 3′-OH-4′-OCH_3 at ring B would contribute to the potency, and the influence of 3′,4′-di-OCH_3 and 3′,5′-di-OCH_3-4′-OH appears to be low. This ordering is not applicable to the case of the 5,6-di-OH-7-OCH_3-flavones.

In the case of MK-1 cells, some differences were observed in the influence of the substituents at ring A among flavones. When we compared the activities of 3′,4′-di-OH-flavones [luteolin, 6-hydroxyluteolin, eupafolin (**11**), and cirsiliol (**8**)], the influence of 5-OH-6,7-di-OCH_3 appears slightly greater, but that of 5,7-di-OH, 5,6,7-tri-OH, and 5,7-di-OH-6-OCH_3 are almost the same. However, the order is 5,7-di-OH>5,7-di-OH-6-OCH_3 for apigenin (**10**) *vs.* hispidulin (**9**), 5,7-di-OH-6-OCH_3>5-OH-6,7-di-OCH_3 for desmethoxycentaureidin *vs.* eupatorin (**2**), and 5,6-di-OH-7-OCH_3·5-OH-6,7-di-OCH_3 for **5** *vs.* **3**.

Table 3. ^{1}H-NMR Chemical Shifts of Flavones from *Lantana montevidensis*[a)]

	10	**9**	**11**	**6**	**4**	**5**	**8**	**2**	**1**	**7**	**3**
C_3-H	6.75 s	6.76 s	6.66 s	6.75 s	6.97 s	6.97 s[b)]	6.72 s	6.80 s	6.93 s[b)]	6.86 s	7.04 s
C_6-H	6.20 d (2)										
C_8-H	6.49 d (2)	6.59 s	6.56 s	6.88 s	6.95 s	6.96 s[b)]	6.87 s	6.91 s	6.94 s[b)]	6.90 s	6.99 s
$C_{2'}$-H	6.75 d (9)	7.92 d (9)	7.39 d (2)	7.46 d (2)	7.59 d (2)	7.35 s	7.44 br s	7.47 d (2)	7.59 br s	7.20 d (2)	7.37 s
$C_{3'}$-H	6.94 d (9)	6.92 d (9)									
$C_{5'}$-H	6.94 d (9)	6.92 d (9)	6.90 d (8)	7.10 d (9)	7.14 d (9)		6.91 d (8)	7.10 d (8)	6.95 d (8)		
$C_{6'}$-H	6.75 d (9)	7.92 d (9)	7.41 dd (2, 8)	7.56 dd (2, 9)	7.71 dd (2, 9)	7.35 s	7.45 dd (2, 8)	7.58 dd (2, 8)	7.60 dd (2, 8)	7.19 d (2)	7.37 s
C_6-OCH_3		3.76 s	3.76 s				3.74 s	3.74 s	3.75 s	3.74 s	3.74 s
C_7-OCH_3				3.93 s	3.93 s	3.94 s	3.93 s	3.94 s	3.93 s[c)]	3.94 s	3.95 s
$C_{3'}$-OCH_3					3.90 s	3.91 s			3.91 s[c)]	3.89 s	3.90 s
$C_{4'}$-OCH_3				3.88 s	3.86 s			3.88 s			
$C_{5'}$-OCH_3						3.91 s					3.90 s
C_5-OH	12.93 s	13.06 s	13.07 s	12.60 s	12.60 s	12.65 s	12.93 s	12.88 s	12.95 s	12.94 s	12.94 s

a) 500 MHz, solvent: DMSO-d_6, internal standard: tetramethylsilane. *b, c*) The assignment in the vertical column is interchangeable. The numbers in the parentheses are the coupling constants in Hz. Compounds are arranged in the order as in Table 2.

Table 4. ^{13}C-NMR Chemical Shifts of Flavones from *Lantana montevidensis*[a)]

	10	**9**	**11**	**6**	**4**	**5**	**8**	**2**	**1**	**7**	**3**
2	163.9	163.7	164.0	163.5	163.3	163.7	164.2	163.8	164.0	164.1	163.9
3	102.9	102.3	102.4	103.1	103.4	103.2	102.7	103.2	103.0	103.1	103.3
4	181.8	182.0	182.0	182.0	182.2	182.2	182.1	182.1	182.2	182.1	182.2
5	161.5	152.7	152.8	146.2	146.1	146.2	152.6	152.0	152.6	152.0	152.0
6	99.0	131.3	131.4	129.9	129.9	129.9	131.9	131.9	131.9	131.8	131.9
7	164.2	157.1	157.2	154.4	154.4	154.4	158.6	158.6	158.6	158.5	158.5
8	94.1	94.1	94.1	91.0	91.2	91.3	91.4	91.5	91.6	91.4	91.7
9	157.4	152.3	152.3	149.6	149.6	149.7	152.1	152.6	152.0	152.5	152.6
10	103.8	104.0	104.1	105.0	105.1	105.1	105.0	105.1	105.1	105.0	105.1
1′	121.3	121.2	121.6	123.2	123.1	120.6	121.5	122.9	121.4	120.3	120.2
2′	128.5	128.4	113.3	113.0	109.5	104.4	113.5	113.1	110.3	102.5	104.5
3′	116.1	115.9	145.7	146.8	149.0	148.2	145.8	146.8	148.1	148.6	148.2
4′	163.9	161.1	149.7	151.0	152.1	139.9	149.8	151.1	150.8	138.8	140.1
5′	116.1	115.9	116.0	112.1	111.7	148.2	116.0	112.1	115.8	146.0	148.2
6′	128.5	128.4	118.9	118.5	119.9	104.4	119.0	118.7	120.5	107.7	104.5
C_6-O$\underline{C}H_3$		59.8	59.9				60.0	59.9	60.0	59.9	59.9
C_7-O$\underline{C}H_3$				56.2	56.3	56.3	56.4	56.4	56.0[b)]	56.4	56.4
$C_{3'}$-O$\underline{C}H_3$					55.9	56.4			56.4[b)]	56.3	56.4
$C_{4'}$-O$\underline{C}H_3$				55.7	55.7			55.8			
$C_{5'}$-O$\underline{C}H_3$						56.4					56.4

a) 125 MHz, solvent: DMSO-d_6, internal standard: tetramethylsilane. *b*) The assignment in the vertical column is interchangeable. Compounds are arranged in the order as in Table 2.

The influence of the substituents at ring B is also not obvious. In six 5,7-di-OH-6-OCH_3-flavones, the order is 3′,4′-di-OH, 3′-OH-4′-OCH_3 and 3′-OCH_3-4′-OH>3′,4′-di-OCH_3>4′-OH>4′-OCH_3, and in five 5-OH-6,7-di-OCH_3-flavones, the order is 3′,4′-di-OH, 3′-OCH_3-4′-OH and 3′,4′-di-OH-5′-OCH_3>3′-OH-4′-OCH_3>3′,5′-OCH_3-4′-OH. No decrease in activity with methylation of 3′-OH in the 3′,4′-di-OH-flavones was observed [eupafolin (**11**) *vs.* jaceosidin, cirsiliol (**8**) *vs.* cirsilineol (**1**)], while the activity decreased when 3′-OH of the 3′-OH-4′-OCH_3 is methylated (desmethoxycentaureidin *vs.* eupatilin, **6** *vs.* **4**). It is apparent at least that 3′,4′-di-OH and 3′,4′-di-OH-5′-OCH_3 have a greater effect on growth-inhibitory activity and that the effect of 3′,5′-di-OCH_3-4′-OH is weak.

The influences of ring A substituents against B16F10 cells were examined in the same manner. By comparison of the activty of four 3′,4′-di-OH-flavones, the order of contribution was found to be 5-OH-6,7-di-OCH_3>5,6,7-tri-OH>5,7-di-OH-6-OCH_3>5,7-di-OH. However, in the 3′-OH-4′-OCH_3-flavones (desmethoxycentaureidin, **2**, and **6**), the order is 5,6-di-OH-7-OCH_3>5-OH-6,7-di-OCH_3>5,7-di-OH-6-OCH_3, and in the 3′,4′-di-OCH_3-flavones (eupatilin and **4**), it is 5,6-di-OH-7-OCH_3>5,7-di-OH-6-OCH_3, and in the 3′-OCH_3-4′-OH-flavones (jaceosidin and **1**), it is 5,7-di-OH-6-OCH_3>5-OH-6,7-di-OCH_3.

In the ring B substituents, 3′,4′-di-OH and 3′,4′-di-OH-5′-OCH_3 showed a greater effect than the others, and the influence of 3′,5′-di-OCH_3-4′-OH appears to be weak, although **5** is the exception.

After investigating the influences of substituents at rings A and B of flavones on cell growth inhibition in a limited number of flavones, we concluded that it is difficult to suggest rules commonly applicable to the three tumor cell lines. There is, however, a general tendency for such polar substitution patterns as 5,7-di-OH, 5,6,7-tri-OH, and 5,7-di-OH-6-OCH_3 at ring A, and catechol functions such as 3′,4′-di-OH

and 3′,4′-di-OH-5′-OCH_3 at ring B to play an important role in potent growth-inhibitory activity.

We intentionally do not discuss the activity of 6- and 7-methoxyflavones and the influence of the "naked" B ring (phenyl group) in chrysin and baicalein. The low activity of 7-methoxyflavone was as expected, although the highly selective inhibition of HeLa cell growth by 6-methoxyflavone and the effect of ring B of chrysin and baicalein on the activity similar to those of 4′-OH and 3′,4′-di-OH groups were outside the scope of this paper. If polar substituents at rings A and B are important for higher activity, it would be difficult to understand the activities of 6-methoxyflavone, as well as contribution of the least polar ring B (phenyl group) to that growth-inhibitory activity.

Cell proliferation involves complex combinations of many biochemical processes, and different flavonoids might influence different biochemical processes or stages in different manners. The less polar (rather lipophilic) flavones such as 6- and 7-methoxyflavones and flavones with a "naked" B ring might inhibit cell growth by mechanisms different from those of the highly oxygenated flavones.

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