JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Comparative Evaluation of Cultivars of *Chrysanthemum morifolium* Flowers by HPLC-DAD-ESI/MS Analysis and Antiallergic Assay

Yuan-yuan Xie,[†] Jia-lin Qu,[†] Qi-long Wang,[†] Yan Wang,[†] Masayuki Yoshikawa,[§] and Dan Yuan*[,†]

[†]College of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, People's Republic of China

[§]Department of Pharmacognosy, Kyoto Pharmaceutical University, Shichono-cho, Misasagi, Yamashina-ku, Kyoto 607-8412, Japan

Supporting Information

ABSTRACT: A multicomponent quantification fingerprint based on HPLC coupled with diode array detection and electrospray ionization tandem mass spectrometry (HPLC-DAD-ESI/MS) technique has been established for the analysis of phenolic compounds in 12 samples originated from 5 different cultivars of *Chrysanthemum morifolium* flowers in China. Four caffeoylquinic acids and 15 flavonoids in the capitulum were identified by comparing the retention times and ultraviolet spectra as well as the mass spectrum and/or matching the empirical molecular formula with that of reference compounds, and the contents of these compounds have been determined simultaneously. The samples from three medicinal cultivars significantly differed in the quality and quantity of flavonoid aglycones and glycosides compared with those from two edible cultivars, which allows the possibility of showing the chemical distinctness of these cultivars and may be useful in their standardization. Moreover, the antiallergic effects of these cultivars were comparatively assayed for the first time. A representative medicinal cultivar, 'huaiju', showed potential activity on the inhibition of antigen-induced degranulation from RBL-2H3 cells and compound 48/80-induced scratching in mice, whereas the in vitro and in vivo antiallergic activities of two edible cultivars were weak. The results suggested that the quality and quantity of some active flavonoid aglycones should be responsible for the pharmacological profiles of these cultivars.

KEYWORDS: *Chrysanthemum morifolium Ramat., caffeoylquinic acids, flavonoids, antiallergic activity, HPLC-DAD-ESI/MS*

INTRODUCTION

Flos Chrysanthemi (Ju Hua in Chinese) is a medicinal and edible cognate that is botanically from the dry capitulum of *Chrysanthemum morifolium* Ramat. As a herbal medicine, it is used to treat the common cold, dim eye sight, dizziness, and skin itch in Traditional Chinese Medicine (TCM).[1] Meanwhile, *Chrysanthemum* tea also has 2000 years of history in China and is a popular beverage prepared from Flos Chrysanthemi in combination with hot or boiling water for health care in summer.[2] Many pharmacological studies reported its extensive biological activities, such as anti-inflammation,[3] anti-human immune deficiency viruses,[4,5,17] antioxidation,[6−8] vaso-relaxant activity,[9] cardiovascular protection,[10,11] anticancer,[12,13] aldose reductase inhibition,[14,15] and antimutagenic activity.[16] Caffeoylquinic acids,[6,7] flavonoids,[4,5,8−11,13−17] and essential oils[3,12] are the major ingredients in Flos Chrysanthemi and should be responsible for these aforementioned activities.

There are many cultivars of *C. morifolium* flowers available in herb or tea markets in China.[18] Five major cultivars, 'Huaiju', 'Boju', 'Chuju', 'Gongju', and 'Xiaoyangju' (commercial name Hangbai Ju in Chinese), were used as medicinal herbs according to the traditional experiences, among which, 'Huaiju' is an original medicinal cultivar in TCM. The cultivars 'Gongju' and 'Xiaoyangju' are often used as tea materials as well, 'Xiaoyangju' being a classical tea material.[2] In fact, these cultivars are recorded under the same monograph of 'Flos Chrysanthemi' in the Chinese Pharmacopoeia[1] and are indiscriminate in medicinal and tea use. So far, no comparative study on their biological activities and chemical profiles has been reported. Thus, there was still no scientific evidence to characterize the rationality of the traditional practice about these cultivars, which is a serious problem with regard to their safety and efficacy. According to the Chinese Pharmacopoeia, chlorogenic acid and luteolin are used as marker compounds for the quality control of Flos Chrysanthemi.[1] During the past few years, holistic chemical profiling methods of Flos Chrysanthemi have been developed by many modern hyphenated techniques, such as GC-MS profiles of essential oils and identification or quantification of flavonoids and caffeoylquinic acids by HPLC, LC-MS, and LC-MS/MS methods.[19−24] The determination of six flavonoid glycosides in 20 cultivars of Flos Chrysanthemi using a HPLC method has been reported.[25] However, a comprehensive chemical profile for the characterization of the medicinal cultivars of *C. morifolium* flowers related with bioactivity has been obscure until now.

The clinical therapy of dermatitis and eye itch using Flos Chrysanthemi suggests its potential antiallergic activity,[1] and it has been reported that the extract of Herba Chrysanthemi Sibirici, a *Chrysanthemum* plant, possessed potent activities in the inhibition of dinitrophenylated bovine serum albumin (DNP-BSA) and compound 48/80-induced degranulation in RBL-2H3 mast cells and in compound 48/80-induced anaphylaxis.[26] Moreover, luteolin and 3,5-dicaffeoylquinic acid, two major components of Flos Chrysanthemi, also showed in vitro and in vivo antiallergic effects.[27,28]

Therefore, the present study aimed at defining the chemical composition and pharmacological characteristics of five cultivars

Received: September 22, 2012
Revised: December 6, 2012
Accepted: December 7, 2012
Published: December 8, 2012

of *C. morifolium* flowers. A multicomponent quantification fingerprinting approach[29,30] based on HPLC coupled with diode array detection and electrospray ionization tandem mass spectrometry (HPLC-DAD-ESI/MS) techniques has been developed, which combined the chemical profiling and simultaneous determination of 4 caffeoylquinic acid derivatives and 15 flavonoids in the different cultivars. Furthermore, the antiallergic activities of these cultivars were comparatively assayed for the first time using rat basophilic leukemia RBL-2H3 cells and a mouse model of pruritus.

MATERIALS AND METHODS

Chemicals. Chlorogenic acid (3-*O*-caffeoylquinic acid, CA) was purchased from China National Institute for the Control of Pharmaceuticals and Biological Products (Bejing, China), and luteolin-7-*O*-*β*-D-glucoside (L-7G), apigenin-7-*O*-*β*-D-glucoside (A-7G), and luteolin (L) were from Funakoshi Co. Ltd. (Tokyo, Japan). Other standard compounds used in the qualitative and quantitative determination were isolated from the extracts of the flowers of *C. morifolium* cv. 'Huaiju' or 'Xiaoyangju' in our previous studies.[13,31] They included luteolin-7-*O*-*β*-D-rutinose (L-7R, scolimoside), 3,5-dicaffeoylquinic acid (3,5-diCQA), 1,3-dicaffeoyl-epi-quinic acid (1,3-diC-epiQA), diosmetin-7-*O*-*β*-D-rutinose (D-7R, diosmin), diosmetin-7-*O*-*β*-D-glucoside (D-7G), diosmetin-7-(6″-*O*-*p*-hydroxyphenyl-acetyl)-*O*-*β*-D-glucoside [D-7-(6″-*O*-*p*-HPA)G], acacetin-7-*O*-*β*-D-rutinose (Ac-7R, linarin), acacetin-7-*O*-*β*- D-glucoside (Ac-7G), apigenin (A), diosmetin (D), acacetin-7-(6″-*O*-acetyl)-*β*-D-glucoside (Ac-7-(6″-Ac) G), and acacetin (Ac). The identities of these compounds were confirmed by melting point, UV, IR, ^{1}H and ^{13}C NMR, and MS,[6,32−38] and their purities evaluated with HPLC-UV at 254 nm were >95%. Other chemical reagents were of analytical or HPLC grade. Ultrapure water (18.2 MΩ) daily prepared with a Milli-Q water purification system (Millipore, Molsheim, France) was used in the mobile phase.

Cell Lines and Biological Materials. The basophilic leukemia RBL-2H3 cells (cell no. JCRB0023) were obtained from the Japan Health Science Foundation (Tokyo, Japan). Antidinitrophenyl (DNP) IgE, Eagle's minimum essential medium (MEM), *p*-nitrophenyl-*N*-acetyl-*β*-D-glucosamine (PNAG), and compound 48/80 (*p*-methoxy-*N*-methylphenethylamine) were purchased from Sigma (St. Louis, MO, USA), fetal calf serum (FCS) was purchased from Gibco (Grand Island, NY, USA), and terfenadine was purchased from Jiangsu Lianhuan Pharmaceutical Co. Ltd. (Yangzhou, Jiangsu, China). The 24- and 96-well microplates were the products of Sumitomo Bakelite (Tokyo, Japan). DNP−BSA was prepared following the method described in the previous literature.[39] Other chemicals were from Wako Pure Chemical Industries (Osaka, Japan).

Animals. Male KM mice (5−6 weeks of age, weighing 18−22 g) were purchased from Changchun Newtec Research Center for Laboratory Animal Medicine (Changchun, China). They were housed under controlled temperature (23−25 °C) and humidity (50 ± 5%) on a 12 h light/dark cycle (lights on from 8:00 a.m. to 8:00 p.m.). Food and water were freely available. Experiments were conducted in accordance with the guidelines of the Guiding Principles for the Care and Use of Laboratory Animals approved by the Committee for Animal Experiments in Shenyang Pharmaceutical University.

Herbal Materials. Twelve samples (samples 1−12, Table 3) were directly obtained from some cultivated farms or herb markets in China, which were harvested in bloom between September and November. All of the samples were authenticated as the dry capitulum of *C. morifolium* Ramat. by Professor Qishi Sun (Shenyang Pharmaceutical University, China). They were divided into different cultivars according to shape, size, and color of flowers, the presence or absence of a scale or bractlet at the base of each individual floret, and the bloom forms that are defined by the way in which the ray and disk florets are arranged.[18] Voucher specimens are kept in the reference library for medicinal herbs in Shenyang Pharmaceutical University.

Instrumentation and Chromatographic Conditions. HPLC analysis was performed on a Hypersil C_{18} column (4.6 × 200 mm, 5 μm, Dalian Elite Analysis Instrument Co, Dalian, China) at 45 °C. The mobile phase consisted of (A) water containing 1% glacial acetic acid and (B) acetonitrile containing 1% glacial acetic acid with gradient elution (linear gradient from 10 to 30% B in 70 min, followed by a linear gradient to 55% B in 5 min, and finally equilibrated with 10% B for 5 min). Re-equilibration duration was 10 min between individual runs. The flow rate was kept at 1.0 mL min^{-1}, and 10 μL of standard and sample solution was injected in each run.

Identification of marker compounds by LC-MS was performed on a Bruker MicroTOF-Q 125 time-of-flight tandem mass spectrometry (TOF-MS) instrument with a Bruker ESI source (Santa Barbara, CA, USA). LC-ESI-MS was performed in the negative mode under the following operating parameters: scanning mode, total ion current (TIC)/selective ion monitoring (SIM); scanning range (m/z), 50−2000; drying gas (N_2) flow rate, 4.0 L/min; drying gas temperature, 230 °C; nebulizer, 0.8 bar; and capillary voltage, 3.2 kV. The online UV spectra of marker compounds at the range of 200−400 nm were obtained with a Waters HPLC system (Milford, MA, USA) consisting of a model 510 pump, an automated gradient controller, a model 2996 photodiode array detector, and Millennium32 software. A Shimadzu LC-2010A^{HT} HPLC system equipped with a Class-VP workstation (Tokyo, Japan) was used for the quantitative determination, and the detection wavelength was set at 348 nm.

Sample Preparation for HPLC Determination. The sample powder (0.2g, passed through a 500 μm mesh sieve) was ultrasonically double-extracted with 20 mL of MeOH/H_2O (75:25) for 20 min once. The mixtures were centrifuged at 3500 rpm for 10 min, and the combined supernatants and washing liquid were evaporated to dryness in vacuo. The residues were dissolved in 20 mL of MeOH/H_2O (75:25) and then filtered through a 0.45 μm membrane filter prior to HPLC analysis.

Primary stock solutions of 16 reference compounds at a final concentration of 0.2 mg/mL were separately prepared by dissolving the accurately weighed reference compounds in MeOH. The stock solutions were then serially diluted until desired concentrations to obtain calibration curves. All of the solutions were stored at 4 °C and were brought to room temperature before use.

For the identification of an unknown compound (peak 11, Figure 1), the sample solution of 'Xiaoyangju' extract was refined with solid phase extraction using a C_{18} cartridge (Varian Bond Elut C_{18}, Varian Co., Ltd., USA). The cartridge was conditioned with 1 mL of methanol and equilibrated with 1 mL of water before the sample was loaded. One milliliter of the sample solution was loaded onto the C_{18} cartridge, and then the cartridge was eluted in turn with 5 mL of water, 5 mL of 10% methanol, 5 mL of 20% methanol, and 10 mL of 30% methanol. The fraction eluted with 30% methanol was evaporated to dryness in vacuo. The residue was reconstituted in 50 μL of methanol, and 20 μL of the solution was injected into the LC-MS system for the identification of peak 11.

Sample Preparation for Bioassay. Seven extracts prepared from five cultivars of *C. morifolium* flowers (samples 1, 3, 4, 5, 7, 9, and 11, Table 4) were used in vitro bioassay experiments. Fifty grams of each sample was refluxed with 750 mL of 75% EtOH for 2 h, and the extract solution was evaporated in vacuo to an adequate concentration. The extract powders were obtained with a freeze-drying method. The yields of these extracts ranged from 20 to 28%. The total contents of five representative phenolic compounds, including CA, LG, L, A, and Ac, in the extracts of different cultivars ranged from 2.04 to 7.13% (Table 4), were determined using the HPLC method as described under Instrumentation and Chromatographic Conditions.

For the in vitro assay, seven extracts, three fractions prepared with the fractionation of 'Huaiju' extract using hexane, ethyl acetate, and *n*-butanol in turn, and Tranilast (used as positive control) were dissolved initially in DMSO and diluted to the tested concentration with Siraganian buffer (containing 119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM PIPES, and 40 mM NaOH, pH 7.2), respectively. Final DMSO concentration was 0.1%, which was not toxic to cells.

For the in vivo experiment on scratching behavior, the extracts of 'Huaiju' and 'Xiaoyangju' (samples 1 and 11, Table 4) were suspended in H_2O at the concentration of 40 mg/mL prior to use.

Measurement of *β*-Hexosaminidase Release in Sensitized RBL-2H3 Cells. The inhibitory activity on *β*-hexosaminidase release from RBL-2H3 cells was measured following the literature method.[39]

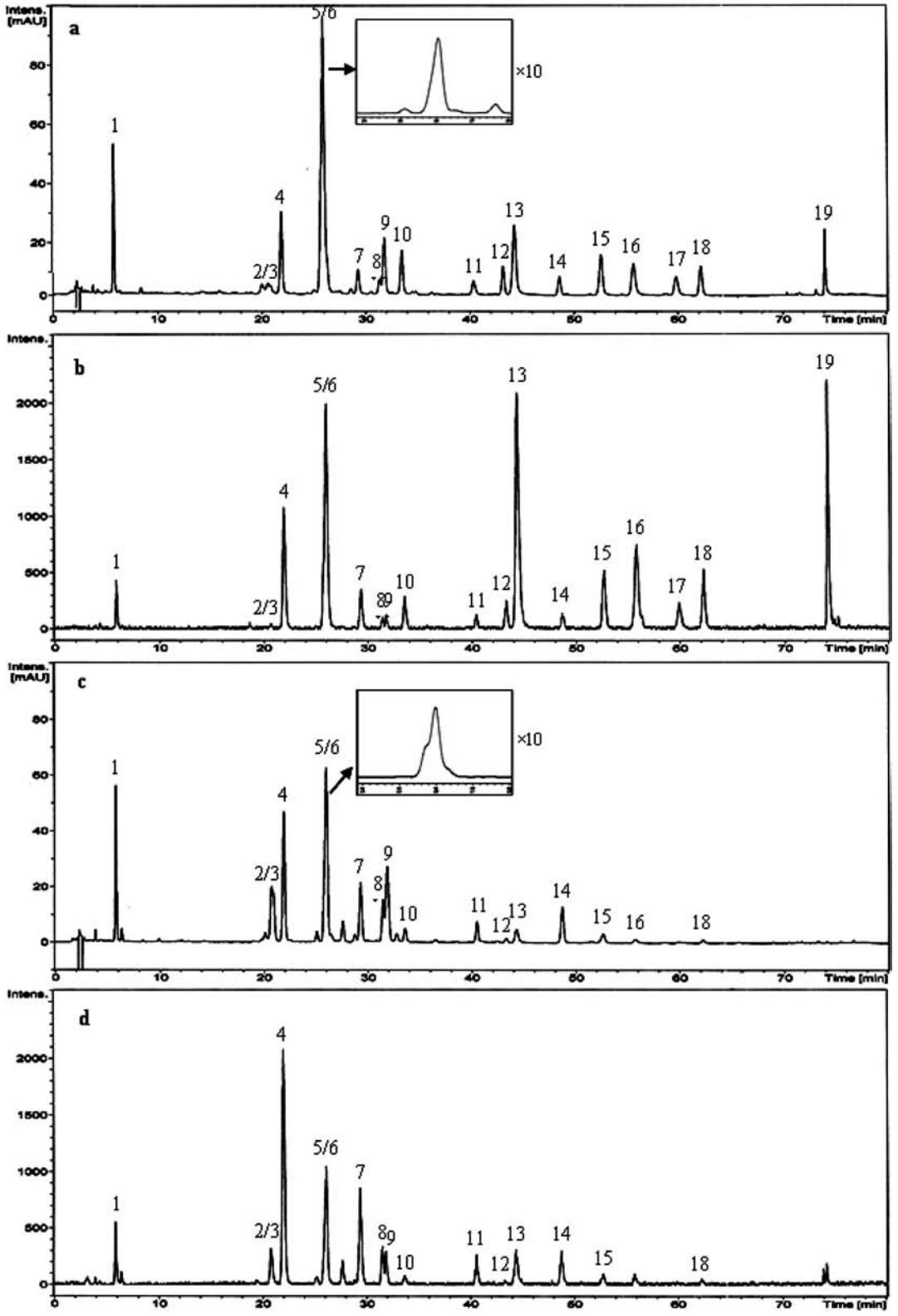


Figure 1. LC-TOF-MS chromatograms of 'Huaiju' (sample 1) and 'Xiaoyangju' (sample 11): (a, c) HPLC chromatograms of the methonolic solutions of 'Huaiju' and 'Xiaoyangju' detected at 348 nm; (b, d) SIM chromatograms of those of 'Huaiju' and 'Xiaoyangju' in negative ionization mode. See Table 1 for the peak numbers and Materials and Methods for HPLC-TOF/MS conditions.

Briefly, RBL-2H3 cells were seeded into a 24-well microplate at the density of 2×10^5 cells/well using MEM containing 10% FCS, penicillin (100 units/mL), and streptomycin (100 μg/mL) and incubated overnight at 37 °C with anti-DNP IgE (0.45 μg/mL) for sensitization of the cells. The cells were then washed twice with Siraganian buffer and incubated in Siraganian buffer supplemented with 5.6 mM glucose, 1 mM $CaCl_2$, and 0.1% BSA for 10 min at 37 °C. Subsequently, 20 μL of the tested sample solution was added and incubated for 10 min, followed by the addition of 20 μL of DNP−BSA (final concentration at 10 μg/mL) as an antigen and incubated for 10 min at 37 °C to evoke degranulation

dx.doi.org/10.1021/jf304080v | J. Agric. Food Chem. 2012, 60, 12574−12583

Table 1. Marker Compounds Identified from the Cultivars of *Chrysanthemum morifolium* Flowers by HPLC-DAD and LC-TOF/MS Methods

peak	identification[a]	t_R (min)	UV (λ_{max}, nm)	$[M-H]^-$ (m/z)	aglycones or diagnostic fragments(m/z)	comparison with standards
1	CA	5.8	243, 300sh, 326	353.1	191, 161	yes
2	L-7GA	20.8	254, 267, 348	461.1	285	no
3	L-7R	21.1	254, 267, 348	593.2	285	yes
4	L-7G	21.9	254, 268, 348	447.1	285	yes
5	3,5-diCQA	26.1	243, 298sh, 327	515.1	353, 191, 161	yes
6	1,3-diC-epi-QA	26.1	243, 298sh, 327	515.1	353, 191, 161	yes
7	A-7G	29.3	267, 337	431.1	269	yes
8	D-7R	31.5	253, 268sh, 348	607.2	299	yes
9	diCQA	31.9	243, 298sh, 327	515.1	353, 191, 161	no
10	D-7G	33.6	252, 268sh, 348	461.1	299	yes
11	A-7-acetyl-H	40.5	267, 338	473.1	431, 269	no
12	D-7-(6″-O-*p*-HPA)G	43.3	252, 267sh, 348	595.1	299	yes
13	L	44.2	252, 267, 345	285.0		yes
14	Ac-7R	48.6	268, 335	591.2	283	yes
15	Ac-7G	52.5	268, 333	445.1	283	yes
16	A	55.7	267, 338	269.1		yes
17	D	59.8	253, 268sh, 348	299.1		yes
18	Ac-7-(6″-acetyl)G	62.2	268, 333	486.2	283	yes
19	Ac	74.0	268, 333	283.1		yes

[a]CA, chlorogenic acid (3-caffeoylquinic acid); L-7GA, luteolin-7-*O*-*β*-D-glucuroside; L-7R, luteolin-7-*O*-*β*-D-rutinose (scolimoside); L-7G, luteolin-7-*O*-*β*-D-glucoside; 3,5-diCQA, 3,5-dicaffeoylquinic acid; 1,3-diC-epi-QA, 1,3-dicaffeoyl-epi-quinic acid; A-7G, apigenin-7-*O*-*β*-D-glucoside; D-7R, diosmetin-7-*O*-*β*-D-rutinose (diosmin); diCQA, dicaffeoylquinic acid; D-7G, diosmetin-7-*O*-*β*-D-glucoside; A-7-acetyl-H, apigenin-7-*O*-(acetyl)-hexoside; D-7-(6″-*O*-*p*-HPA)G, diosmetin-7-(6″-*O*-*p*-hydroxyphenyl-acetyl)-*O*-*β*-D-glucoside; L, luteolin; Ac-7R, acacetin-7-*O*-*β*-D-rutinose (linarin); Ac-7G, acacetin-7-*O*-*β*-D-glucoside; A, apigenin; D, diosmetin; Ac-7-(6″-Ac)G, acacetin-7-(6″-*O*-acecyl)-*β*-D-glucoside; Ac, acacetin.

reaction. The reaction was stopped by cooling in an ice bath for 10 min. The supernatant (50 μL) was transferred into a 96-well microplate and incubated with an equal volume of PNAG (1 mM in 0.1 M citrate buffer, pH 4.5) at 37 °C for 1 h. The reaction was stopped by adding 200 μL of a stop solution (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). The optical density was measured at 405 nm on a microplate reader (model 550, Bio-Rad Laboratories, Hercules, CA, USA). Under these conditions, 40−70% of *β*-hexosaminidase relative to the total amount of cellular *β*-hexosaminidase should be released from the cells in the antigen stimulation control group. The total amount of *β*-hexosaminidase was estimated by ultrasonication of the cell suspension. The inhibitory effects were calculated using the equation

$$\text{inhibition}\,(\%) = [1 - (T - B - N)/(C - N)] \times 100$$

where T is the absorbance of the test group in which both DNP−BSA and test sample were added, B is that of the blank group in which only test sample was added, N is that of the normal group in which neither DNP−BSA nor test sample was added, and C is that of the control group in which only DNP−BSA was added. IC_{50} values for inhibition of *β*-hexosaminidase release were determined graphically from the concentration−response data.

Compound 48/80-Induced Scratching Behavior. Scratching behavior was observed following the method used by Kuraishi et al.[40] To acclimate the animals to experimental environment, they were put individually in the cell of an acrylic cage composed of four cells (13 × 9 × 30 cm) for at least 1 h. The extracts of 'Huaiju' and 'Ziaoyangju' at 50, 100, or 200 mg/kg and terfenadine at 30 mg/kg (dissolved in saline at the concentration of 6 mg/mL) were orally administered to mice 1 h before the stimulation of compound 48/80, respectively. Later, compound 48/80 (10 μg/0.05 mL/site) was intradermally injected into the rostral part of the shaved back of the mice. Immediately after the injection, the mice were put individually into one of the four cells of an acrylic cage (26 × 18 × 30 cm) for 40 min for the observation of scratching behavior. The scratching behaviors were recorded using an 8 mm video camera (CCD-700 V, Sony, Tokyo, Japan) under unmanned conditions, and play-back of the video served for measuring the counts of scratching.

Statistical Analysis. Data are presented as the mean ± SEM of three independent batches of cells in in vitro assay or of more than six animals in in vivo experiments. One-way analysis of variance (ANOVA) followed by Dunnett's test was used for statistical analysis. Probability (p) values of <0.05 were considered to be significant.

RESULTS AND DISCUSSION

HPLC-UV and LC-TOF/MS Identification of 19 Marker Compounds from the Cultivars of *C. morifolium* Flowers. To characterize the major components, the extracts of 'Huaiju' and 'Xiaoyangju' (samples 1 and 11), two representative cultivars of *C. morifolium* flowers, were subjected to HPLC-UV and LC-ESI/MS analysis. Nineteen specific peaks (labeled peaks 1−19, Figure 1) in the HPLC chromatograms were characterized by typical UV absorptions obtained with a Waters PDA detector, with the maximum absorptions at 252−268 and 333−348 nm for flavone or at 243 and 326−327 nm for caffeoylquinic acid derivatives. By comparison of the retention time, UV spectra, and m/z of characteristic molecular ions and fragment ions with those of authentic standards that were isolated in our previous studies (Table 1),[13,31] the compounds corresponding to 16 peaks were identified as CA (peak 1), L-7R (peak 3), L-7G (peak 4), 3,5-diCQA (peak 5), 1,3-diC-epiQA (peak 6), A-7G (peak 7), D-7R (peak 8), D-7G (peak 10), D-7-(6″-*O*-*p*-HPA)G (peak 12), L (peak 13), Ac-7R (peak 14), Ac-7G (peak 15), A (peak 16), D (peak 17), Ac-7-(6″-acetyl)G (peak 18), and Ac (peak 19).

Because of the absence of reference compounds, peaks 2, 9, and 11 were tentatively identified by MS/MS fragment ion assignment together with the characteristic ultraviolet absorption spectra using the previously reported methods.[24] In the HPLC-DAD and ESI-TOF/MS determination of 'xXiaoyangju' extract, peak 2 showed UV absorptions of a flavone at 254, 267(sh), and 348 nm, a negative molecular ion at m/z 461.1 $[M-H]^-$, and a fragment ion at m/z 285.0 (loss of a glucuronyl group, 176 mass units), suggesting that it is a glucuronyl flavone. Acoding to the

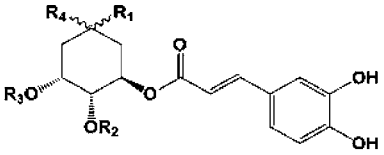
same UV absorbance spectrum of the glucuronyl flavone as that of L-7G together with the same molecular mass of a major fragment ion as that of luteolin, the compound corresponding to peak 2 was tentatively identified as luteolin-7-*O*-*β*-D-glucuroside (L-7GA). Peak 9 showed the same UV absorption spectrum at 243, 298 (sh), and 327 nm as that of CA, and its MS chromatograms exhibited a negative molecular ion at m/z 515.1 $[M-H]^-$, three diagnostic fragment ions at m/z 353 (loss of a caffeoyl group, 162 mass units), 191 (loss of two caffeoyl groups, 324 mass units), and 161 (loss of a caffeoyl quinic acid, 354 mass units). The same fragmentation was also detected in the ESI-TOF/MS determination of its isomers 3,5-diCQA and 1,3-diC-epiQA. The compound corresponding to peak 9, thus, was tentatively identified as a dicaffeoylquinic acid (diCQA). However, the configuration and substituted position of the dicaffeoyl groups could not be confirmed on the basis of the data mentioned above.

The UV absorptions of peak 11 displayed at 267 and 338 nm, but its quasi-molecular and characteristic fragment ions did not clearly display in the LC-TOF/MS determination of 'Xiaoyangju' extract. As a consequence, a fraction mainly containing peak 11 was refined from 'Xiaoyangju' extract with solid phase extraction using a C_{18} cartridge (see Sample Preparation for HPLC Determination under Materials and Methods) and then subjected to LC-MS and LC-TOF/MS determinations for the identification of peak 11. The base ion at m/z 473.1 corresponded to the deprotonated molecule ion $[M-H]^-$ of peak 11. Two characteristic fragment ions at m/z 431.1 and 269 displayed in further MS/MS determination were produced by selecting the base ion as the precursor ion. The compound corresponding to peak 11 is a flavone glycoside containing an acetylated sugar, as evidenced by the loss of an acetyl group (42 mass units) from the base ion to produce its glycoside fragment ion at m/z 431 and the loss of acetyl and hexosyl groups (204 mass units) to produce its aglycone fragment ion at m/z 269. According to the same UV absorbance spectrum as that of A-7G together with the same molecular mass of the aglycone ion as that of A, the compound corresponding to peak 11 was tentatively identified as apigenin-7-*O*-(acetyl)-hexoside (A-7-acetyl-H). However, the structure of the hexosyl group and the substituted position of the acetyl group could not be confirmed in the present study.

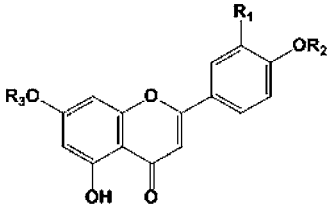
The structures of the identified or tentatively identified compounds are shown in Figure 2, and they were satisfactorily separated under the HPLC-UV condition except for L-7GA (**2**), L-7R (**3**), 3,5-diCQA (**5**), and 1,3-diC-epiQA (**6**). The detection wavelength for quantification of these compounds was chosen as 348 nm by comparison of the chromatograms of sample solutions at different wavelengths.

Method Validation. Good linear calibration curves and the linear range were obtained with 15 tested reference standards (see Table 2). Due to unsatisfactory separation, the total content of L-7GA and L-7R was calculated by the calibration of L-7R and that of 3,5-diCQA and 1,3-diC-epiQA was calculated by the calibration of 3,5-diCQA. Because there were no available reference compounds, the contents of diCQA and A-7-acetyl-H were calculated by the calibration of 3,5-diCQA and A-7G, respectively. The detection limits for 15 marker compounds were ca. 0.51−19.2 ng.

The precision and recovery tests were done for 13 major marker compounds, CA, L-7G, 3,5-diCQA, A-7G, D-7R, D-7G, L, Ac-7R, Ac-7G, A, D, Ac-7-(6″-Ac)-G, and Ac. This method exhibited good reproducibility with intraday variations (RSD) of <1.3% and interday variations (RSD) of <2.1%, respectively. Recovery data were obtained from the comparison of the



Comps.	R_1	R_2	R_3	R_4
chlorogenic acid (**1**)	*β*-COOH	H	H	*α*-OH
3,5-dicaffeoyl-quinic acid (**5**)	*β*-COOH	H	Caf	*α*-OH
1,3-dicaffeoyl-epi-quinic acid (**6**)	*α*-COOH	H	H	*β*-O-Caf



Comps.	R_1	R_2	R_3
luteolin-7-O-*β*-D-glucuroside (**2**)	OH	OH	H
scolimoside (**3**)	OH	OH	glc-rham
luteolin-7-O-*β*-D-glucoside (**4**)	OH	OH	glc
apigenin-7-O-*β*-D-glucoside (**7**)	H	OH	glc
diosmin (**8**)	OH	OCH_3	glc-rham
diosmetin-7-O-*β*-D-glucoside(**10**)	OH	OCH_3	glc
apigenin-7-(acetyl-O)-hexoside (**11**)	H	OH	acetyl-hexoside
diosmetin-7-(6"-O-*p*-hydroxy phenyl-acetyl)-O-*β*-D-glucoside (**12**)	OH	CH_3	6-*p*-hydroxy phenyl-acetyl-glc
luteolin (**13**)	OH	OH	H
linarin (**14**)	H	OCH_3	glc-rham
acacetin-7-O-*β*-D-glucoside (**15**)	H	OCH_3	glc
apigenin (**16**)	H	OH	H
diosmetin (**17**)	OH	CH_3	H
acacetin-7-(6"-acecyl-O)-*β*-D-glucoside (**18**)	H	OCH_3	6-acecyl-glc
acacetin (**19**)	H	CH_3	H

Figure 2. Structures of marker compounds identified in Flos Chrysanthemi.

responses between the test samples and the spiked samples. The recovery for these markers ranged from 98.4 to 101%, with RSD ranging from 1.7 to 2.8% (data not shown). Thus, this analytical procedure is accurate and sufficiently sensitive for the quantitative determination of the major flavones and caffeoylquinic acid derivatives from the cultivars of *C. morifolium* flowers.

Chemical Profiling and Quantification of 19 Components of the Cultivars of *C. morifolium* Flowers. Chemical profiling and quantification of the 19 marker compounds from 5 cultivars of *C. morifolium* flowers with HPLC-UV method were carried out. Figure 3 presents typical HPLC chromatograms of their methanolic extracts.

There is a significant difference in the chemical profiling patterns between the five cultivars. Fifteen components were commonly found in them, including 4 caffeoylquinic acids, CA, 3,5-diCQA, 1,3-diC-epiQA, and DCA, and 10 flavones, L-7G, A-7G, D-7R, D-7G, A-7-(6″-Ac)-G, D-7-(6″-*O*-*p*-HPA)-G, L, Ac-7R, Ac-7G, and A. Two luteolin glycosides, L-7GA and L-7R, and two flavone aglycones, D and Ac, might contribute to chemically distinguish the different cultivars. The HPLC-UV chromatograms of 'Xiaoyangju' and 'Gongju' for both medicinal and edible usage demonstrated a clear detection of L-7GA and L-7R, but no clear detection of D and Ac. On the other hand, in three medicinal *Chrysanthemum* cultivars, 'Huaiju', 'Boju', and 'Chuju', D and Ac were clearly detected, without a clear detection of

Table 2. Calibrations and Detection Limits for Marker Compounds

compound	calibration curve[a]	r	linear range (mg/mL)	LOD[b] (ng)	LLOQ[b] (ng)
CA (**1**)	$Y = 1.458 \times 10^7 x + 3.180 \times 10^4$	0.9998	4.35×10^{-3}–8.70×10^{-2}	0.64	2.13
L-7R (**3**)	$Y = 1.884 \times 10^7 x + 1.831 \times 10^4$	0.9991	1.59×10^{-3}–4.77×10^{-2}	0.51	1.70
L-7G (**4**)	$Y = 2.463 \times 10^7 x - 8.011 \times 10^2$	0.9999	3.60×10^{-3}–7.20×10^{-2}	1.30	4.32
3,5-diCQA (**5**)	$Y = 1.261 \times 10^8 x - 1.651 \times 10^5$	0.9994	3.70×10^{-2}–7.40×10^{-1}	18.8	62.7
A-7G (**7**)	$Y = 1.725 \times 10^7 x - 5.994 \times 10^4$	0.9996	6.75×10^{-3}–1.35×10^{-1}	12.9	43.1
D-7R (**8**)	$Y = 1.431 \times 10^7 x - 1.249 \times 10^4$	0.9997	5.46×10^{-3}–1.09×10^{-1}	4.20	14.0
D-7G (**10**)	$Y = 1.763 \times 10^7 x + 4.934 \times 10^3$	0.9995	2.00×10^{-3}–4.00×10^{-2}	1.61	5.36
D-7-(6″-O-*p*-HPA)G (**12**)	$Y = 1.367 \times 10^7 x + 4.934 \times 10^3$	0.9995	2.58×10^{-3}–5.16×10^{-2}	2.07	6.92
L (**13**)	$Y = 2.954 \times 10^7 x - 2.936 \times 10^4$	0.9995	3.05×10^{-3}–6.10×10^{-2}	9.63	32.1
Ac-7R (**14**)	$Y = 2.388 \times 10^7 x - 2.882 \times 10^3$	0.9986	3.00×10^{-3}–6.00×10^{-2}	2.16	7.19
Ac-7G (**15**)	$Y = 1.529 \times 10^7 x - 3.425 \times 10^4$	1.000	5.17×10^{-3}– 1.03×10^{-2}	16.6	55.2
A (**16**)	$Y = 2.273 \times 10^7 x - 1.619 \times 10^4$	0.9995	3.50×10^{-3}–7.00×10^{-2}	15.3	51.1
D (**17**)	$Y = 1.674 \times 10^7 x - 6.208 \times 10^3$	0.9995	1.67×10^{-3}–3.33×10^{-2}	19.2	64.0
Ac-(acycel)G (**18**)	$Y = 1.600 \times 10^7 x - 1.919 \times 10^4$	0.9998	2.82×10^{-3}–5.64×10^{-2}	18.1	60.4
Ac (**19**)	$Y = 1.866 \times 10^7 x - 2.750 \times 10^4$	0.9992	2.50×10^{-3}–5.00×10^{-2}	5.11	17.0

[a] Y and X are, respectively, the peak areas and concentrations (mg/mL) of the analytes. [b] The LLOQ was defined as the concentration that could be detected at a signal-to-noise ratio of 10 and the LOD was defined as the concentration that could be detected at a signal-to-noise ratio of 3.

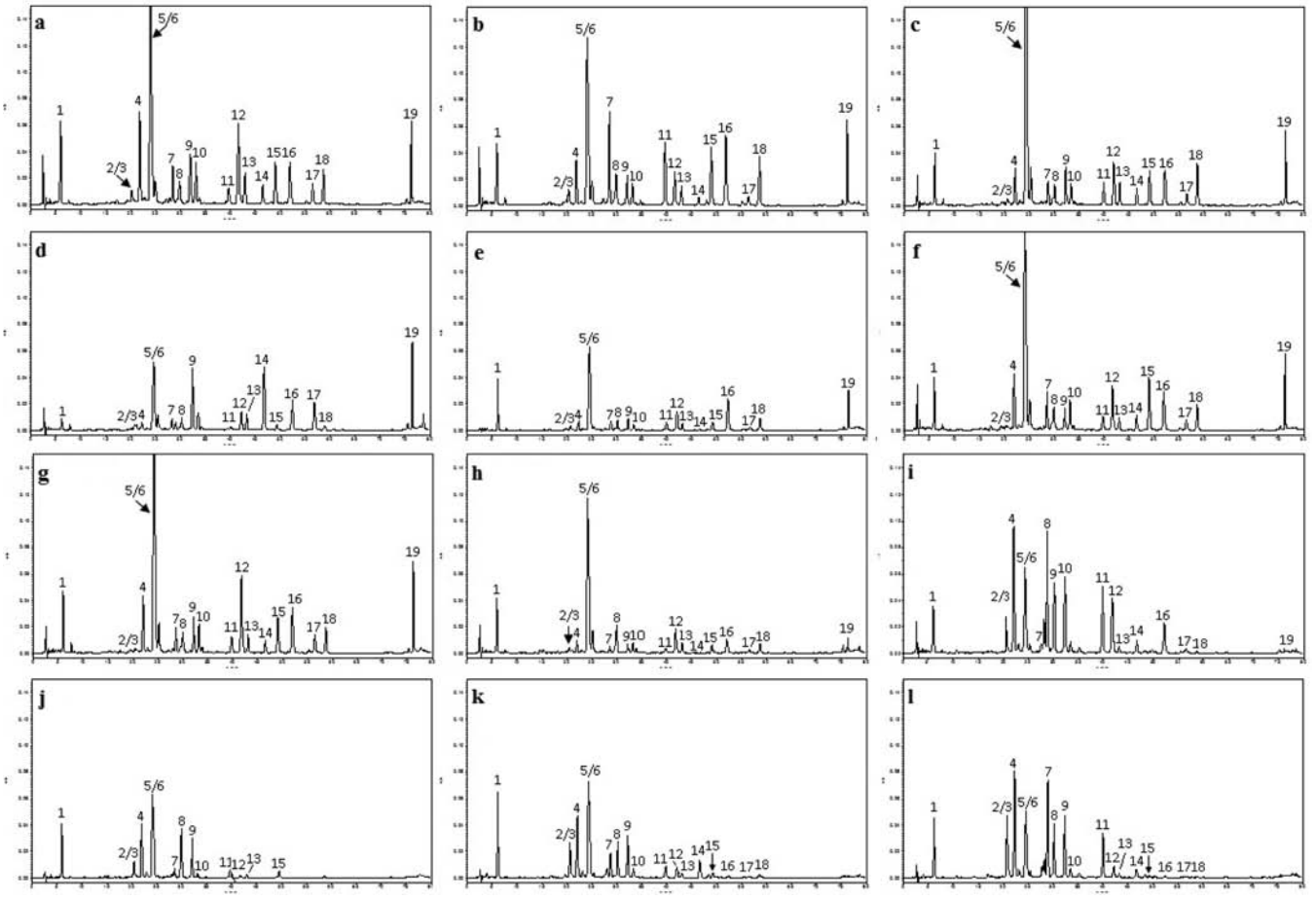


Figure 3. HPLC chromatograms of methanolic solutions of five cultivars of *Chrysanthemum morifolium* flowers: (a, b) 'Huaiju' (samples 1, 2); (c–f) 'Boju' (samples 3–6); (g, h) 'Chuju' (samples 7, 8); (i, j) 'Gongju' (samples 9, 10); (k, l) 'Xiaoyangju' (samples 11, 12). See Table 1 for the peak numbers, see Materials and Methods for HPLC-MS conditions, and see Table 3 for plant material.

L-7GA and L-7R. Thus, the comprehensive HPLC-UV profiles displayed in our present study may serve for the chemical characterization of three medicinal cultivars and two edible ones.

The contents of 19 compounds in 12 samples originating from the 5 cultivars were quantitatively determined (Table 3). The results also clearly indicated significant differences in the quality and quantity of the luteolin glycosides and the flavone aglycones between two edible cultivars and three medicinal cultivars.

In Vitro and in Vivo Antiallergic Effects of the Cultivars of *C. morifolium* Flowers. RBL-2H3 cells were used to assess in vitro antiallergic activity. The optimal release of β-hexosaminidase from RBL-2H3 cells was achieved by stimulation with 45 μg/mL

Table 3. Content (Milligrams per Gram) of 19 Marker Components in the Cultivars of *Chrysanthemum morifolium* Flowers[a]

		caffeoylquinic acids			flavone glycosides									flavone aglycones						
cultivar (commercial name)	collection region/date	1	5 + 6	T_{Caf}	2 + 3	4	7	9	10	13	14	15	18	T_{FG}	12	16	17	19	T_{FA}	T_A
1 'Huaiju' (Huai Ju)	Henan/Dec 2002	7.01	39.6	46.6	1.49	5.33	3.33	5.96	3.33	3.24	1.24	4.78	3.44	32.2	4.67	3.44	2.17	3.23	13.5	92.3
2 'Huaiju' (Huai Ju)	Henan/Sept 2004	5.61	28.2	33.8	1.37	2.42	7.72	3.24	1.74	1.84	0.52	6.55	4.71	30.1	2.00	5.54	0.97	3.29	11.8	75.8
3 'Boju' (Qi Ju)	Hebei/Nov 2003	3.89	41.2	45.1	0.57	1.94	2.18	4.11	1.69	2.23	1.10	4.04	4.11	22.0	2.53	2.86	1.29	2.82	9.51	76.6
4 'Boju' (Ji Ju)	Shandong/Oct 2003	0.61	12.6	13.2	0.35	0.48	0.90	5.87	1.32	1.59	3.97	0.76	0.56	15.8	1.12	2.39	2.85	3.20	9.56	38.6
5 'Boju' (Bo Ju)	Anhui/Dec 2002	2.42	13.9	16.3	0.17	0.57	1.03	1.08	0.37	0.67	nd	1.02	1.22	6.13	1.21	2.57	0.40	1.56	5.74	28.2
6 'Boju' (Bo Ju)	Anhui/Sept 2004	4.54	38.1	42.6	nd	3.12	3.30	2.58	2.36	1.32	0.92	5.92	2.55	22.1	2.61	3.04	1.14	2.96	9.75	74.4
7 'Chuju' (Chu Ju)	Anhui/Dec 2003	4.57	37.9	42.5	nd	2.91	2.29	3.72	2.27	1.77	0.77	4.04	2.50	20.3	4.52	3.63	2.01	3.45	13.6	76.4
8 'Chuju' (Chu Ju)	Anhui/Dec 2004	3.46	23.9	27.3	0.35	0.56	0.83	0.92	0.74	0.89	nd	0.96	0.93	6.19	1.47	1.13	0.33	0.62	3.56	37.1
9 'Gongju' (Gong Ju)	Anhui/Dec 2002	4.24	13.4	17.6	3.75	6.89	10.0	8.39	0.87	0.67	0.77	tr	0.27	31.6	3.21	2.50	0.67	nd	6.38	55.6
10 'Gongju' (Gong Ju)	Anhui/Sept 2004	3.17	13.9	17.1	1.09	2.67	1.11	3.64	0.26	0.29	n.d.	0.76	0.28	10.1	tr	nd	tr	nd	tr	27.2
11 'Xiaoyangju' (Hangbai Ju)	Zhejiang/Oct 2002	5.08	15.0	20.1	2.66	3.68	2.29	5.01	0.66	0.43	1.12	0.67	0.40	25.1	0.66	tr	tr	nd	0.66	45.9
12 'Xiaoyangju' (Hangbai Ju)	Jiangsu/Nov 2004	4.08	11.0	15.1	4.70	6.16	7.87	6.48	0.65	0.40	0.51	0.33	tr	27.1	0.76	tr	tr	nd	0.76	42.9

[a]All of the cultivar samples are botanically from *Chrysanthemum morifolium* Ramat. T_{Caf}, T_{FG}, T_{FA} represent the sum quantities of caffeoylquinic acids, flavone glycosides and flavone aglycones, respectively. nd, not detectable (< limit of detection). tr, trace (> limit of detection but < minimum of the linear range).

antigen for 10 min (data not shown), which is consistent with our previous results.[39] The inhibitory activity of the extracts of the five cultivars of *C. morifolium* flowers on antigen-induced degranulation in RBL-2H3 cells was determined (Table 4). The extracts of 'Huaiju' (sample 1) and 'Boju' (samples 3 and 5) substantially inhibited β-hexosaminidase release from RBL-2H3 cells stimulated by DNP−BSA antigen, with their IC_{50} values being 63, 70, and 68 μg/mL, respectively. The extracts of 'Xiaoyangju' (samples 8 and 11), 'Gongju' (sample 9), and 'Chuju' (sample 7) showed weak inhibition for the degranulation, with their IC_{50} values being >100 μg/mL. Furthermore, three fractions prepared with the polarity-based fractionations of 'Huaiju' extract were assayed. Among them, the ethyl acetate fraction showed the highest antiallergic activity (IC_{50} = 10.1 μg/mL), which contains mainly flavone components according to our HPLC analysis (data not shown). In a parallel experiment, luteolin, an active flavone widely present in *Chrysanthemum* sp., significantly suppressed DNP−BSA antigen-induced degranulation from RBL-2H3 cells (IC_{50} = 2.8 μM), which was consistent with previous literature.[27] The results suggest that two cultivars, 'Huaiju' and 'Boju', exhibited potent antiallergic activity in vitro.

The in vivo antiallergic activity of two representative cultivars, 'hHuaiju' and 'Xiaoyangju', was assayed using compound 48/80-induced scratching animal model. It was demonstrated that an intradermal injection of compound 48/80 (10 μg/site) evoked scratching of the skin around the injection site by the hind paws within 5 min and that the effect peaked during the initial 10 min period (Figure S1, Supporting Information), which was consistent with the previous literature.[40] The scratching behavior was measured in 40 min after the compound 48/80 injection because no obvious itching had been observed since then. The scratching induced by compound 48/80 was dose-dependently inhibited by the pretreatment of the 'Huaiju' extract, with significant inhibition displayed at doses of 50, 100, and 200 mg/kg. The 'Xiaoyangju' extract showed a significant inhibition of the scratching behaviors only at the highest dose of 200 mg/kg. Terfenadine, an inhibitor of H1-receptor antagonist, showed a significant inhibitory effect at the dose of 30 mg/kg, which was consistent with the previous literature (Figure 4).[41]

In the present study, a total of 19 compounds present in 5 cultivars of *C. morifolium* flowers were characterized by HPLC-DAD and LC-TOF/MS methods. Of these, 15 common components were obtained in these cultivars. Thus, the difference in the flavone pattern between the medicinal and edible cultivars is of a little interest.

There have been several reports on HPLC fingerprints of unauthenticated cultivars of *C. morifolium* flowers in China, in which only some common components such as chlorogenic acid, luteolin, quercetin, apigenin, 3′,5′,5-trihydroxyflavanone 7-*O*-β-D-glucopyranoside, luteolin 7-*O*-β-D-glucopyranoside, apigenin 7-*O*-β-D-glucopyranoside, diosmetin 7-*O*-β-D-glucopyranoside, linarin, and acacetin 7-*O*-β-D-glucopyranoside were identified and quantified.[20,25] In addition, an LC-DAD-ESI/MS method was successfully used to identify 46 flavonoids and 17 caffeic acid derivatives from the authenticated cultivars of *C. morifolium* flowers,[23] in which study many compounds were present in trace or small amount, and both major components D-7-(6″-*O*-*p*-HPA)-G and Ac-7-(6″-acetyl)-G were not identified. The present study focused on the major and characteristic compounds that could be used to chemically characterize the similarity and distinctness between the different cultivars, serving for their quality control and biological assessment.

Table 4. Inhibitory Effects of Five Cultivars of *Chrysanthemum morifolium* Flowers on the Release of β-Hexosaminidase from RBL-2H3 Cells Stimulated by Antigen

	inhibition[a] (%) at						
sample[b] (plant material)	0 μg/mL	10 μg/mL	30 μg/mL	60 μg/mL	100 μg/mL	IC_{50} (μg/mL)	sum content[c] (mg/g)
'Huaiju' (1) extract	0.0 ± 6.7	7.6 ± 3.9	21.9 ± 3.6**	47.8 ± 4.1**	66.9 ± 2.6**	63	71.3
n-hexane fraction	0.0 ± 1.3	−2.3 ± 1.7	2.8 ± 2.6**	6.9 ± 1.5*	39.2 ± 1.7**	>100	
EtOAc fraction	0.0 ± 2.9	43.0 ± 1.6	88.8 ± 1.0**	99.9 ± 0.4**	101.0 ± 0.8**	10	
n-BuOH fraction	0.0 ± 1.4	3.3 ± 1.1	7.8 ± 1.6**	13.2 ± 1.4**	25.7 ± 1.4**	>100	
H_2O fraction	0.0 ± 1.3	−1.9 ± 2.6	1.5 ± 3.3	−4.4 ± 2.8	3.0 ± 1.4	>100	
'Boju' (3) extract	0.0 ± 8.0	9.0 ± 3.3	24.2 ± 5.7*	44.8 ± 6.0**	65.3 ± 2.6**	70	40.6
'Boju' (4) extract	0.0 ± 5.4	10.2 ± 4.0	16.6 ± 4.7	42.6 ± 4.8**	53.8 ± 1.7**	97	20.4
'Boju' (5) extract	0.0 ± 7.7	0.0 ± 9.8	36.7 ± 8.8**	44.7 ± 5.0**	56.7 ± 2.2**	68	22.8
'Chuju' (7) extract	0.0 ± 5.1	−12.8 ± 2.8	5.7 ± 3.4	9.7 ± 4.1	20.0 ± 4.4**	>100	49.7
'Gongju' (9) extract	0.0 ± 4.9	−18.4 ± 3.8	15.7 ± 4.4**	25.5 ± 2.8**	42.8 ± 3.2**	>100	50.6
'Xiaoyangju' (11) extract	0.0 ± 2.2	1.9 ± 2.8	−0.3 ± 3.2	−1.0 ± 2.6	1.2 ± 2.2	>100	31.6

	inhibition (%) at					
	0 μM	1 μM	3 μM	10 μM	30 μM	IC_{50}
luteolin	0.0 ± 0.6	9.8 ± 1.9**	54.1 ± 2.4**	97.4 ± 0.7**	100.0 ± 1.0**	2.8 μM

[a]Sensitized RBL-2H3 cells were incubated with samples for 10 min followed by stimulation by DNP−BSA for 10 min. The inhibition (%) of the release of β-hexosaminidase was determined, and IC_{50} values were determined graphically. Each value was expressed as the mean ± SEM (n = 4). Statistical significance was tested by a one-way analysis of variance (ANOVA) followed by Dunnett's test for in vitro assay. *, $p < 0.05$;, and **, $p < 0.01$, vs control group (0 μg/mL). [b]The plant materials for the preparation of these extracts were described under Preparation of Chrysanthemum Extracts for in Vitro and in Vivo Assay. For plant material, see Table 3. [c]The sum content refers to the total contents of five phenolic compounds, including CA, LG, L, A, and Ac, in different cultivars.

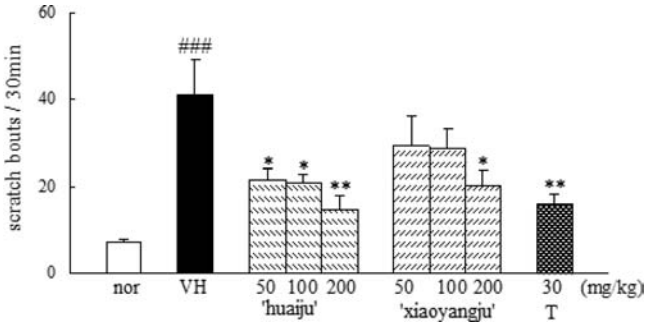


Figure 4. Effects of 'Huaiju' and 'Xiaoyangju' extracts on scratching induced by compound 48/80 in mice. The 'Huaiju' and 'Xiaoyangju' extracts and saline (VL) were administered orally 60 min before the intradermal injection of compound 48/80 (10 ng/site). To normal group mouse (nor), only saline was orally given, and successively intradermally injected. Tefenate (T) was used as positive control. Values are the mean ± SEM (n = 6−8). ###, $p < 0.001$ as compared with the normal group; *, $p < 0.05$, and **, $p < 0.01$, as compared with the vehicle control (VH) by Dunnett's test.

The pharmacological effects of *C. morifolium* flower have been well studied except for the antiallergic effect. Because it has been traditionally used to treat eye redness and itching,[42] we comparatively investigated the antiallergic effect of its five cultivars widely available in China. The cultivars 'Huaiju' and 'Boju' demonstrated potent inhibition of antigen-induced degranulation in RBL-2H3 cells, of which, 'Huaiju' possessing the strongest potency is the earliest medicinal cultivar. On the other hand, the cultivars 'Xiaoyangju', 'Gongju', and 'Chuju' showed weak or little inhibitory activity in vitro. Chemically, these cultivars mainly contain caffeoylquinic acids and flavonoid aglycones and glycosides. Several studies indicated that some flavonoid aglycones, such as luteolin, apigenin, diosmetin, and quercetin, potently inhibited antigen-induced degranulation and TNF-α and IL-4 production from RBL-2H3 cells[27] and that the related glycosides as well as chlorogenic acid possessed weak or little inhibitory potency. Thus, the weakness of in vitro activities of 'Xiaoyangju', 'Gongju', and 'Chuju' should be ascribed to the low content or absence of active flavone aglycones, such as apigenin, diosmetin, and acacetin (see Table 3).

Flavonoid-*O*-glycosides would be metabolized into the related aglycones by microbial hydrolysis in the gastrointestinal tract after oral administration.[43,44] Thus, the cultivars possessing high content of flavonoid-*O*-glycosides but low in flavone aglycones, may exert antiallergic effects in vivo by oral administration. Our result indicated that the pretreatment of mice with the 'Huaiju' extract produced a significant inhibition of compound 48/80-evoked scratching behaviors in a dose-dependent manner (Figure 4). The 'Xiaoyangju' extract without significant in vitro antiallergic activity showed a significant inhibition only at the high dose of 200 mg/kg. The topical application of luteolin produced potent effects on histamine-, serotonin-, and compound 48/80-evoked scratching behavior in mice, which are mainly due to its inhibition of mediator release from activated mast cells and direct antagonist effects on the released mediators, which may act as local pruritogens.[45] The attenuation of exacerbation of atopic dermatitis AD-like symptoms by apigenin in NC/Nga mice was attributed in part to the reduction of serum IgE level and IFN-γ expression.[46] In addition, caffeic acid, a major metabolite of chlorogenic acid,[47] significantly inhibited scratching behavior and vascular permeability changes induced by compound 48/80 in mice only at an oral dose of 500 mg/kg.[48] Thus, the in vivo antiallergic effects of both *Chrysanthemum* cultivars should be ascribed mainly to flavonoid aglycones and glycosides as well as chlorogenic acid. The difference in inhibitory potency between both cultivars should be related to the difference in quality and quantity of some active flavonoid aglycones. Thus, a relationship between the chemical composition and antiallergic properties for different *Chrysanthemum* cultivars has been partly demonstrated. However, for the quality control of Flos Chrysanthemi, the similarities of the chemical fingerprints of five cultivars of *C. morifolium* flowers should be concerned. Of course, a reliable fingerprint of plant herbs should be established on the basis of analysis of a large number of samples,

which is a limitation in the present study, and needs to be studied in the near future.

In summary, the present study developed an HPLC-DAD-ESI/MS method for the chemical characterization of five cultivars of *C. morifolium* flowers. The difference in chemical profiles between medicinal and edible cultivars is helpful for the standardization and quality control of plant materials of Flos Chrysanthemi. Moreover, a medicinal cultivar, 'Huaiju', possesses potential antiallergic activity in vitro and in vivo. The difference in antiallergic activity between medicinal and edible cultivars might be ascribed to the variations of flavonoid aglycone profiles.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone/fax: +86-24-2398-6502. E-mail: yuandan_kampo@163.com.

Funding

This study was supported by the Program for Excellent Talents in Liaoning Province University (2007R45) and Building Program of Liaoning Provincial Key Laboratory (20060857), China.

Notes

The authors declare no competing financial interest.

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