Cytotoxic Flavonoids from the Whole Plants of *Chrysanthemum zawadskii* Herbich var. *latilobum* Kitamura

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Two known flavonoids, luteolin (**1**) and acacetin (**2**) were isolated from a $CHCl_3$ soluble fraction of the whole plants of *Chrysanthemum zawadskii* Herbich var. *latilobum* Kitamura, and their structures were determined by NMR analysis. The luteolin (**1**) was isolated from this plant for the first time. These compounds were examined for their *in vitro* cytotoxic activities against four human cancer cell lines including HCT116 (colon), UO-31 (renal), PC-3 (prostate) and A549 (lung) by sulforhodamine B (SRB) assay. Acacetin (2) showed significant cytotoxic activity against HCT116 and UO-31 cells with an IC_{50} of 2.44 and 2.89 μg/ ml, respectively.

Key words – *Chrysanthemum zawadskii* Herbich var. *latilobum* Kitamura, luteolin, acacetin, sulforhodamine B, cytotoxic activity

Introduction

Chrysanthemum zawadskii Herbich var. *latilobum* Kitamura (Compositae), known as "Gu-Jul-Cho" in Korea, is a white flower blooming from August to October throughout Korea. The whole plant of *C. zawadskii* is used for a traditional folk medicine in order to cure several symptoms, especially pneumonia, bronchitis, cough, common cold, pharyngitis, bladder-related disorders, gastroenteric, disorders and hypertension[1]. Recently, we reported the isolation and structural determination of two sesquiterpene lactones (SQLs) from this plant. And also investigated their medicinal properties such as *in vitro* cytotoxicity, anti-bacterial activities, farnesyl-protein transferase inhibitory activities, nitric oxide and cholesterol acyltransferase inhibitory activities[2,3,4,5,6]. But there are small fractions has been studied for their medicinal properties. Thus, there has been considerable interested in phytochemical investigations of *C. zawadskii* during our continuing search for biologically active substances from medicinal plants.

In the present paper, two flavonoids, luteolin (**1**) and acacetin (**2**) were isolated from a $CHCl_3$ soluble fraction of the whole plants of *C. zawadskii* by repeated silica gel column chromatography. Their structures were identified by comparisons of spectroscopic data with those of the corresponding compounds in the literature. These two flavonoids were evaluated for their *in vitro* inhibitory activities on the growth of human cancer cell lines including HCT116 (colon), UO-31 (renal), PC-3 (prostate) and A549 (lung) by the sulforhodamine B (SRB) assy. In this paper, we report isolation and structural elucidation of compounds **1** and **2** from *C. zawadskii* and their *in vitro* cytotoxic activities against four human cancer cell lines.

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Materials and Methods

Plant material

The whole plants of *Chrysanthemum zawadskii* was collected on October 2000, in Gyengsangnam-do Agricultural Research & Extension Service, Institution of Medicinal Plants, hamyang district of Korea.

Instruments

Optical rotations were obtained using a Perkin-Elmer polarimeter. UV spectra were measured on a Beckman DU650 spectrophotometer. NMR experiments were conducted on Bruker AM 500 (^{1}H-NMR at 500 MHz, ^{13}C-NMR at 125 MHz) spectrometer with tetramethylsilane (TMS) as the internal standard. EIMS were recorded on a Jeol

JMS-700 instrument operated at 70 eV. TLC analysis was performed on Kieselgel 60 F_{254} (Merk) plates. Silica gel (Merck 60A, 70-230 or 230-240 mesh ASTM) and Sephadex LH-20 (Amersham Biosciences) were used for column chromatography.

Extraction and isolation

The air-dried whole plants (2.0 kg) were extracted with MeOH (30 *l*) at room temperature for three days. The MeOH extract (3.9 g) was evaporated to dryness and suspended in 500 m*l* of H_2O, and then it was extracted successively with $CHCl_3$, EtOAc, and BuOH to yield three fractions, $CHCl_3$ (2.5 g), EtOAc (700 mg) and BuOH (500 mg). The $CHCl_3$ fraction was chromatographed on a silica gel (0.8 kg, 70-230 mesh) column eluted with a gradient of 100% $CHCl_3$ to 100% MeOH to afford twelve fractions (F1-F12). The Fraction F3 (670 mg) was chromatographed over silica gel (700 g, 230-400 mesh) using *n*-hexane:Me_2CO (9:1) and then increasing the polarity to the final ratio of 1/1 volume percentage. Altogether, 50 fractions (100 m*l* each) were collected and combined to give twelve major subfractions (F3-1 through F3-12), based on the comparison of TLC profiles after examination by shortwave UV light (254 nm) and by spraying with 10% v/v sulfuric acid in water. F3-5 and F3-6 were combined and then subjected to column chromatography on Sephadex LH-20, and then, compound **1** (96 mg, R_f = 0.4, $CHCl_3$:EtOAc=1:1) was purified by preparative TLC with n-hexane:Et_2O (1:1).

Subfraction F3-4 was purified by repeated silica gel column chromatography using $CHCl_3$:EtOAc gradient (4:1→ 1:1) to afford four sub-subfractions (F3-4-1 to F3-4-4). Finally, compound **2** (80 mg, R_f =0.63, $CHCl_3$:MeOH=19:1) was isolated from F3-4-3 by preparative TLC with $CHCl_3$:EtOAc (2:3).

Compound 1

Yellow amorphous powder; UV, λ_{max} (MeOH) 350, 288 nm; EI/MS (70 eV) *m/z* 286 $[M]^+$; IR ν_{max} 3421, 3150, 1654, and 1503 cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 6.20 (1H, d, *J* = 2.0 Hz, H-8), 6.46 (1H, d, *J* = 2.0 Hz, H-6), 6.67 (1H, s, H-3), 6.91 (1H, d, *J* = 8.1 Hz, H-5′), 7.40 (1H, d, *J* = 2.2 Hz, H-2′), 7.41 (1H, dd, *J* = 2.2, 8.1 Hz, H-6′), 12.97 (1H, s, 5-OH); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 92.3 (C-6), 97.3 (C-8), 101.3 (C-3), 102.2 (C-10), 111.8 (C-2′), 114.5 (C-5′), 117.4 (C-6′), 119.9 (C-1′), 144.2 (C-3′), 148.1 (C-4′), 155.7 (C-5), 159.9 (C-9), 162.3 (C-2), 162.6 (C-7), 180.1 (C-4).

Compound 2

Yellow needle crystal; UV, λ_{max} (MeOH) 328, 285 nm; EI/MS (70 eV) *m/z* 284 $[M]^+$; IR ν_{max} 3445, 3150, 1652, and 1508 cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 3.86 (3H, s, 4′-OMe), 6.21 (1H, d, *J* = 1.9 Hz, H-8), 6.50 (1H, d, *J* = 1.9 Hz, H-6), 6.84 (1H, s, H-3), 7.10 (2H, d, *J* = 8.8 Hz, H-3′, 5′), 8.01 (2H, d, *J* = 8.8 Hz, H-2′, 6′), 12.91 (1H, s, 5-OH); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 55.5 (OMe), 93.9 (C-6), 98.9 (C-8), 103.5 (C-3), 103.7 (C-10), 114.5 (C-3′, 5′), 112.9 (C-1′), 128.2 (C-2′, 6′), 157.3 (C-5), 161.4 (C-9), 162.3 (C-4′), 163.3 (C-2), 164.3 (C-7), 181.7 (C-4).

Sulforhodamine B (SRB) assay

Antiproliferative SRB assay was performed to assess the growth inhibition by a colorimetric assay, which estimates cell number indirectly by staining total cellular protein with dye SRB[7]. In brief, cells were fixed by layering 100 ice-cold 15% trichloroacetic acid (TCA, Aldrich Chemical) on top of the growth medium. They were then incubated at 4°C for 1 h, after the plates were washed five times with cold water, the excess water drained off and the plates left to dry in air. SRB stain (100 μ*l*: 0.4 in 1% acetic acid, Sigma) was added to each well and left in contact with the cells for 1 h, after which they were washed with 1% acetic acid and rinsed four times until only dye adhering to cells was left. The plates were dried and 1 μ*l* of 10 mM Tris base (pH 10.5, Sigma) were added to each well to dissolve the dye. The plates were shaken gently for 20 min on a gyratory shaker, and the absorbance (OD) of each well was read on spectrophotometer at 520 nm. Cell survival was measured as the percentage absorbance compared to the control. Cancer cell lines were used HCT116 (colon), UO-31 (renal), PC-3 (prostate) and A549 (lung).

Results and Discussions

Dried whole plants of *C. zawadskii* were extracted with MeOH and then defatted with *n*-hexane. Silica gel column chromatography of the dried $CHCl_3$ soluble fraction led to the isolation of the flavonoids **1** and **2** (Fig. 1).

Compound **1** was obtained as yellow amorphous powder and a molecular ion peak at *m/z* 286 $[M]^+$. The molecular formula $C_{15}H_{10}O_6$ was deduced from its HREIMS and NMR. The IR spectrum showed the presence of hydroxyl (3421 cm^{-1}), aromatic C=C (1503 cm^{-1}) and carbonyl (1654 cm^{-1}). The ^{13}C-NMR data with DEPT experiments showed

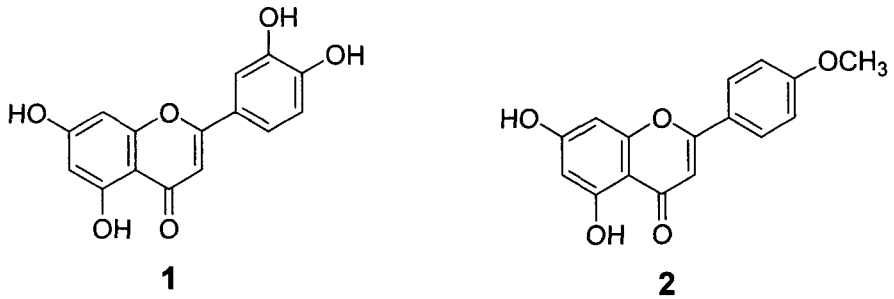

Fig. 1. Structures of compounds **1** and **2**.

the presence of fifteen carbon atoms as six methins [92.3 (C-6), 97.3 (C-8), 101.3 (C-3), 111.8 (C-2'), 114.5 (C-5'), 117.4 (C-6')], one carbonyl (180.1, C-4) and eight quaternary carbons [102.2 (C-10), 119.9 (C-1'), 144.2 (C-3'), 148.1 (C-4'), 155.7 (C-5), 159.9 (C-9), 162.3 (C-2), 162.6 (C-7)]. The ^{1}H-NMR spectra of compound **1** showed two *meta*-coupled doublets protons at δ 7.40 (d, *J* = 2.2 Hz, H-2'), 6.46 (d, *J* = 2.0 Hz, H-6) and 6.20 (d, *J* = 2.0 Hz, H-8). And an *ortho*, *meta*-coupled doublet-doublet and an *ortho*-coupled doublet protons showed at δ 7.41 (dd, *J* = 2.2, 8.1 Hz, H-6') and 6.91 (d, *J* = 8.1 Hz, H-5'), respectively. The ^{1}H and ^{13}C-NMR signals was almost identical to those in published data[8]. Thus compound **1** was identified as the known 3',4',5,7-tetrahydroxy flavone, luteolin. This compound have been isolated from many Compositae plants[9,10], however, it is the first report from *C. zawadskii*.

Compound **2** was isolated as a yellow needle crystal from MeOH. The EIMS of **2** exhibited a molecular ion peak at *m/z* 284 $[M]^+$. The molecular formula $C_{16}H_{12}O_5$ was deduced from its HREIMS and NMR. The IR spectrum of **2** showed absorption bands of hydroxyl (3445 cm^{-1}), carbonyl (1652 cm^{-1}) and aromatic rings (1508 cm^{-1}). The ^{13}C-NMR data with DEPT experiments showed the presence of sixteen carbon atoms as seven methins [93.9 (C-6), 98.9 (C-8), 103.5 (C-3), 114.5 (C-3', 5'), 128.2 (C-2', 6')], one carbonyl (181.7, C-4), one methoxy (55.5) and seven quaternary carbons [103.7 (C-10), 112.9 (C-1'), 157.3 (C-5), 161.4 (C-9), 162.3 (C-4'), 163.3 (C-2), 164.3 (C-7)]. The ^{1}H-NMR spectrum of **2** showed four *ortho*-coupled doublets of two protons each at δ 7.10 (d, *J* = 8.8 Hz, H-3', 5') and 8.01 (d, *J* = 8.8 Hz, H-2', 6'), two *meta*-coupled doublets of one proton each at δ 6.21 (d, *J* = 1.9 Hz, H-8) and 6.50 (d, *J* = 1.9 Hz, H-6), a singlet of one proton at δ 6.84 (s, H-3) and a methoxy singlet signal at δ 3.86. Accordingly, structure of compound **2** was identified as the known 5,7-dihydroxy-4-methoxyflavone, acacetin[10,11,12,13].

These compounds were examined for *in vitro* cytotoxic activities against a panel of human cancer cell lines including HCT116 (colon), UO-31 (renal), PC-3 (prostate) and A549 (lung) by the sulforhodamine B (SRB) assay. The IC_{50} values for compounds **1** and **2** are presented in Table 1. Compound **2** showed significant cytotoxic activity against HCT116 (IC_{50} 2.44 μg/m*l*) and UO-31 (IC_{50} 2.89 μg/m*l*) cells. Whereas compound **1** did not showed the cytotoxicity up to 100 μg/m*l*. This results were showed the structure activity relationships of flavonoids. The previous works have been that the inhibitory potencies are different from one structural moiety to another[13]. In this paper, the isolated compounds are the two flavones with the 5,7-dihydroxy group in A ring. Acacetin possessing the 4'-methoxyl group was more active than luteolin with 3',4'-dihydroxy group. This result clearly indicated that the methoxy moiety at 4'-position gave effective cytotoxic activity against human cancer cell lines.

This work were isolated two flavonoids, luteolin (**1**) and

Table 1. Inhibition of tumor cell proliferation by compounds **1** and **2** from *C. zawadskii*.

Compound	Cell line[a] [IC_{50} (μg/m*l*)]			
	HCT 116	UO-31	PC-3	A 549
1	>10	>10	>10	>10
2	2.44±0.27	2.89±0.38	5.18±0.69	>10
Adriamycin	2.74±0.37	0.72±0.63	0.76±0.39	0.72±0.32

[a] IC_{50} A values of compound against each human cancer cell lines (HCT 116: colon, UO-31: renal, PC-3: prostate, A 549: lung) were defined as concentrations (μg/m*l*) that caused 50% inhibition of cell proliferation *in vitro*.

acacetin (**2**) from the whole plants of *C. zawadskii*. Among the isolated compounds, **1** is the first isolation from this plant. The **2** showed significant cytotoxic activity against human cancer cell lines.

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초록 : 구절초에서 분리한 Flavonoids의 인체암 세포주에 대한 세포독성 효과

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구절초 전초의 chloroform 분획물로부터 2종의 flavonoid화합물을 분리하여 NMR을 통해서 구조를 확인한 결과, luteolin (**1**)과 acacetin (**2**)으로 구조 동정되었다. 이들의 화합물 중에서 luteolin (**1**)은 구절초에서 처음으로 분리하였다. 분리된 화합물은 sulforhodamine B (SRB) assay법에 따라 인체암세포주인, HCT116 (결장암) , UO-31 (신장암), PC-3 (전립선암) 와 A549 (폐암)등에 대한 *in vitro*에서의 세포독성을 실험하였다. 그 결과, acacetin (**2**)이 HCT116 (IC_{50} 2.44 μg/m*l*)과 UO-31 (IC_{50} 2.89 μg/m*l*)에서 유의할만한 세포독성을 나타내었다.