Antiproliferative Constituents from Umbelliferae Plants VII.[1] Active Triterpenes and Rosmarinic Acid from *Centella asiatica*

Miyako YOSHIDA, Masahiro FUCHIGAMI, Tsuneatsu NAGAO, Hikaru OKABE, Kazuhisa MATSUNAGA, Jiro TAKATA, Yoshiharu KARUBE, Ryota TSUCHIHASHI, Junei KINJO, Kunihide MIHASHI, and Toshihiro FUJIOKA*

Faculty of Pharmaceutical Sciences, Fukuoka University; 8–19–1 Nanakuma, Jonan-ku, Fukuoka 814–0180, Japan.
Received August 25, 2004; accepted October 18, 2004; published online October 19, 2004

The antiproliferative constituents in the MeOH extract from the aerial parts of *Centella asiatica* were investigated. Activity-guided fractionation of MeOH extract resulted in the isolation of ursolic acid lactone, ursolic acid, pomolic acid, 2α,3α-dihydroxyurs-12-en-28-oic acid, 3-epimaslinic acid, asiatic acid, corosolic acid, and rosmarinic acid. Antiproliferative activity of the isolated compounds against human gastric adenocarcinoma (MK-1), human uterine carcinoma (HeLa), and murine melanoma (B16F10) cells was estimated.

Key words *Centella asiatica*; antiproliferative activity; Umbelliferae; triterpene

The Umbelliferae *Centella asiatica*, which has many common names including gotu kola, hydrocotyle, Indian pennywort, marsh penny, thick-leaved pennywort and white rot, has been widely cultivated as a vegetable or spice in China, Southeast Asia, India, Sri Lanka, Africa, and Oceanic countries. In the course of our search to identify the antiproliferative constituents in the Umbelliferae plants, we have found that the MeOH extract from the aerial parts of *C. asiatica* inhibits *in vitro* the growth of human gastric adenocarcinoma (MK-1), human uterine carcinoma (HeLa), and murine melanoma (B16F10) cells, and these findings caused us to check the active constituents in this plant.

MATERIALS AND METHODS

Instruments All the instruments and materials used were the same as those shown in the previous paper.[2] ^{1}H- (500 MHz) and ^{13}C- (125 MHz) NMR spectra were measured in pyridine-d_5. Chemical shifts are expressed on the δ scale with tetramethylsilane as an internal standard. The signal assignment was based on comparison with data reported for compounds having similar structures, and confirmed with the aid of NMR spectral techniques (^{1}H–^{1}H COSY, ^{1}H–^{13}C COSY, HMQC and HMBC).

Materials *Centella asiatica* was cultivated in the medical plant garden of Fukuoka University, and the aerial parts were collected in July 2000 and air-dried.

Tumor Cells MK-1 cells were provided by Professor M. Katano of the Faculty of Medicine, Kyushu University, and HeLa and B16F10 cells were supplied by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University.

Measurement of Antiproliferative Activity against Tumor Cell Lines The inhibition of the cellular growth was estimated using 3-(dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Mosmann.[3]

Extraction and Isolation The air-dried aerial parts of *Centella asiatica* (722.7 g) were extracted with MeOH at room temperature. The MeOH solution was concentrated *in vacuo* to give dark green syrup (109.0 g). The MeOH extract was distributed with $CHCl_3$ and H_2O. The $CHCl_3$ solution was concentrated *in vacuo* to give dark green syrup (CAW-C; 30.6 g). The H_2O solution was passed through a column of styrene polymer, Dianion HP-20. The H_2O elute was concentrated *in vacuo* to give a yellow syrup (CAW-W; 51.2 g). The column was then washed with MeOH and the eluate was concentrated to give a brown powder (CAW-M; 19.4 g). CAW-C (GI_{50}: 50—100 μg/ml, >100 μg/ml, 50—100 μg/ml) and CAW-M (GI_{50}: 100 μg/ml, 50—100 μg/ml, 50—100 μg/ml) had dose-dependent antiproliferative activity for MK-1, HeLa and B16F10 cells. CAW-C was separated into 15 fractions (CAW-C1—C15) by silica gel column chromatography using $CHCl_3$–MeOH (1 : 0→0 : 1) as the eluting solvent, CAW-C1 (2.0 g), C2 (0.7 g), C3 (1.1 g), C4 (3.0 g), C5 (4.2 g), C6 (1.1 g), C7 (1.2 g), C8 (3.2 g), C9 (0.9 g), C10 (0.9 g), C11 (1.5 g), C12 (1.1 g), C13 (2.3 g), C14 (1.3 g) and C15 (0.7 g). In these fractions, CAW-C4 (GI_{50}: 50—100 μg/ml), C5 (GI_{50}: 25—100 μg/ml), C6 (GI_{50}: 6.25—25 μg/ml), C7 (GI_{50}: 25—50 μg/ml), C8 (GI_{50}: 50—100 μg/ml), C9 (GI_{50}: 25—100 μg/ml) had dose-dependent antiproliferative activity for MK-1, HeLa and B16F10 cells. At first, CAW-C5 was purified by MCI-gel CHP20P column using H_2O–MeOH (1 : 1→0 : 1), silica gel using hexane–acetone (1 : 1→0 : 1) and HPLC (ODS, 90% MeOH) to give **1** (2.4 mg). CAW-C6 was chromatographed on YMC gel column (ODS) using H_2O–MeOH (1 : 1→0 : 1) and HPLC (ODS, 90% MeOH) to give **2** (240 mg) and **3** (4.0 mg). CAW-C7 was chromatographed on YMC gel column (ODS) using H_2O–MeOH (1 : 1→0 : 1) and HPLC (ODS, 90% MeOH) to give **4** (60 mg) and **5** (25 mg). CAW-8 was chromatographed on silica gel using hexane–acetone (1 : 1→0 : 1) and YMC gel column (ODS) using H_2O–MeOH (1 : 1→0 : 1) and HPLC (ODS, 90% MeOH) to give **6** (40 mg) and **7** (8.4 mg). CAW-C4 was chromatographed on silica gel using $CHCl_3$–MeOH (1 : 0→0 : 1) and then on MCI-gel CHP20P column using H_2O–MeOH (1 : 1→0 : 1) to give **8** (200 mg). CAW-C9 was chromatographed on YMC gel column (ODS) using H_2O–MeOH (1 : 1→0 : 1) and silica gel using hexane–acetone (1 : 1→0 : 1) and HPLC (ODS, 100% MeOH) to give **9** (20 mg). CAW-M was separated into 9 fractions (CAW-M1—M9) by silica gel chromatography using $CHCl_3$–MeOH (1 : 0→0 : 1) as the eluting solvent. In these fractions, only

* To whom correspondence should be addressed. e-mail: fujioka@fukuoka-u.ac.jp
© 2005 Pharmaceutical Society of Japan

CAW-M9 had dose-dependent antiprolifeative activity for MK-1 (GI_{50}: 25—50 μg/ml), HeLa (GI_{50}: 12.5—25 μg/ml) and B16F10 (GI_{50}: 6.25—12.5 μg/ml) cells. CAW-M9 was further chromatographed on YMC column (ODS) using H_2O–MeOH (1 : 0→0 : 1), silica gel using $CHCl_3$–acetone (1 : 0→0 : 1), and YMC column (ODS) using H_2O–MeOH (1 : 0→0 : 1) to give **10** (38 mg). Compounds **1**—**10** were identified as ursolic acid lactone (**1**),[4] ursolic acid (**2**), pomolic acid (**3**),[5,6] 2α,3α-dihydroxyurs-12-en-28-oic acid (**4**),[7,8] 3-epimaslinic acid (**5**),[8,9] asiatic acid (**6**),[10] corosolic acid (**7**),[8,9] 8-acetoxy-1,9-pentadecadiene-4,6-diyn-3-ol (**8**),[11,12] β-sitosterol 3-*O*-β-glucopyranoside (**9**), and rosmarinic acid (**10**),[13] respectively, by analyses of MS and NMR spectra and comparison of their physical data with those reported.

RESULTS AND DISCUSSION

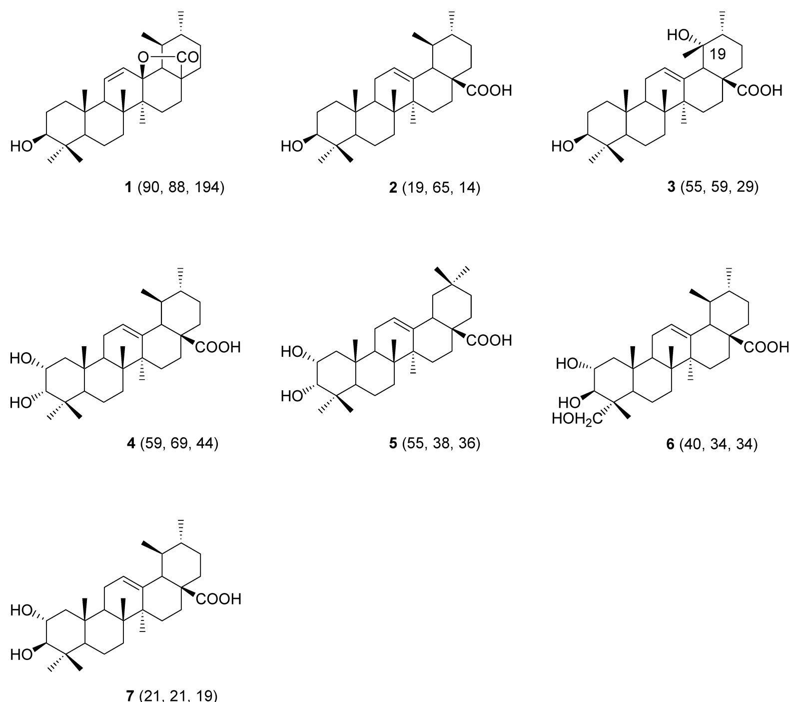
Antiproliferative activity-guided fractionation of the $CHCl_3$ (CAW-C) and MeOH (CAW-M) extracts from the aerial parts of *Centella asiatica* resulted in the isolation of 10 compounds. Compounds **1**—**10** were identified as 11,12-dehydroursolic acid lactone (**1**), ursolic acid (**2**), pomolic acid (**3**), 2α,3α-dihydroxyurs-12-en-28-oic acid (**4**), 3-epimaslinic acid (**5**), asiatic acid (**6**), corosolic acid (**7**), 8-acetoxy-1,9-pentadecadiene-4,6-diyn-3-ol (**8**), β-sitosterol 3-*O*-β-glucopyranoside (**9**), and rosmarinic acid (**10**), respectively, by analyses of MS and NMR spectra and comparison of their physical data with those reported.

The antiproliferative activity of the isolated compounds was determined against MK-1, HeLa, B16F10 cells. The 50% growth inhibition (GI_{50}) values are listed in Table 1. As for these compounds, antiproliferative activities against MK-1, HeLa, and B16F10 cells were concentration-dependently increased. Among these compounds, **2** was given in largest amount of yield in CAW-C, and it had high antiproliferative activity (GI_{50}: 19 μM for MK-1, 65 μM for HeLa, and 14 μM for B16F10). Therefore, it is possible that **2** accounts for the main part of antiproliferative activity in CAW-C. When a lactone ring was formed by the C-13 and C-28 of **2**, as seen in **1**, antiproliferative activity was decreased as compared with **2** (Fig. 1). When a hydroxyl group was attached to the C-2 of **2**, as seen in **7**, only the activity against HeLa cell was increased as compared with **2**. When a hydroxyl group attached to the C-19 of **2**, as seen in **3**, the activity against MK-1 and B16F10 was decreased as compared with **2**. When a hydroxyl group attached to the C-23 of **7**, as seen in **6**, the activity was just decreased as compared with **7**. When a hydroxyl group attached to the C-3α instead of C-3β of **7**, as seen in **4**, the activity was decreased as compared with **7**. There was almost no difference of the antiproliferative activity between the ursane type triterpene compound (**4**) and oleanane type triterpene compound (**5**).

Antiproliferative activity-guided fractionation of CAW-M resulted in the isolation of rosmarinic acid (**10**). Antiprolifer-

Table 1. Antiproliferative Activities (GI_{50}, μM) against MK-1, HeLa, and B16F10 *in Vitro*

Compound	MK-1	HeLa	B16F10
1	90	88	194
2	19	65	14
3	55	59	29
4	59	69	44
5	55	38	36
6	40	34	34
7	21	21	19
8	91	102	109
9	>173	121	8
10	119	75	16



The values in parentheses are GI_{50} values against MK-1, HeLa, and B16F10, in order.

Fig. 1

ative activities of **10** was concentration-dependently increased against only HeLa and B16F10 cells, as seen in the antiproliferative activities of CAW-M. Therefore, it is possible that **10** is the main part of antiproliferative activity in CAW-M.

Acknowledgements We express our appreciation to Ms. Y. Iwase and Mr. H. Hanazono of Fukuoka University for measurement of the NMR and MS spectra.

REFFERENCES

1) Part VI: Fujioka T., Yoshida K., Fujii H., Nagao T., Okabe H., Mihashi K., *Chem. Pharm. Bull.*, **51**, 365—372 (2003).
2) Part III: Ikeda R., Nagao T., Okabe H., Nakano Y., Matsunaga H., Katano M., Mori M., *Chem. Pharm. Bull.*, **46**, 871—874 (1998).
3) Mosmann T., *J. Immunol. Methods*, **65**, 55—63 (1983).
4) Wang H., Fujimoto Y., *Phytochemistry*, **33**, 151—153 (1993).
5) Brieskorn C. H., Wunderer H., *Chem. Ber.*, **100**, 1252—1265 (1967).
6) Nakanishi T., Terai H., Nasu M., Miura I., Yoneda K., *Phytochemistry*, **21**, 1373—1377 (1982).
7) Biessels H. W. A., van der Kerk-van Hoof A. C., Kettenes-van den Bosh J. J., Salemink C. A., *Phytochemistry*, **13**, 203—207 (1974).
8) Kojima H., Ogura H., *Phytochemistry*, **25**, 729—733 (1986).
9) Seo S., Tomita Y., Tori K., *J. Am. Chem., Soc.*, **103**, 2075—2080 (1981).
10) Kojima H., Ogura H., *Phytochemistry*, **28**, 1703—1710 (1989).
11) Bohlmann F., Zdero C., *Chem. Ber.*, **208**, 511—514 (1975).
12) Fujioka T., Furumi K., Fujii H., Okabe H., Mihashi K., Nakano Y., Matsunaga H., Katano M., Mori M., *Chem. Pharm. Bull.*, **47**, 96—100 (1999).
13) Kelly C. J., Harruff R. C., Carmack M., *J. Org. Chem.*, **41**, 449—454 (1976).