Natural Product Sciences
21(2) : 71-75 (2015)

Chemical Constituents from the Aerial Parts of *Bupleurum falcatum* L. and Biological Evidences

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Abstract – In this study, phytochemical investigation on the aerial parts of *Bupleurum falcatum* resulted in the isolation of fourteen compounds including three quinic acid derivatives (**1 - 3**), five flavonoids (**4 - 8**), three monoterpene glycosides (**9 - 11**), and three saikosaponins (**12 - 14**). Compound **1** was first isolated from nature and unambiguously determined to be 3-*O*-feruloyl 5-*O*-caffeoylquinic acid on the basis of the extensive spectroscopic evidence. Biological testing revealed that saikosaponin A (**12**) and saikosaponin D (**13**) showed moderate anti-proliferative effects on HL-60 and HepG2 cancer cell lines.
Keywords – *Bupleurum falcatum*, Umbellifeae, Quinic acid, Saikosaponin, Anti-proliferative activity

Introduction

Bupleuri Radix, the dried roots of *Bupleurum falcatum* L. (Umbelliferae), which is a perennial medicinal herb distributed mainly in China, Korea, and Japan, is one of the most important ingredients in traditional Kampo and Chinese medicines.[1,2] Principal bioactive constituents of Bupleuri radix include a group of oleanane-type triterpene glycosides, commonly known as saikosaponins such as saikosaponins A-D.[3-5] On the other hand, the aerial part of *B. scorzonerifolium* Willd. has been used in China and, recently, Nakahara *et al.* reported low concentrations of several saikosaponins in the aerial parts and seeds of *B. falcatum*.[6] Previously, we prepared monoclonal antibody (MAb) against saikosaponin A in order to control the quality of *Bupleurum* species.[7] Our ongoing study on *B. falcatum* led to isolate eight phenolic compounds (**1 - 8**) for the first time including three quinic acid derivatives (**1 - 3**) along with six terpenoid constituents (**9 - 14**) from the aerial parts of the title plant. This paper herein deals with the chemical profile of the aerial parts of *B. falcatum* and biological evidences of the obtained components.

Experimental

General procedures – Optical rotations were obtained using a DIP-360 digital polarimeter (JASCO, Easton, USA). NMR spectra were recorded on a JEOL ECX 400 NMR spectrometer (JEOL, Tokyo, Japan). HR-ESI-TOFMS experiments utilized a JEOL Accu TOF^{TM} LC 1100 mass spectrometer (JEOL, Tokyo, Japan). Column chromatography was performed on silica gel 60 (230 - 400 mesh, Nacalai Tesque Inc., Kyoto, Japan), YMC ODS-A gel (50 μm, YMC Co. Ltd., Kyoto, Japan), and Diaion HP-20 (Mitsubishi Chemical Ltd., Tokyo, Japan). TLC was performed on Kieselgel 60 F_{254} and TLC Silica gel 60 RP-18 F_{254S} (Merck, Damstadt, Germany) plates. Spots were visualized by spraying with 1% $Ce(SO_4)_2$-10% aqueous H_2SO_4 solution, followed by heating.

Plant material – The aerial parts of *B. falcatum* were collected in Genkai-cho, Saga prefecture in 2012 and authenticated by one of the authors (Y.S.). A voucher specimen has been deposited in the Department of Pharmacognosy, Nagasaki International University.

Extraction and isolation – The air and shade-dried sample (1,100 g) was extracted with 95% EtOH (4.0 L × 3 times) at 40 °C under sonication. The combined extracts were concentrated to give dark brown syrup (122 g). The obtained crude extract (120 g) was suspended in water (1000 mL), then partitioned with CH_2Cl_2 (1000 mL × 3), and the water layer was subjected to a Diaion HP-20 column eluted with a stepwise gradient of MeOH-H_2O (25, 50, 75, and 100% MeOH; v/v) to afford four fractions (fr.1 – 4). Fr.2 (5.9 g) was chromatographed using a silica gel column with $CHCl_3$-MeOH-H_2O (7:3:0.4, v/v/v) to give eight fractions (fr.2.1 – 2.8). Fr.2.3 (450 mg) was

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further chromatographed over a reversed-phase (RP) column with MeOH-H_2O (1:2, v/v), followed by a silica gel column with $CHCl_3$-MeOH-H_2O (5:1:0.1, v/v/v) to yield **3** (80 mg) and **10** (13 mg). Fr.2.8 (230 mg) was loaded onto a RP column with MeOH-H_2O (1:2, v/v), followed by a silica gel column with EtOAc-MeOH (3:1, v/v) to afford **2** (26 mg). Fr.3 (10.1 g) was subjected to a silica gel column with stepwise gradient of $CHCl_3$-MeOH (8:1 → 0:1, v/v) to give five fractions (fr.3.1 – 3.5). Fr.3.2 (600 mg) was then chromatographed over a RP column with MeOH-H_2O (3:2, v/v) to obtain **4** (310 mg), **13** (8 mg), and **14** (6 mg). Fr. 3.4 (4.8 g) was repeatedly chromatographed over a RP column with MeOH-H_2O (1:1, v/v) to furnish **6** (17 mg), **7** (550 mg), and **8** (32 mg). Fr.3.5 (270 mg) was loaded onto a RP column with MeOH-H_2O (1:3, v/v), followed by a silica gel column with EtOAc-MeOH (5:2, v/v) to afford **1** (9 mg). Fr.3.3 (2.2 g) was further subjected to a silica gel column with $CHCl_3$-MeOH (4:1, v/v) to give five fractions (fr.4.1 – 4.5). Fr.4.1 (400 mg) was then repeatedly chromatographed over a RP column with MeOH-H_2O (1:1, v/v) to yield **9** (13 mg), **11** (8 mg), and **12** (19 mg). Finally, compound **5** (45 mg) was purified from fr.4.3 (180 mg) by a RP column with MeOH-H_2O (3:4, v/v).

3-*O*-feruloyl 5-*O*-caffeoylquinic acid (1) – Yellowish powder; $[\alpha]_D^{20}$ −114° (*c* 0.13, MeOH); HR-ESI-TOFMS: *m/z*531.1507 $[M+H]^+$ (calcd. for $C_{26}H_{27}O_{12}$, 531.1502); ^{1}H-NMR (CD_3OD, 400 MHz) and ^{13}C-NMR (CD_3OD, 100 MHz): see Table 1.

Cell culture and sample treatment – The cell lines were obtained from RIKEN BioResource Center Cell Bank. HL-60 and HepG2 cells were maintained in RPMI1640 and DMEM media, respectively. All cell cultures were supplemented with 10% FBS, 1% penicillin-streptomycin, and then incubated at 37 ºC under 5% CO_2 under fully humidified conditions. For cell treatment, the samples were dissolved in DMSO as 100 mM stock solution and were stored at −20 ºC before use. DMSO concentrations in the cell culture medium did not exceed 0.2% (v/v) and the controls were always treated with the same amount of DMSO as used in the corresponding experiments.

MTT assay – Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[8] Cells in 96-well plates (HL-60: 1×10^4 cells/well and HepG2: 0.5×10^4 cells/well) were treated with each compound at various concentrations for 24 h. At the end of treatment, MTT solution was added to each well, and the cells were incubated for another 4h. The precipitated MTT-formazan was dissolved with 0.04 N HCl-isopropanol, and the amount of formazan was measured at 595 nm using microplatereader (ImmunoMiniNJ-2300, Nihon InterMed, Tokyo, Japan). Cell viability was expressed as a percentage of the control culture.

Table 1. NMR Data for Compound **1**

Position	^{13}C (CD_3OD, 100 MHz)	^{1}H (CD_3OD, 400 MHz)
1	76.4	
2	37.7	2.24 (1H, dd, *J* = 14.8, 4.0 Hz) 2.06 (1H, dd, *J* = 14.8, 5.2 Hz)
3	74.5	5.34 (1H, dt, *J* = 4.8, 3.6 Hz)
4	73.3	3.88 (1H, dd, *J* = 9.2, 3.2 Hz)
5	72.5	5.50 (1H, m)
6	41.0	2.10-2.16 (2H, m)
-COOH	181.7	
1'	128.1	
2'	111.6	7.14 (1H, d, *J* = 1.6 Hz)
3'	149.6	
4'	150.4	
5'	116.0	6.81 (1H, d, *J* = 8.0 Hz)
6'	122.9	7.03 (1H, br d, *J* = 7.6 Hz)
7'	146.8	7.58 (1H, d, *J* = 16.0 Hz)
8'	115.5	6.36 (1H, d, *J* = 16.0 Hz)
9'	169.4	
3'-OCH_3	56.4	3.63 (3H, s)
1"	127.8	
2"	115.1	6.96 (1H, d, *J* = 1.6 Hz)
3"	146.7	
4"	149.5	
5"	116.5	6.74 (1H, d, *J* = 7.6 Hz)
6"	122.9	6.85 (1H, br d, *J* = 7.2 Hz)
7"	146.9	7.54 (1H, d, *J* = 16.0 Hz)
8"	115.5	6.26 (1H, d, *J* = 16.0 Hz)
9"	169.0	

Assignments were established by HMQC, DQF-COSY, and HMBC spectra. *J* values (in Hz) are given in parentheses.

Results and discussion

The ethanolic extract of the aerial part of *B. falcatum* was suspended in water and then partitioned with CH_2Cl_2, followed by column chromatography to afford fourteen compounds (**1 - 14**) (Fig. 1).

Compound **1** was purified as a yellowish amorphous powder and its molecular formula was determined to be $C_{26}H_{26}O_{12}$ based on the observed molecular peak at *m/z* 531.1507 $[M+H]^+$ (calcd. for $C_{26}H_{27}O_{12}$, 531.1502) in its HR-ESI-TOF-MS spectrum. The ^{1}H NMR spectrum displayed signals of two methylenes [δ 2.10-2.16 (2H, m,

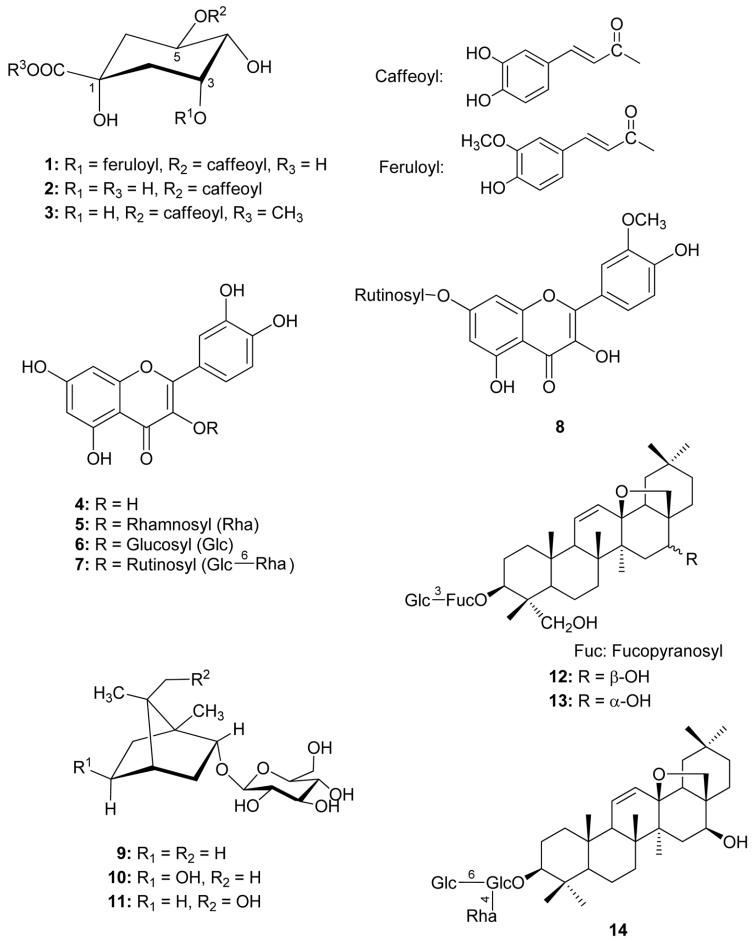

Fig. 1. Structures of compounds **1** - **14**.

H-6), 2.06 (1H, dd, J = 14.8, 5.2 Hz, H-2_{eq}), and 2.24 (1H, dd, J = 14.8, 4.0 Hz, H-2_{ax})] and three oxymethine protons [δ 3.88 (1H, dd, J = 9.2, 3.2 Hz, H-4), 5.34 (1H, dt, J = 4.8, 3.6 Hz, H-3), and 5.50 (1H, m, H-5)]. The ^{13}C NMRspectrum showed two methylene carbons at δ 37.7 and 41.0, four oxygenated carbons at δ 72.5, 73.3, 74.5, and 76.4, and a free carboxylic carbon at δ 181.7, respectively (Table 1). In addition, ^{1}H and ^{13}C NMR spectra of **1** suggested the presence of a caffeoyl and a feruloyl groups.[9,10] The above spectroscopic data of **1** were very similar with those of 3,5-di-*O*-caffeoyl quinic acid except for the 3-*O*-feruloyl moiety in the molecule.[11,12] Comprehensive analyses of NMR data of **1** using HMQC, HMBC, and DQF ^{1}H-^{1}H COSY permitted complete assignments of individually the 3-*O*-feruloyl and 5-*O*-caffeoyl groups and verified the interlinkage positions between the quinic acid and the 3-*O*-feruloyl and 5-*O*-caffeoyl moieties. Namely, long-range correlations were observed between the following proton and carbon pairs: H-3 and C-9', H-5 and C-9" (Fig. 2). Furthermore, the

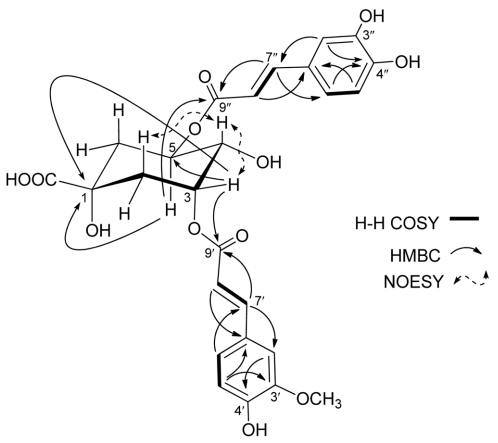

Fig. 2. Selected H-H COSY, HMBC, and NOESY correlations of **1**.

configuration of the quinic acid moiety was determined by comparison of NMR data of **1** with 3,5-dicaffeoylquinic acid[12] and nuclear Overhauser enhancement spectroscopy (NOESY) experiment. The NOEs were observed between the following pairs of protons: H-4 and

H-2_{ax}, H-4 and H-3 (Fig. 2), respectively, suggested that the orientation of H-4 and H-5 was axial and the configuration of H-3 was equatorial in respect to that of 3,5-dicaffeoylquinic acid and the other quinic acid derivatives.[12] On the basis of the above evidence, the structure of **1** was determined as 3-*O*-feruloyl 5-*O*-caffeoylquinic acid.

This is the first isolation of compound **1**, though it was previously detected by LC-MS from green coffee beans[13] and *Artemisia annua*.[14] Because of the structural similarities and complexities of quinic acid derivatives, the structural characterization of quinic acid derivatives, especially stereo chemistry, requires extensive spectroscopic evidences.[15,16] Other compounds were characterized as 5-*O*-caffeoyl quinic acid (chlorogenic acid) (**2**),[17] chlorogenic acid methyl ester (**3**),[18] quercetin (**4**),[19] quercitrin (**5**),[19] isoquercitrin (**6**),[19] rutin (**7**),[20] isorhamnetin 7-rutinoside (**8**),[21] (1*S*,2*R*,4*S*)-borneol 2-*O*-*β*-D-glucopyranoside (**9**),[22] (−)-angelicoidenol 2-*O*-*β*-D-glucopyranoside (**10**),[23] (2*R*)-bornane-2,9-diol 2-*O*-*β*-D-glucopyranoside (**11**),[23] saikosaponin A (**12**),[6] saikosaponin D (**13**),[6] and saikosaponin C (**14**),[6] respectively, by comparing their physical and spectroscopic data with those reported in the literatures. To our knowledge, this is the first report of phenolic constituents from the aerial parts of *B. falcatum*. In addition, quinic acid derivatives (**1 - 3**) and three monoterpene glycosides (**9 - 11**) have not been isolated previously from the *Bupleurum* spp. In agreement with the literature, several saikosaponins with low yields as compared relatively with those in the roots have been isolated from the aerial parts of *B. falcatum*.[5,6] It is likely that the aerial parts of *B. falcatum* contain phenolic compounds as major constituents.

The anti-proliferative activity against human leukemia cells (HL-60) and hepatoma cells (HepG2) of three quinic derivatives (**1-3**), saikosaponin A (**12**), saikosaponin D (**13**), and saikosaponin C (**14**) were examined. Compounds **1-3** did not exhibit inhibitory effects on the growth of the cancer cell lines at concentrations up to 40 μM. Of the three saponins, saikosaponin A (**12**) and saikosaponin D (**13**) showed moderate anti-proliferative effects on the two cancer cell lines. At the concentration of 40 μM for 24h incubation, compounds **12** and **13** significantly inhibited both HL-60 cell growth by 96.8% and 94.8% and HepG2 cell growth by 94.4% and 85.4%, respectively.

These data was well correlated with the previous studies regarding anticancer activity of individual saikosaponins A and D.[24-26] In addition, anti-inflammation,[27] corticosterone secreting,[28] plasma-cholesterol lowering action,[2] hemolytic activity,[1] and protective action against hepatic damage,[29,30] were reported for saikosaponins A and D. However, no such biological activities are observed for saikosaponin C. It is necessary to analyze the saikosaponin concentration by ELISA using anti-saikosapon in a MAb[7] of which sensitivity is approximately 1,000 times higher than HPLC for the quality control of *B. falcatum* because of lower concentrations of saikosaponins. Chlorogenic acid,[31,32] rutin,[33] quercetin,[34] and quercitrin,[33] are common dietary polyphenols contained in green tea, coffee beans, cocoa, citrus, etc., as well as various medicinal plants and based on their compelling pharmacological profiles, they have been recognized as key healthy constituents in the functional foods and used as ingredients of certain herbal remedies.[35,36]

In conclusion, the present study pointed out the chemical constituents of the aerial parts of *B. falcatum*, which contains major phenolic components together with low concentrations of the saikosaponins. These findings support utilization of the aerial parts of the title plant as medicinal material.

Acknowledgements

N.H.T. is grateful to the Japan Society for the Promotion of Science (JSPS) for a postdoctoral fellowship (P10423) at the Nagasaki International University.

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Received September 15, 2013
Revised January 16, 2015
Accepted January 20, 2015