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ISOLATION AND CYTOTOXICITY OF TWO NEW FLAVONOIDS FROM *CHRYSOSPLENIUM GRAYANUM* AND RELATED FLAVONOLS

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ABSTRACT.—In view of the observation that a $CHCl_3$-soluble fraction of the MeOH extract of *Chrysosplenium grayanum* has shown cytotoxic activity in the KB cell in vitro system, the fraction has been further fractionated to furnish five known flavonoids (chrysosplenols B, D, and E, retusin, and brickellin), 3,5-dihydroxy-4-methoxybenzoic acid methyl ester, and two new flavonoids named chrysosplenols F [**1**] and G [**2**]. The structures of the new flavonoids have been elucidated to be 5,4′,5′-trihydroxy-3,7,2′-trimethoxyflavone [**1**] and 5,5′-dihydroxy-3,7,2′,4′-tetramethoxyflavone [**2**] on the basis of spectral data. Certain of the isolated compounds and related flavonol aglycones showed marginal activities against KB cells in vitro.

Chrysosplenium grayanum Maxim. (Saxifragaceae) is an herbaceous perennial. A Chinese crude drug, "Jín gián kǔ yè cǎo" is the fresh whole plant of *C. grayanum*, and it has been used for treatment of swellings and rashes (1). Previously, several flavonol glycosides, chrysosplenosides A (2), B (3), C (4), D (5), and E (6), and pendulin (2) were isolated from the genus *Chrysosplenium*, and their aglycones, oxyayanin A, chrysosplenols B, C, D, and E, and penduletin were tested for antiviral activity (7). In the continuation of our research on cytotoxic compounds in the plant, we found that the $CHCl_3$-soluble fraction of the MeOH extract of the fresh plant markedly inhibited the growth of cultured KB cells. In this paper we report the isolation, structure elucidation, and cytotoxicity of eight compounds, including two new flavonols and related flavonol aglycones from *C. grayanum*.

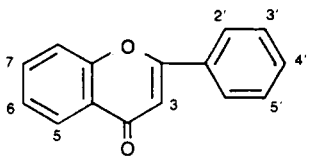
RESULTS AND DISCUSSION

The $CHCl_3$-soluble fraction was partitioned between 95% MeOH and petroleum ether. The 95% MeOH extract, which was cytotoxic against KB cells in vitro (Table 1), was separated on a Si gel column by elution with a MeOH/$CHCl_3$ mixture. The 1% MeOH/$CHCl_3$ eluent from the column was further separated on a Si gel column by elution with a EtOAc/*n*-hexane mixture. The 15% EtOAc/*n*-hexane eluent was separated on preparative layer chromatography to afford six known compounds and two new flavonols, **1** and **2** (Table 2). Four of the known compounds, chrysosplenols B, D, E, and brickellin (10,11), were identified by direct comparison with the authentic samples. Retusin (8,9) and 3,5-dihydroxy-4-methoxybenzoic acid methyl ester were identified by their spectral data.

TABLE. 1. Cytotoxic Activity of the Fractions of *Chrysosplenium grayanum* (KB cell growth inhibition % at 50 μg/ml, in vitro).

	Fraction					
	MeOH	H_2O	$CHCl_3$	Insoluble part	95% MeOH	Petroleum ether
Inhibition %	87.0	33.0	94.0	25.3	98.4	73.4

TABLE 2. Structures of Flavonols from the Genus *Chrysosplenium*.



Flavonol	Position							
	3	5	6	7	2′	3′	4′	5′
Chrysosplenol B	OMe	OH	OMe	OMe		OMe	OH	
Chrysosplenol D	OMe	OH	OMe	OMe		OH	OH	
Chrysosplenol E	OMe	OH		OMe	OH		OMe	OMe
Retusin	OMe	OH		OMe		OMe	OMe	
Brickellin	OMe	OH	OMe	OMe	OH		OMe	OMe
Chrysosplenol F [1]	OMe	OH		OMe	OH		OH	OMe
Chrysosplenol G [2]	OMe	OH		OMe	OMe		OMe	OH
Chrysosplenol C	OMe	OH	OH	OMe		OMe	OH	
Oxyayanin A	OMe	OH		OMe	OH		OMe	OH

A new compound [**1**], mp 162–165°, hreims *m/z* 360.0818, calcd 360.0844 for $C_{18}H_{16}O_8$, was shown to be a flavonoid from its color reactions and uv absorption. The uv absorption spectra on addition of $AlCl_3$, $AlCl_3$/HCl, NaOMe, and NaOAc/H_3BO_3 indicated the presence of a hydroxyl group at the 5 and 4 positions and the absence of orthophenols. The ^{1}H nmr of **1** (in Me_2CO-d_6) showed the presence of three methoxyl groups (δ 3.90, 3.94, and 3.96 ppm) and four aromatic protons. The aromatic proton signals at δ 6.13 and 6.54 ppm (each 1H, d, $J = 2.3$ Hz) are assigned to the protons at the 6 and 8 positions. Two signals at δ 6.99 and 7.11 ppm, each appearing as a singlet, indicated the two protons in the B ring to be in the para position. The eims spectrum of **1** showed the molecular ion peak at *m/z* 360 and prominent peaks at *m/z* 343 $[M-OH]^+$, 329 $[M-OMe]^+$, 317 $[M-Me-CO]^+$ and 167 $[C_8H_7O_4]^+$ originating from both A and B rings. Based on these data, the structure of **1** was determined to be 5,4′,5′-trihydroxy-3,7,2′-trimethoxyflavone and named chrysosplenol F [**1**].

The second new compound **2**, mp 153–155°, hreims *m/z* 374.0979, calcd 374.1001 for $C_{19}H_{18}O_8$, was also shown to be a flavonoid from its color reactions and uv absorption. The uv absorption spectra on addition of $AlCl_3$/HCl and NaOMe indicated the presence of a hydroxyl group at the 5 position and the absence of a 4′-hydroxyl group. The ^{1}H-nmr of **2** (in Me_2CO-d_6) closely resembled that of **1**, except for the appearance of four signals of methoxyl groups at δ 3.80, 3.84, 3.90, and 3.92 ppm instead of three methoxyl signals. Two singlet signals for the protons on the 2-hydroxy-4,5-dimethoxyphenyl moiety of chrysosplenol E and brickellin appeared at δ 6.63 and 7.12 ppm. However, the two signals for the protons on the phenyl moiety of **2** appeared at δ 7.11 and 7.13 ppm. In the eims spectrum of **2**, the molecular ion peak was observed at *m/z* 374, and prominent peaks were exhibited at *m/z* 359 $[M-Me]^+$, 357 $[M-OH]^+$, 343 $[M-OMe]^+$, and 331 $[M-Me-CO]^+$. Further, an ion peak at *m/z* 167 $[C_8H_7O_4]^+$, which would originate from the A ring, was observed. Based on the above evidence, the structure of **2** was determined to be 5,5′-dihydroxy-

3,7,2′,4′,-tetramethoxyflavone and named chrysosplenol G [**2**].

The isolated compounds and related flavonols, oxyayanin A and chrysosplenol C, were tested for cytotoxic activity in the KB cell culture system. Only oxyayanin A was significantly cytotoxic, with an ED_{50} value of 1.99 ± 0.75 μg/ml (Table 3).

plots of sample concentration (μg/ml) vs. the percent of viable cells. The assay represents three determinations. The cytotoxicities of the fractions were shown by % KB cell growth inhibition at 50 μg/ml (Table 1), and the cytotoxicities of ten compounds were shown as ED_{50} values (Table 3). ED_{50} values of < 20 μg/ml for crude extracts and < 4 μg/ml for pure compounds are considered significant (12).

EXTRACTION AND SEPARATION.—The fresh whole plants (4.2 kg) were thrice extracted with MeOH at room temperature for 3 days. The residue of the MeOH extract (147 g) was partitioned between $CHCl_3$ and H_2O to afford the $CHCl_3$ extract (20 g) and the H_2O extract (124 g). The $CHCl_3$ extract was further partitioned between 95% MeOH and petroleum ether to afford the 95% MeOH extract (18.1 g) and the petroleum ether extract (1.7 g). The cytotoxic 95% MeOH extract (Table 1) was chromatographed on Si gel by stepwise elution with $CHCl_3$ and a MeOH/$CHCl_3$ mixture. The fraction eluting with 1% MeOH/$CHCl_3$, which was most active against KB cells (91.0% inhibition at 20 μg/ml) among the eluted fractions, was rechromatographed on Si gel by elution with a 15% EtOAc/*n*-hexane mixture, and preparative layer chromatography separations were performed to afford six known compounds, chrysosplenols B (7 mg), D (5 mg), and E (25 mg), retusin (4 mg), brickellin (30 mg), and 3,5-dihydroxy-4-methoxy-benzoic acid methyl ester (2 mg), and two new compounds, chrysosplenols F [**1**] (3 mg) and G [**2**] (2 mg).

TABLE 3. Cell growth Inhibition of Isolated Compounds and Related Flavonols Against KB Cells in vitro.

Compound	ED_{50}, μg/ml[a]
Chrysosplenol B	7.42 ± 0.43
Chrysosplenol D	13.95 ± 0.25
Chrysosplenol E	>25
Retusin	>25
Brickellin	>25
3,5-Dihydroxy-4-methoxybenzoic acid methyl ester	8.62 ± 0.21
Chrysosplenol F [**1**]	>25
Chrysosplenol G [**2**]	8.61 ± 0.43
Chrysosplenol C	11.20 ± 0.32
Oxyayanin A	1.99 ± 0.75

[a]Mean ± SE of three time determinations.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All mp's were determined on a Yanagimoto micro mp apparatus and are uncorrected. Uv and ir spectra were recorded on a Hitachi 220 S double beam spectrophotometer and 260-10 ir spectrometer, respectively, with polystyrene calibration at 1601 cm^{-1}. ^{1}H-nmr and ^{13}C-nmr spectra were taken on a JEOL JNM-GX 270 spectrometer at 270 MHz and Varian XL-200 spectrometer at 50.3 MHz, respectively, with TMS as standard. The chemical shifts are recorded in δ (ppm) values. Eims spectra were obtained on a JEOL JMS-D-200 mass spectrometer operating at 70 eV.

PLANT MATERIALS.—*C. grayanum* was collected at Ooiwa, Kamiichi-machi, Toyama prefecture, Japan, in April 1990, and a voucher specimen is on deposit at our Institute.

IN VITRO CYTOTOXICITY ASSAY.—The cytotoxicity assays were mainly carried out according to the standard National Cancer Institute guidelines (12,13). The KB cells were maintained on MEM media supplemented with 5% fetal calf serum. The assays were performed on tissue culture plates with 24 flat-bottomed wells in a CO_2 incubator. After 72 h the KB cells were assayed according to the literature procedure (14). The ED_{50} values were estimated from semi-log

CHRYSOSPLENOL F [**1**].—Yellow needles, mp 162–165° (MeOH); positive to $FeCl_3$ and Mg-HCl; uv λ max (MeOH) (log ε) 262 (4.26), 300 (sh) (3.88), 350 nm (3.94); λ max (MeOH/$AlCl_3$ and -$AlCl_3$/HCl) 270, 312, 385 nm; λ max (MeOH/NaOMe) 255, 295, 335 nm; λ max (MeOH/NaOAc and -NaOAc/H_3BO_3) 255, 300,

335 nm; ^{1}H nmr (Me_2CO-d_6) δ 3.90 (3H, s, OMe), 3.94 (3H, s, OMe), 3.96 (3H, s, OMe), 6.31 (1H, d, J = 2.3 Hz, H-6), 6.54 (1H, d, J = 2.3 Hz, H-8), 6.99 (1H, s, H-3′), 7.11 ppm (1H, s, H-6′); eims *m/z* $[M]^+$ 360, 343, 329, 317, 167, 166; hreims *m/z* 360.0818 ($C_{18}H_{16}O_8$ requires 360.0844).

CHRYSOSPLENOL G [**2**].—Yellow needles, mp 153–155° (MeOH); positive to $FeCl_3$ and Mg-HCl; uv λ max (MeOH) (log ε) 252 (4.29), 297 (sh) (3.70), 337 nm (3.89); λ max (MeOH/$AlCl_3$ and -$AlCl_3$/HCl) 265, 315, 387 nm; λ max (MeOH/NaOMe, -NaOAc, and -NaOAc/H_3BO_3) same as original spectrum in MeOH; ^{1}H nmr (Me_2CO-d_6) δ 3.80 (3H, s, OMe), 3.84 (3H, s, OMe), 3.90 (3H, s, OMe), 3.92 (3H, s, OMe), 6.32 (1H, d, J = 2.4 Hz, H-6), 6.53 (1H, d, J = 2.4 Hz, H-8), 7.11 (1H, s, H-3′), 7.13 ppm (1H, s, H-6′); eims *m/z* $[M]^+$ 374, 359, 357, 343, 331, 167; hreims *m/z* 374.0979 ($C_{19}H_{18}O_8$ requires 374.1001).

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