Flavonoids from *Artemisia sacrorum* Ledeb. and their cytotoxic activities against human cancer cell lines

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Abstract. Flavonoids have been demonstrated to have cytotoxic activities toward numerous human cancer cells, whereas they have little or no effect on normal cells. The numerous flavonoids in traditional Chinese herbs may be promising candidates for the development of novel anti-cancer drugs. Our previous study demonstrated that CH_2Cl_2 and 95% ethanol eluate (EE) fractions have the strongest cytotoxic activities against human cancer cell lines of the 9 fractions separated from *Artemisia sacrorum* Ledeb., which is widely used to prevent and treat diverse diseases in Northeast China. In the present study, 8 flavonoids were isolated from the 95% EE fraction of *Artemisia sacrorum* Ledeb. The chemical structures of the compounds were elucidated by extensive spectroscopic analyses. The following 5 flavonoids were isolated for the first time from this plant: Jaceosidin, kaempferol, quercetin, luteolin and quercitrin. A total of 2 flavonoids from the CH_2Cl_2 fraction and 8 flavonoids from the 95% EE fraction were examined to evaluate their cytotoxic activities against human SK-HEP-1 hepatoma cancer cells and human HeLa cervical cancer cells, respectively. The results revealed that 2 flavonoids had marked cytotoxic activities against HeLa cells.

Introduction

The majority of approved anticancer drugs are either natural products or have been developed based on knowledge gained from natural products, which have been important in the search for novel anticancer drugs compared with other areas of drug development (1-5). Traditional Chinese medicine has been historically used to treat disease using natural products. Although certain traditional Chinese herbs should no longer be used, other traditional Chinese herbs that have been proven to be effective have been gradually incorporated into modern medicine (6).

Among these traditional Chinese medicinal plants, *Artemisia sacrorum* Ledeb (ASL; *Compositae*) is of particular interest as it is widely used to prevent and treat diverse diseases in the Yanbian area of Northeast China (7,8). Previous studies have indicated that the water-soluble parts of the *A. sacrorum* extract were protective against carbon tetrachloride and acetaminophen-induced hepatotoxicity in mice, and the underlying mechanisms have been investigated (9-11). In a previous study, *A. sacrorum* was demonstrated to prevent N-acetyl-p-aminophenol (APAP)-induced apoptosis and necrosis, as indicated by liver histopathological and immunohistochemical analysis, and DNA laddering (9-11). According to the results from a western blot analysis, the ethanol eluate precipitation (EEP) decreased APAP-induced caspase-3 and -8 protein expression levels in mouse livers (9-11). Furthermore, ASL was able to inhibit adipocyte differentiation and adipogenesis through the activation of 5'-adenosine monophosphate-activated protein kinase (AMPK) in 3T3-L1 adipocytes and hepatocellular carcinoma (HepG2) cells, respectively (12,13). Petroleum ether fraction of *A. sacrorum* Ledeb, another extract from *A. sacrorum*, inhibited glucose production via the AMPK-glycogen synthase kinase-cAMP response element binding protein signaling pathway in HepG2 cells (14). Notably, the cytotoxicities of the 9 fractions separated from *A. sacrorum* via the two extraction methods was investigated in our previous study, and the results demonstrated that the CH_2Cl_2 and 95% ethanol eluate (EE) fractions revealed the strongest cytotoxic activities against 3 human cancer cell lines. In addition, a subsequent study demonstrated that 10 compounds, of which 2 were flavonoids, were isolated from the CH_2Cl_2 fraction (15).

The present study aimed to investigate the isolation and structure of the compounds from the 95% EE fraction of *A. sacrorum*. The results demonstrated that this fraction was markedly rich in flavonoids. Flavonoids are cancer-preventive agents, and have been shown to be particularly important (16-18). Therefore, the present study was further conducted to evaluate the cytotoxic activities of all flavonoids from the CH_2Cl_2 and 95% EE fractions.

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Materials and methods

General experimental procedures. The nuclear magnetic resonance (NMR) spectra of compounds 1, 2, 3, 5, 6, and 7 were recorded on a Bruker 500 MHz NMR spectrometer (Bruker Corporation, Billerica, MA, USA), operating at a frequency of 500 MHz for ^{1}H and 125 MHz for ^{13}C nuclei at room temperature, and with TMS as the internal standard. The NMR spectra of compounds 4 and 8 were recorded on a Bruker 300 MHz NMR spectrometer, operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C nuclei at room temperature with tetramethylsilane (TMS) as the internal standard. Chemical shifts (δ) were expressed in parts per million (ppm) relative to TMS. The chemical shifts (δ) and coupling constant values were reported in ppm and Hz, respectively. Compounds 1, 2, 3, 4, 6, and 7 were dissolved in CD_3OD, 5 was dissolved in dimethyl sulfoxide (DMSO), and 8 was dissolved in acetone-d_6. Column chromatography (CC) was performed on a Sephadex LH-20 (18-111 μm; GE Healthcare Biosciences, Pittsburgh, PA, USA), YMC-GEL octadecyl (ODS)-A (50 μm; YMC Co., Ltd., Kyoto, Japan) and silica gel (200-300 mesh, Qingdao Marine Chemical, Ltd., Qingdao, China) columns. D-101 macroporous absorption resin was purchased from Tianjin Haiguang Chemical Co., Ltd. (Tianjin, China). Silica gel 60 F254 plates (20x20 cm; 0.2 mm thick; Merck, Darmstadt, Germany) and silica gel GF 254 (Qingdao Marine Chemical Co., Ltd.) were used for thin-layer chromatography (TLC) analysis. The CH_3OH used for column chromatography was high performance liquid chromatography-grade (Jiangsu Hanbang Science and Technology Co., Ltd., Jiangsu, China). All other chemicals and reagents used in the present study were of analytical grade.

Plant material. The aerial parts of ASL (*Compositae*) were collected in July 2008 from Xidong, Yanji in Jilin. The plant was taxonomically identified and authenticated by Professor Huizi Lv (College of Pharmacy, Yanbian University of China, Yanji, China). A specimen of the plant was deposited at the College of Pharmacy, Yanbian University.

Extraction and isolation. A total of 5 kg of the aerial parts of *A. sacrorum* were collected, air-dried in the shade, and dissolved in boiling water twice (Figs. 1 and 2). The extract was then separated on a D-101 macroporous absorption resin to elute water, 50% EE and 95% EE fractions. From this separation, the 95% EE fraction (16.03 g) was chromatographed on a silica-gel column (200-300 mesh) with petroleum ether-EtOAc in a step-wise gradient elution (50:1-0:100). The eluates were combined based on the TLC results in order to produce 8 fractions (1-8).

The fractions were chromatographed as follows: Fraction 2 was chromatographed on an ODS column by eluting with gradient mixtures of CH_3OH and H_2O (8:2 to 10:0) in order to obtain 7 subfractions (2.1-7). Subfraction 2.3 was rechromatographed on a Sephadex LH-20 column by eluting with gradient mixtures of CH_3OH and H_2O (8:2 to 10:0) to obtain compound A (3.0 mg). Fraction 3 was rechromatographed on an ODS column by eluting with gradient mixtures of CH_3OH and H_2O (7:3 to 10:0) to afford compound 1 (23.2 mg). Fraction 4 was rechromatographed on an ODS column by eluting with gradient mixtures of CH_3OH and H_2O (6:4 to 10:0) to obtain 11 subfractions (4.1-11). Subfraction 4.3 was rechromatographed on a Sephadex LH-20 column by eluting with gradient mixtures of CH_3OH and H_2O (8:2 to 10:0) to obtain compound 2 (2.6 mg) and compound 3 (1.5 mg). Subfraction 4.6 was subjected to a Sephadex LH-20 column by eluting with gradient mixtures of CH_3OH and H_2O (7:3 to 10:0) to obtain compound 4 (8.2 mg). Fraction 5 was rechromatographed on a Sephadex LH-20 column by eluting with CH_3OH-H_2O (7:3 to 10:0) to obtain compound 5 (2.0 mg) and 12 subfractions (5.1-12). Subfraction 5.10 was subjected to a Sephadex LH-20 column by eluting with gradient mixtures of CH_3OH and H_2O (8:2 to 10:0) to obtain compound 6 (1.5 mg). Fraction 6 was rechromatographed on an ODS column by eluting with gradient mixtures of CH_3OH and H_2O (6:4 to 10:0) to obtain eight subfractions (6.1-8). Subfraction 6.6 was subjected to a Sephadex LH-20 column repeatedly to obtain compound 7 (2.6 mg). Fraction 7 was rechromatographed on a Sephadex LH-20 column by eluting with gradient mixtures of CH_3OH-H_2O (4:6 to 10:0) to produce 5 subfractions (7.1-5). Finally, subfraction 7.2 was subjected to a Sephadex LH-20 column once more by eluting with gradient mixtures of CH_3OH and H_2O (6:4 to 10:0) to afford compound 8 (6.0 mg).

Cell culture and MTS assay. The two human cancer cell lines (SK-HEP-1 and HeLa) and one normal human cell line (HEK293) used in the cytotoxic assay were purchased from American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin and kept at 37˚C in a humidified atmosphere containing 5% CO_2.

A 200-μl aliquot of adherent cells was used to seed 96-well cell culture plates at $3x10^4$ cells/well and allowed to adhere for 6 h prior to drug addition. Each cell line was treated with 0, 5, 10, 25, 50, 100 and 200 μM of flavonoids for 48 h. Subsequently, 20 μl MTS reagent was added into each well and incubated for 1 h. The cell viability was detected by the CellTiter 96 AQueous One solution Cell Proliferation Assay kit (Promega Corporation, Madison, WI, USA).

Results and Discussion

To observe the cytotoxic activity of the herb, *A. sacrorum* was extracted and isolated by 95% EtOH and water, as described in our previous study (15). Following extraction with 95% EtOH extract with various solvents, and separation of the water extract with a D-101 macroporous resin column, 9 fractions of *A. sacrorum* were obtained, of which the 95% EE and CH_2Cl_2 fraction revealed the strongest cytotoxity against 3 human cancer cell lines. In a subsequent study, 2 flavonoids were isolated from the CH_2Cl_2 fraction (15). In the present study, 9 compounds were isolated from the 95% EE fraction.

Compound 1 was isolated as yellow, needle-like crystals (acetone). The ^{1}H-NMR and ^{13}C-NMR spectra of the compound revealed the characteristic signals of a flavonoid. The ^{1}H-NMR spectrum exhibited two proton signals at δ 6.56 (1H; s) and 6.61 (1H; s), which represented the proton signals of C3-H and C8-H of the A ring, respectively. Signals at δ 7.45 (1H; d; *J*=2.0 Hz), 6.92 (1H; d; *J*=8.35 Hz) and 7.49 (1H; dd; *J*=2.0; 8.35 Hz) represented the proton signals of C2'-H, C5'-H and C6'-H of the B ring. δ 3.96 (3H; s) and 3.88 (3H; s) corresponded to the

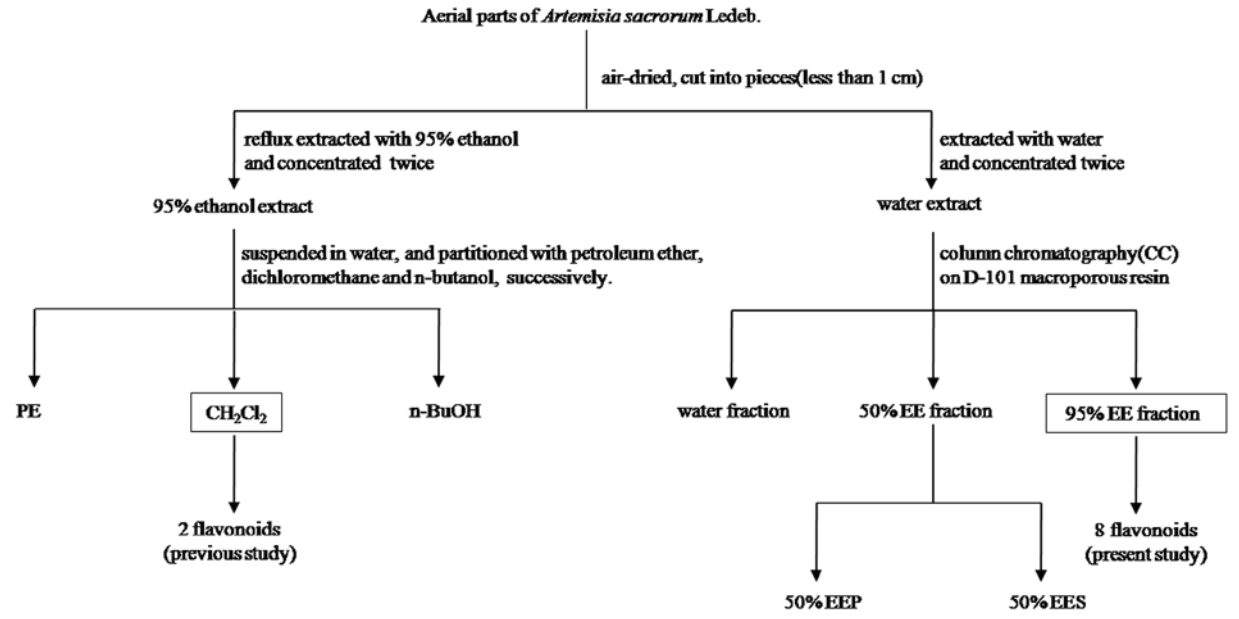

Figure 1. Extraction scheme for the isolation of 10 flavonoids from the 2 most cytotoxic fractions of *Artemisia sacrorum* Ledeb. By shade-dried we mean that the aerial parts of the herb were air-dried in the shade. EE, ethanol eluate; PE, petroleum ether; EEP, ethanol eluate precipitation; EES, ethanol eluate supernatant.

proton signals of the 2 methoxy groups. The ^{13}C-NMR spectrum showed 17 carbon signals of which δ_C 184.23 was the carbonyl signal, whereas 56.71 and 60.94 were assigned to 2 methoxy groups. By analyzing these data and comparing them with those in the literature (19), the structure of compound 1 was determined as jaceosidin (Fig. 3).

Compound 2 was obtained as a yellow powder (MeOH). The ^{1}H-NMR spectrum demonstrated 2 *meta*-coupled proton signals at δ 6.18 (1H; d; *J*=2.0 Hz; H-6) and 6.40 (1H; d; *J*=2.0 Hz; H-8) of the A ring. Two groups of *meta*-coupled proton signals at δ 6.90 (2H; d; *J*=9.0 Hz; H-3'; H-5') and 8.09 (2H; d; *J*=9.0 Hz; H-2'; H-6') were assigned to 4 protons in the B ring. The ^{13}C-NMR spectrum revealed carbon signals of δ_C 148.15, 137.17, 177.44, 158.35, 99.38, 165.77, 94.55, 162.53, and 104.56 for the A ring, and 123.81, 130.69, 116.35, 160.57, 116.35, and 130.69 for the B ring. Based on the above spectral data and comparison with a previous study (20), the chemical structure of compound 2 was determined as kaempferol.

Compound 6 was obtained as yellow, needle-like crystals (MeOH). The ^{1}H-NMR spectrum revealed 2 isolated proton signals at δ 6.58 (1H; s; H-3) and 6.67 (1H; s; H-8), which were attributed to H-3 (C-ring) and H-8 (A-ring), respectively. Two groups of *meta*-coupled proton signals at δ 6.92 (2H; d; *J*=8.75 Hz; H-3'; H-5') and 7.84 (2H; d; *J*=8.75 Hz; H-2'; H-6') were assigned to 4 protons of the B ring. The ^{13}C-NMR spectrum showed δ_C values of 164.46, 102.46, 184.31, 152.69, 131.70, 157.66, 94.17, 152.69, and 104.87 for the A ring, and 122.14, 128.45, 116.15, 161.32, 116.15, and 128.45 for the B ring. These spectroscopic data were characteristic of a flavonoid. Based on the above spectral data and comparison with previous studies (21,22), the chemical structure of compound 6 was determined as hispidulin.

Compound 8 was obtained as a yellow powder (acetone). ^{1}H-NMR revealed, in the aromatic region, 2 *meta*-coupled proton signals at δ 6.25 (1H; d; *J*=2.07 Hz; H-6) and 6.46 (1H; d; *J*=2.07 Hz; H-8) for the A ring. ^{1}H-NMR also demonstrated 2 *meta*-coupled proton signals at δ 7.49 (1H; d; *J*=2.07 Hz; H-2'), 7.39 (1H; dd; *J*=2.07; 8.34 Hz; H-6'), and 1 isolated proton signal at δ 6.98 (1H; d; *J*=8.34 Hz; H-5') for the B ring. These 3 proton signals exhibited the typical three-spin system of the 1',3',4'-trisubstituted B ring. δ 5.28 (1H; d; *J*=1.5 Hz; H-1'') was the anomeric proton signal of sugar. The ^{13}C-NMR spectrum revealed a carbonyl proton signal at δ_C 179.18, which was assigned to C-4, and 17.63-102.57 ppm for a group of 6-carbon sugar signals revealing the presence of an aglycone moiety. Based on the above spectral data and a comparison with previous studies (23,24), the chemical structure of compound 8 was determined as quercitrin.

In addition to the above-mentioned compounds, 5 other compounds were also identified as chrysoeriol (compound 3) (25), quercetin (compound 4) (26), apigenin (compound 5) (27), luteolin (compound 7) (28,29) and scopoletin (compound A) (30). These were identified by interpretation of their spectroscopic data and comparison of the data with the reported values. Among them, compounds 1-8 were flavonoids, and flavonoids 1, 2, 4, 7 and 8 were isolated for the first time from *A. sacrorum*. The structures of the 8 flavonoids are depicted in Fig. 3 and all ^{13}C-NMR spectra data were listed in Table I.

A total of 8 flavonoids isolated from the 95% EE fraction of *A. sacrorum* were designated as flavonoids 1-8, and 2 flavonoids (genkwanin and acacetin) previously isolated from the CH_2Cl_2 fraction were designated as flavonoids 9 and 10. Although these 10 flavonoids were not novel compounds, their possible biological activities should not be ignored because they have previously been identified. Furthermore, not every novel compound is examined for all its activities (even *in vitro* experiments before it is considered to be old). Conversely, the majority of novel compounds have only undergone *in vitro* activity tests.

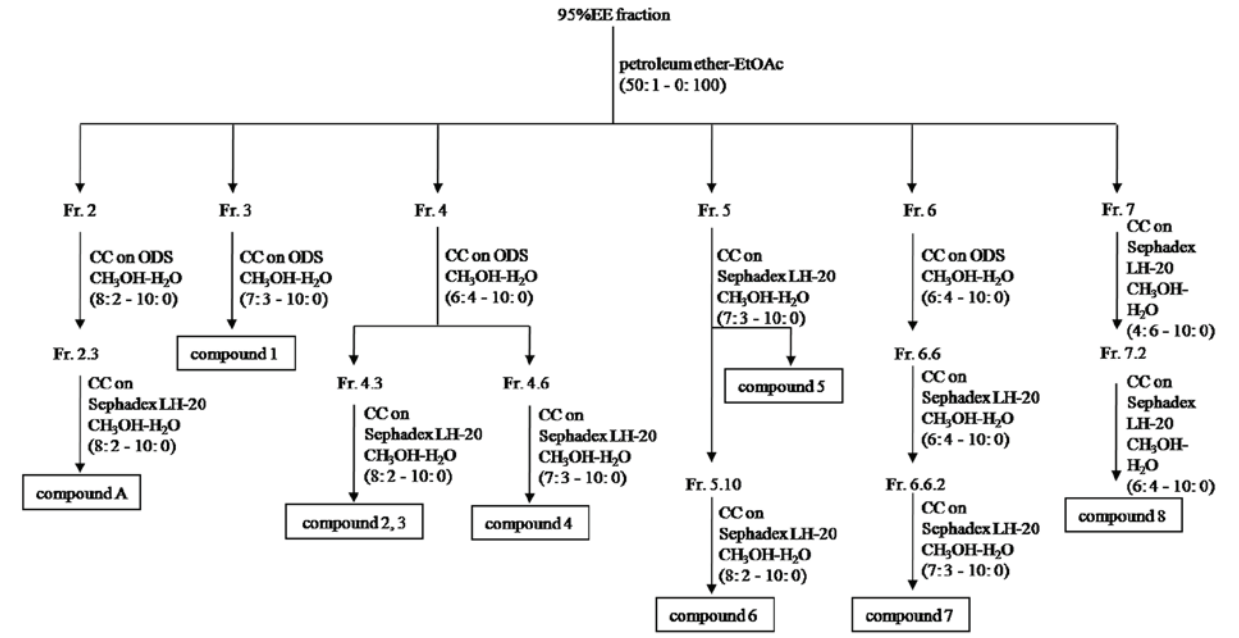

Figure 2. Extraction and separation of 8 flavonoids and 1 coumarin from the 95% EE fraction. Fr, fraction; EE, ethanol eluate.

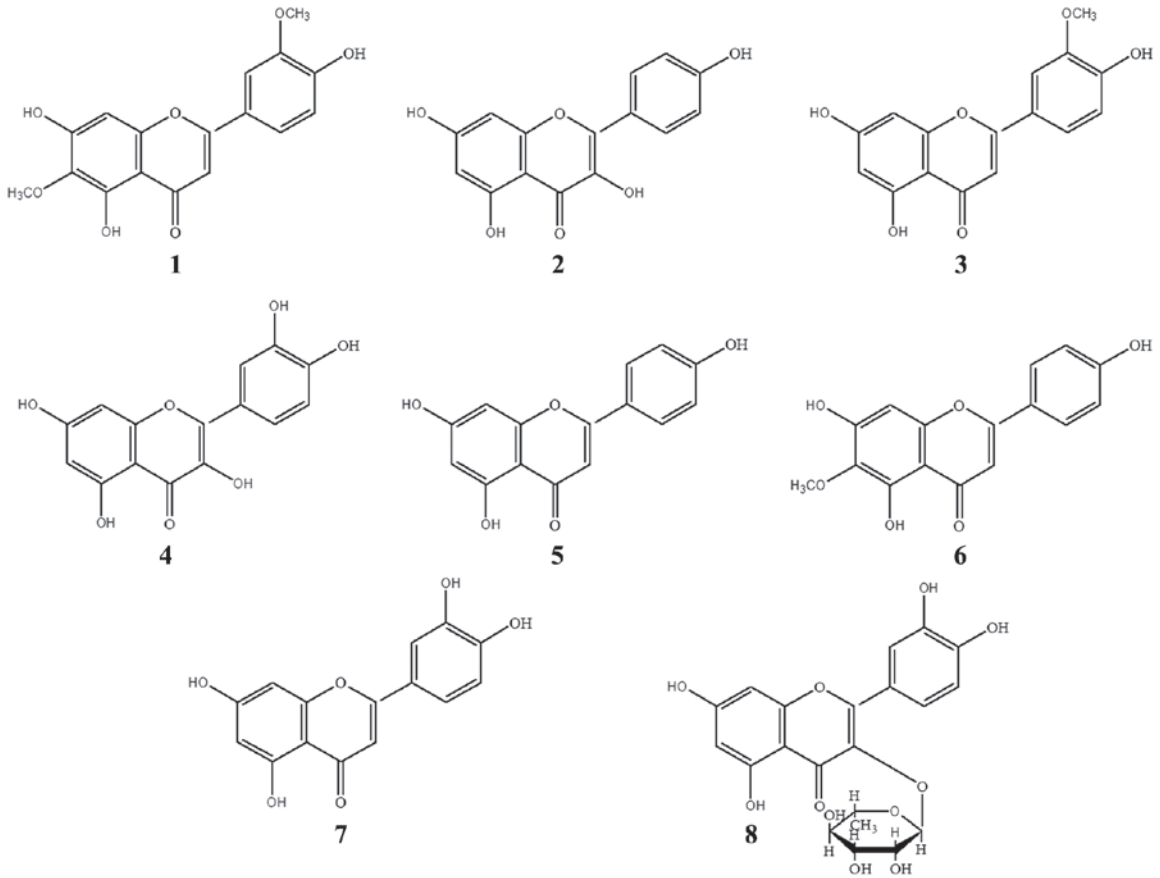

Figure 3. Chemical structures of 8 flavonoids from the 95% EE fraction from *Artemisia sacrorum* Ledeb. 1-8 represent the compound numbers. EE, ethanol eluate.

In the present study, the cytotoxic activities against the 10 flavonoids on SK-HEP-1, HeLa and HEK293 cell growth were determined using an MTS assay (31). Their IC_{50} values were presented in Table II, in which apigenin (flavonoid 5) and luteolin (flavonoid 7) exhibited strong cytotoxic activities against human cervical cancer HeLa cells. Conversely, they had

Table I. ^{13}C-NMR spectra data of compounds 1-8 (δ, ppm; compounds 4 and 8 were detected at 75 MHz, others at 125 MHz; compounds 1, 2, 3, 4, 6 and 7 dissolved in CD_3OD, compound 5 dissolved in DMSO, and compound 8 dissolved in acetone-d_6; OMe, OCH3).

Compound no.	1	2	3	4	5	6	7	8
2	166.24	148.15	160.89	147.99	164.17	164.46	164.73	158.15
3	103.80	137.17	104.15	137.24	103.04	102.46	104.62	135.63
4	184.23	177.44	183.79	177.34	181.77	184.31	183.66	179.18
5	154.70	158.35	163.13	158.23	157.48	152.69	162.33	163.06
6	132.97	99.38	123.75	99.22	98.90	131.70	99.78	99.35
7	158.90	165.77	166.09	162.52	163.88	157.66	165.61	164.77
8	95.38	94.55	95.31	94.39	94.16	94.17	94.75	94.35
9	154.70	162.53	156.19	165.58	161.22	152.69	158.25	157.63
10	105.78	104.56	105.39	104.52	103.77	104.87	103.89	105.75
1'	123.78	123.81	124.69	148.77	121.38	122.14	122.31	122.43
2'	110.75	130.69	110.70	115.98	128.56	128.45	114.22	115.99
3'	149.52	116.35	149.58	124.14	116.05	116.15	146.78	145.68
4'	152.13	160.57	152.52	146.22	161.06	161.32	150.71	148.85
5'	116.81	116.35	116.86	116.22	116.05	116.15	116.01	116.56
6'	121.76	130.69	121.77	121.67	128.56	128.45	120.46	122.73
OMe	56.71		56.72			59.85		
OMe	60.94							
1"								102.57
2"								71.26)
3"								71.97)
4"								72.86)
5"								71.19
6"								17.63

The table values represent ^{13}C-NMR spectra data of compounds 1-8. OMe represents OCH_3. NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide.

no cytotoxic activity against normal human embryonic kidney HEK293 cells.

The structures of the 10 flavonoids are similar. However, certain flavonoids revealed different cytotoxic activity against the two human liver cancer SK-HEP-1 and human cervical cancer HeLa cells. Although certain flavonoids have similar chemical structures, there are still compound-specific effects on certain neoplasms that are relevant to adjust specific biochemical processes to treat certain neoplasms differentially (32,33). In the present study, the majority of the 10 flavonoids demonstrated no cytotoxic activity against normal human embryonic kidney cells.

Flavonoids may serve as clinically significant chemotherapeutic agents in the treatment of cancer. Indeed, flavonoids have been demonstrated to reveal cytotoxic activities towards numerous human cancer cells, whilst having little or no effect on normal cells. This has led to interest in the development of potential flavonoid-based chemotherapeutics for anticancer treatment (34-37). Traditional Chinese herbs, which depend predominantly on empirical medication, have been used for thousands of years in the treatment of numerous diseases, often with few adverse effects. Furthermore, many of the active flavonoids in traditional Chinese herbs have been the subject of studies aiming to develop novel anti-cancer drugs by demonstrating their activity through *in vivo* and *in vitro* experiments (38-40). The diverse flavonoids in these herbal medicines may be promising candidates in the development of novel anti-cancer drugs.

Table II. Cytotoxities of 10 flavonoids isolated from *Artemisia sacrorum* (IC_{50}, μM).

Flavonoids	SK-HEP1	HeLa	HEK293
1	>50	>50	14.1380
2	>50	45.809	>50
3	47.19	>50	>50
4	45.96	>50	>50
5	46.56	6.092	>50
6	49.93	>50	>50
7	>50	16.254	>50
8	>50	>50	>50
9	>50	47.834	>50
10	>50	>50	>50

All compounds with IC_{50} values >50 μM were considered inactive.

Acknowledgements

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