Journal of Enzyme Inhibition and Medicinal Chemistry, June 2009; 24(3): 676–679

Calpain inhibitory flavonoids isolated from *Orostachys japonicus*

CHOONG JE MA[1,2], WON JOO JUNG[1], KI YONG LEE[1], YOUNG CHOONG KIM[1], & SANG HYUN SUNG[1]

[1]*College of Pharmacy and Research Institute of Pharmaceutical Science, Seoul National University, Seoul 151-742, Republic of Korea, and* [2]*School of Bioscience and Biotechnology, Kangwon National University, Chuncheon 200-701, Republic of Korea*

(*Received 11 March 2008; accepted 20 May 2008*)

Abstract

The *n*-butanol (*n*-BuOH) fraction of *Orostachys japonicus* A. Berger (Crassulaceae) significantly inhibited calpain activity. Through the activity-guided isolation from the *n*-BuOH fraction, herbacetin 8-*O*-α-D-ribopyranoside (**1**), kaempferol (**2**), quercetin (**3**), afzelin (**4**), astragalin (**5**), isoquercetin (**6**) and quercitrin (**7**) were obtained. Their structures were determined by spectroscopic techniques. Among them, compound **3** and **5** had significant calpain inhibitory activities.

Keywords: Orostachys japonicus, *flavonoid, calpain, neuroprotection, inhibition*

Introduction

Calcium initiates major changes in cell architecture and function during development and adult plasticity, but it may trigger a regressive event, such as irreversible degeneration when its level exceeds a certain threshold. Changes in calcium levels are executed through an array of calcium-dependent enzymes such as calpain, protein kinase C, calcium and calmodulin-dependent protein kinase, calcineurin and calcium ATPase [1]. Among them, calpain is a cytosolic cysteine protease that requires calcium for its activation. Calpain is over-activated in case of stroke, Alzheimer's disease (AD), muscular dystrophy and cataract. Therefore, calpain inhibitors can be candidates of treatment of them [2–4]. In the course of searching for calpain inhibitors from natural products, the methanolic extract of *Orostachys japonicus* A. Berger (Crassulaceae) showed a significant inhibitory effect. The *O. japonicus* is a perennial herb, which is found fairly ubiquitously in Korea, China, and Japan. Dried whole plants of this species have been used as a Chinese crude drug for the treatment of fever, hemostasis, hepatitis, arthritis, eczema and intoxication and have also been used in folk medicine as an anti-cancer agent [5]. Many flavonoids, phenolic acids, triterpenoids and sterols have all been previously reported as components of *O. japonicus* [6]. We previously reported that gossypetin 8-*O*-α-D-lyxopyranoside, a novel flavonol lyxoside was isolated from *O. japonicus* through the phytochemical study [7]. In addition to this flavonoid we attempted to isolate more calpain inhibitory compounds from the *n*-butanol (*n*-BuOH) fraction of methanolic extract. As a result, we isolated and identified a new flavonoid, herbacetin 8-*O*-α-D-ribopyranoside (**1**), and six known compounds. The six known compounds were identified as kaempferol (**2**), quercetin (**3**), afzelin (**4**), astragalin (**5**), isoquercetin (**6**) and quercitrin (**7**).

Materials and methods

General experimental procedures

The ^{1}H- and ^{13}C-NMR measurements were carried out in a Bruker AMX 400 spectrometer operating at 400 and 100 MHz, respectively. TMS or solvent

Correspondence: S. H. Sung, College of Pharmacy and Research Institute of Pharmaceutical Science, Seoul National University, San56-1, Shilim-Dong, Gwanak-Gu, Seoul 151-742, Republic of Korea. Tel: 82 2 880 7859. Fax: 82 2 877 7859. E-mail: shsung@snu.ac.kr

ISSN 1475-6366 print/ISSN 1475-6374 online © 2009 Informa UK Ltd.
DOI: 10.1080/14756360802328075

signals were used as internal standard. FT-IR spectra were recorded on a Perkin-Elmer 1710 spectrophotometer. UV spectra were recorded on a Shimadzu UV-2100 spectrophotometer. FABMS spectra were obtained on a VG Trio II spectrometer. Column chromatography was performed on Merck (9025) silica gel 60 (0.04-0.063 mm). Analytical TLC was performed on precoated Merck F_{254} silica gel plates and visualized by spraying with anisaldehyde-H_2SO_4. An HPLC system (Hitachi L-6200, Japan) equipped with a UV-visible detector and Microsorb C_{18} semi-preparative column (Rainin Inst. Co.) was used for isolation. A Hewlett-Packard model HP 5985 series II GC system equipped with a flame ionization detector and Ultra-2 capillary column (25 m × 0.32 mm) was used for sugar detection after acid hydrolysis.

Plant materials

Aerial part of *O. japonicus* were purchased from Kyoungdong Oriental medicine Market, Seoul, Korea and identified by the late Dr. Dae S. Han, an emeritus professor of the College of Pharmacy, Seoul National University. Voucher specimen has been deposited in the Herbarium of Medicinal Plant Garden, College of Pharmacy, Seoul National University.

Extraction and isolation

The air-dried and powdered aerial part (10 kg) of *O. japonicus* was extracted with 80% methanol (MeOH) in an ultrasonic apparatus which, upon removal of the solvent *in vacuo*, yielded a methanolic extract (600 g). The MeOH extract was then suspended in distilled water and partitioned successively with CH_2Cl_2 and *n*-BuOH. The *n*-BuOH fraction (205 g) was subjected to column chromatography over Lichoprep RP-18 (600 g, 10 × 80 cm) and eluted with H_2O, H_2O-MeOH and MeOH to six fractions (B1-B6). The H_2O-MeOH (4:1) fraction (B2) was applied on silica gel column (500 g, 8 × 60 cm) using CH_2Cl_2-MeOH-H_2O and yielded to 15 subfractions (B2-1 to B2-15). Following Sephadex LH-20 column (100 g, 3 × 40 cm) chromatography of B2-5 with ethanol yielded compound **1** (4 mg). To determine sugar composition of compound **1**, we conducted acid hydrolysis of compound **1**. A methanolic solution of **1** (2.2 mg) in 10% HCl (3.5 mL) was heated at 100°C for 100 min. After the addition of NH_4OH, the reaction mixture was evaporated to dryness. This was suspended in H_2O and fractioned with EtOAc. The aqueous fraction was subjected to GC analysis with a sugar standard. For the analysis by GC, an equal amount of aqueous fraction and of a silylating agent (Trans-Sil BSA in DMF, Pierce Chemicals, Rocford, IL, USA) were vortexed and incubated at 50°C for 1 h. The column temperature was initiated at 150°C for 1 min and then increased by the rate of 5°C/min. Other GC conditions were as follows: injector temperature of 200°C, detector temperature of 280°C and a flow rate of 3.7 mL/min using He as a carrier gas. Compounds **2** (98 mg) and **3** (118 mg) were obtained as crystals from B2-2 and B2-3, respectively. Compounds **4** (2 mg) and **5** (589 mg) were isolated from B2-6 and compounds **6** (7 mg) and **7** (5 mg) were isolated from B2-9 through silica gel (120 g, 5 × 55 cm) chromatography. The solvent systems CH_2Cl_2-MeOH-H_2O (5:1:1 to 6:5:1) and CH_2Cl_2-MeOH-H_2O (70:18:12 to 6:5:1) were used in purification for compounds **4** to **7**, respectively.

Herbacetin 8-O-α-D-ribopyranoside (**1**). Yellow powder. UV (MeOH) λ_{max}: 271, 287sh, 325, 337sh, 369; + $AlCl_3$ 271, 322sh, 356, 383sh, 430; + $AlCl_3$/HCl 272, 319sh, 355, 379sh, 430; + NaOH 283, 359sh, 431; + NaOAc 278, 292sh, 316, 349sh, 387; + NaOAc/H_3BO_3 271, 295sh, 319, 340sh, 373 nm. IR (KBr) ν_{max}: 3385, 2935, 1650, 1599, 1455, 1373, 1180, 974, 934 cm^{-1}. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.45 (1H, dd, J = 1.7, 7.3 Hz, H-5″), 3.58 (1H, dd, J = 3.1, 7.6 Hz, H-3″), 3.73 (1H, br s, H-4″), 3.80 (1H, dd, J = 1.5, 5.3 Hz, H-2″), 3.84 (1H, d, J = 4.9 Hz, H-5″), 4.83 (1H, d, J = 5.5 Hz, H-1″), 6.26 (1H, s, H-6), 6.91 (2H, d, J = 8.8 Hz, H-3′, H-5′), 8.23 (2H, d, J = 8.8 Hz, H-2′, 6′), 12.27 (1H, br s, 5-OH) ppm, ^{13}C-NMR: see *Table I*. Positive FABMS: 435 $[M + H]^+$, 303 $[M + H\text{-}132]^+$.

Calpain activity assay

To evaluate calpain activity, reaction mixtures containing 50 mM Tris-HCl (pH 7.4), 100 μM EGTA, 0.2% casein, 10 mM dithiothreitol, 0.1 unit

Table I. Comparison of ^{13}C-NMR data of compound **1** and herbacetin (solvent; DMSO-d_6).

C	Compound **1**	Herbacetin [12]
2	147.2	145.0
3	136.1	135.5
4	176.3	176.3
5	156.5	152.4
6	98.8	98.2
7	157.4	152.9
8	124.4	124.8
9	148.7	146.7
10	103.2	102.9
1′	122.2	122.1
2′	130.2	129.8
3′	116.7	115.5
4′	159.6	159.3
5′	116.7	115.5
6′	130.2	129.8
1″	105.1	
2″	72.1	
3″	71.0	
4″	66.7	
5″	65.2	

calpain, 4 mM calcium chloride incubated at 25°C for 30 min. The reaction terminated by adds of 5% TCA and cooling on ice for 10 min. And reaction mixtures centrifuged at 4000 × g for 5 min. UV absorbance of the supernatant of reaction mixtures was measured at 278 nm [8]. Leupeptin (*N*-acetyl-L-leucyl-L-leucyl-L-argininal), an inhibitor of calpain was used for a positive control of calpain assay.

Statistical analysis

Data are expressed as the percentage protection relative to control cultures. Data were evaluated for statistical significance by "ANOVA" test using a computerized statistical package. The confidence level for statistical significance was set at a probability value of 0.05.

Results and discussion

The methanolic extract of *O. japonicus* exhibited a significant calpain inhibitory activity. The methanolic extract was suspended in H_2O and partitioned successively with CH_2Cl_2 and *n*-BuOH. At a concentration of 100 μg/ml, calpain inhibitory activities of CH_2Cl_2, *n*-BuOH and H_2O fraction were 24.6, 49.4 and 18.9%, respectively. *n*-BuOH fraction showed most potent calpain inhibitory activity. Further fractionation and separation of the *n*-BuOH fraction by several chromatographic methods yielded six fractions (B1-B6). As shown in Figure 1, B2 fraction (100 μg/ml) effectively inhibited calpain activity and were used for the isolation of active compounds to yield seven flavonoids. Spectral data of compounds **2**-**7** matched those of kaempferol (**2**), quercetin (**3**), afzelin (**4**), astragalin (**5**), isoquercetin (**6**) and quercitrin (**7**) (Figure 2) [9–11].

Compound **1** was obtained as yellow powders. The positive fast atom bombardment ionization mass spectroscopy (FABMS) exhibited a quasimolecular ion at *m/z* 435 $[M + H]^+$ and a fragmentation ion at *m/z* 303 $[M + H\text{-}132]^+$, which is in accord with the molecular formula $C_{20}H_{18}O_{11}$ and was further corroborated by the ^{13}C-NMR spectrum (20 carbon atoms). It contained a hydroxyl group at position 4 of B ring as established by the presence of four protons (δ 8.23 (2H, d, $\mathcal{J} = 8.8$ Hz) and δ 6.91 (2H, d, $\mathcal{J} = 8.8$ Hz)). A singlet signal at δ 6.26 was attributed to the C-6 proton. This peak showed 8-hydroxy-kaempferol, herbacetin containing three hydroxyl groups at 5, 7 and 8 position as an aglycone [12]. According to FABMS and ^{13}C-NMR data, we assumed the ribopyranoside moiety in compound **1**. Acid hydrolysis of compound **1** yielded D-ribose confirmed by co-gas chromatography with authentic standard. The site of glycosylation was determined by HMBC correlation of carbon signal at δ 124.4 (C-8) and proton signal at δ 4.83 (H-1″) in the HMBC spectrum (Figure 3). A proton doublet signal (δ 4.83 (1H, d, $\mathcal{J} = 5.5$ Hz)) was assigned to a ribopyranosyl anomeric proton. The configuration of the anomeric center of the ribopyranoside in compound **1** was determined to be a from the presence of anomeric carbon signal δ104.8 in its ^{13}C-NMR spectrum and also from the coupling constant ($\mathcal{J} = 5.5$ Hz) for the anomeric proton signal in its ^{1}H-NMR spectrum [13]. From the spectroscopic data above, the structure of compound **1** was concluded to be herbacetin 8-*O*-α-D-ribopyranoside.

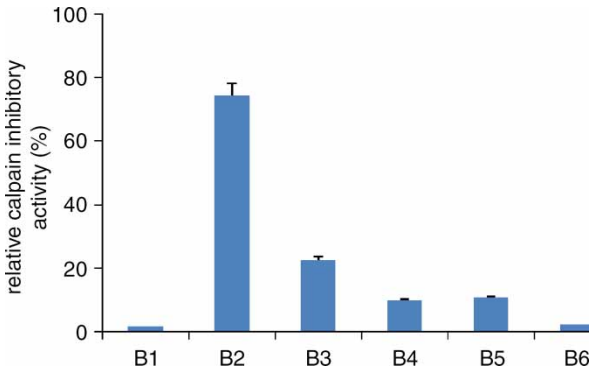

Figure 1. Calpain inhibitory activity of subfractions (B1-B6, 100 μg/ml) of *n*-BuOH fraction.

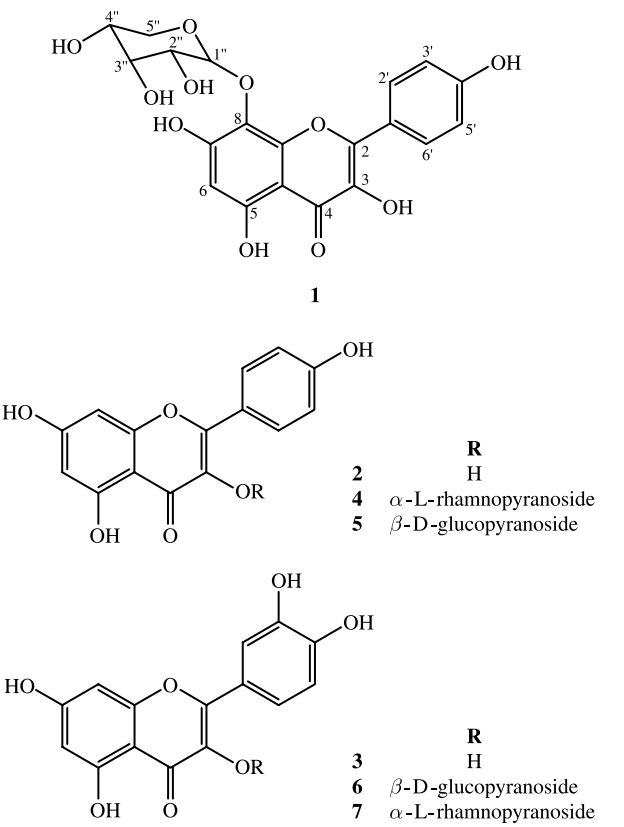

Figure 2. Structures of compounds **1**-**7** isolated from the B2 fraction of *O. japonicus*.

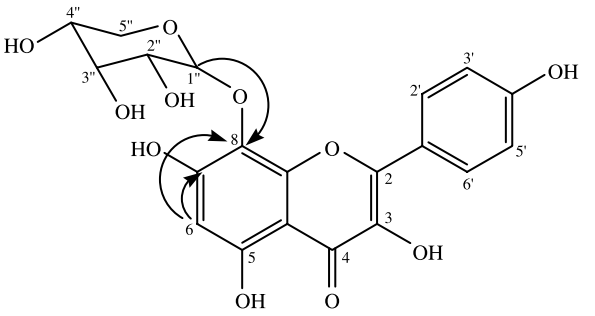

Figure 3. Selected HMBC correlations of **1**.

Compounds **1**-**7** were tested for calpain inhibitory activity. The IC_{50} value of inhibition of the seven compounds was measured, from data of triplicate experiment. Among them, quercetin (**3**) and astragalin (**5**) showed significant calpain inhibitory activities and their IC_{50} value were 210.9 μM and 171.3 μM, respectively, while the IC_{50} value of a positive control, leupeptin, was 5.4 μM. Quercetin (**3**) is one of the most frequently found and studied flavonoids and has many known biological activities [14]. It was reported that many flavonoids including quercetin (**3**) and astragalin (**5**) protect neuronal cells from oxidative stress induced by glutamate in the mouse hippocampal cell line HT-22 [15]. Also, quercetin has been shown to have reactive oxygen species scavenging activity, reduction of oxidative DNA damage and lipid peroxidation decrease in many cell-free *in vitro* experimental systems [16]. Taken together, quercetin and astragalin may be beneficial for the prevention and treatment of neurological disorders. Furthermore, neuroprotective activities of these two compounds will be studied in primary cultures of rat cortical cells against glutamate-induced neurotoxicity in our laboratory.

Acknowledgements

This work was supported by Research Settlement Fund for the new faculty of SNU.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Astma DE, Bastiaanse EML, Jerzewski A, Van der Valk LJM, Van der Laarse A. Role of calcium-activated neutral protease (calpain) in cell death in cultured neonatal rat cardiomyocytes during metabolic inhibition. Circul Res 1999;76:1071–1078.

[2] Park SY, Tournell C, Sinjoanu RC, Ferreira A. Caspase-3- and calpain-mediated tau cleavage are differentially prevented by estrogen and testosterone in beta-amyloid-treated hippocampal neurons. Neuroscience 2007;144:119–127.

[3] Wang KKW, Larner SF, Robinson G, Hayes R. Neuroprotection targets after traumatic brain injury. Curr Opin Neurol 2006;19:514–519.

[4] Xu W, Wong TP, Chery N, Gaertner T, Wang YT, Baudry M. Calpain-mediated mGluR1a truncation: A key step in excitotoxicity. Neuron 2007;53:399–412.

[5] Kim JB. Ilustrated Natural Drugs Encyclopedia., Vol 2. Seoul: Namsandang; 1984. p 447.

[6] Yoon NY, Min BS, Lee HK, Park JC, Choi JS. A potent anticomplementary acylated sterol glucoside from *Orostachys japonicus*. Arch Pharm Res 2005;28:892–896.

[7] Sung SH, Jung WJ, Kim YC. A novel flavonol lyxoside of *Orostachys japonicus* herb. Nat Prod Lett 2002;16:29–32.

[8] Burokerkilgore M, Wang KKW. A Coomassie brilliant blue G-250-based colorimetric assay for measuring activity of calpain and other proteases. Anal Biochem 1993;208:387–392.

[9] Park HJ, Lim SC, Lee MS, Young HS. Triterpene and steroids from *Orostachys japonicus*. Kor J Pharmacog 1994;25:20–23.

[10] Park JG, Park JC, Hur JM, Park SJ, Choi DR, Shin DY. Phenolic compounds from *Orostachys japonicus* having anti-HIV-1 protease activity. Nat Prod Sci 2000;6:117–121.

[11] Park HJ, Young HS, Park KY, Rhee SH, Chung HY, Choi JS. Flavonoids from the whole plants of *Orostachys japonicus*. Arch Pharm Res 1991;14:167–171.

[12] Markham KR, Geiger H. ^{1}H nuclear magnetic resonance spectroscopy of flavonoids and their glycosides in hexadeuterodimethylsulfoxide. In: Harborne JB, editor. The flavonoids: Advances in Research Since 1986. London: Chapman & Hall; 1994. p 441–497.

[13] Bennini B, Chulia AJ, Kaouadji M. Flavonol glycosides from *Erica cinerea*. J Nat Prod 1994;57:178–180.

[14] Formica JV, Regelson W. Review of the biology of quercetin and related bioflavonoids. Food Chem Toxicol 1995;33: 1061–1080.

[15] Ishige K, Schubert D, Sagara Y. Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. Free Rad Biol Med 2001;30:433–446.

[16] Dok-Go H, Lee KH, Kim HJ, Lee EH, Lee J, Song YH. Neuroprotective effects of antioxidative flavonoids, quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether, isolated from *Opuntia ficus-indica* var *saboten*. Brain Res 2003;965: 130–136.