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Cytotoxic Saponins from the Root of *Dipsacus asper* Wall

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Cytotoxic activitiy of seven hederagenin saponins isolated from the root of *Dipsacus asper* were investigated *in vitro* against L1210, HL-60 and SK-OV-3 tumor cell lines by the MTT method. 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl hederagenin (**2**), 3-*O*-β-D-xylopyranosyl-(1→3)-α-L-Rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl hederagenin (**6**) and 3-*O*-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl hederagenin (**7**) exhibited the potent cytotoxicity against the three tumor cell lines with IC_{50} values ranging from 4.7 to 8.7 μg/mL, with the exception of compound **7**, which exhibited weak cytotoxic activity against SK-OV-3 (IC_{50} 22.5 μg/mL). Other compounds did not exhibit any cytotoxic activity (IC_{50} > 30 μg/mL).

Key words: *Dipsacus asper,* Dipsacaceae, Hederagenin saponins, Cytotoxicity, MTT assay

INTRODUCTION

Dipsacus asper Wall (Dipsacaceae) is a perennial herb found in China. Its roots have been used in Chinese traditional medicine as an analgesic, an anti-inflammatory agent, to enhance liver activity, and to treat fractures (Namba, 1986). Previous researchers reported the isolation of several triterpene glycosides and iridoid glycosides from this plant (Kouno *et al.*, 1990; Jung *et al.*, 1993; Tomita and Mouri, 1996). Recently, saponins isolated from the root of this plant were found to have anti-inflammatory (Jung, 1995) and anticomplementary qualities (Oh *et al.*, 1999). Dipsacus saponin C was found to possess a strong antinociceptive effect (Suh *et al.*, 1996; Suh *et al.*, 2000). In addition, the extract of this plant reduced cognitive deficits and caused the overexpression of β-amyloid protein which was induced by aluminum exposure (Zhang *et al.*, 2003). However, no studies have investigated the effects of isolated saponins in cytotoxic activity. Thus, we report herein on the structure-cytotoxicity relationship of hedragenin glycosides isolated from *D. asper* against L1210, HL-60, and SK-OV-3 tumor cell lines *in vitro*.

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MATERIALS AND METHODS

Plant material

The dried root of *Dipsacus asper* was purchased in Kumsan, Chungnam, 2003. Botanical identification was performed by Professor KiHwan Bae and a voucher speciment (CNU 2023-1) was deposited in the herbarium of the College of Pharmacy, Chungnam National University, Korea.

Chemicals

The organic solvents were supplied by DaeJung Chemical and Metals Co. Ltd, Korea. Standard sugars were purchased from Aldrich Chemical Co., USA. Column chromatographic packing materials: Kieselgel 60 (0.040-0.063) mm (Meck, Germany), Sephadex LH-20, Pre-coated TLC: Kieselgel 60 F_{254}, RP-18 F_{254} (Merck, Germany). Melting point was measured on an Electrothermal apparatus. The IR spectra were determined using a Hitachi 270-30 type spectrometer with KBr discs. FAB-MS was taken in MeOH and obtained using a JEOL JMS-DX 300 spectrometer. ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) spectra were recorded on a Bruker-AM600 FT-NMR unit and chemical shift are expressed as δ values using TMS as an internal standard. Spots were detected by UV light (254 nm) and by spraying 10% H_2SO_4 followed by heating. Preparative MPLC was performed on a YAMAZEN MPLC system with pump 540; detector Prep UV-10V; ULTRA PACK ODS-S-50A (11 mm×300 mm).

Fraction, isolation and identification

The dried-root (2 kg) was refluxed with hot MeOH three times and concentrated in vacuo to a dry residue (380 g). The residue was suspended in water and successively partitioned with *n*-hexane, EtOAc and *n*-BuOH, and then evaporated to yield a hexane fraction, an EtOAc fration, and a BuOH fraction. Bioassay followed revealed that the EtOAc and *n*-BuOH fraction exhibited cytotoxic activity against HL-60 cells (data not showed), and TLC analysis suggested that it contained several saponins. The EtOAc-soluble fraction (65 g) was chromatographed on a silica gel column and eluted with $CHCl_3$-MeOH (50:1 to 1:1), which produced 18 fractions (E.1~E.18). Fraction E.14 (2 g) was chromatographed on a silica gel column eluted with $CHCl_3$-MeOH (5:1) which yielded two subfractions and compound **1** (20 mg). Fraction E.15 (1.2 g) was re-chromatographed on silica gel column using stepwise gradient elution with $CHCl_3$-MeOH-H_2O which produced three sub-fractions (E.15.1~E.15.3). Compound **2** (50 mg) was obtained by YMC reverse phase column using MeOH-H_2O (10:1) as eluted solvent from fraction E.15.1.

The *n*-BuOH fraction (120 g) was subjected to a silica gel column chromatography and eluted with $CHCl_3$-MeOH-H_2O which produced 20 subfractions (B.1~B.20). Fraction B.2 was chromatographed on YMC reverse phase and eluted with MeOH-H_2O (5:1) which yielded compound **3** (20 mg) and **4** (15 mg). Fraction B.5 was passed through an ODS Sep-Pak cartridge and then purified by ODS-MPLC under the following conditions: column, ODS-S-50A column (11 mm×300 mm, Yamazen Corporation); solvent, MeOH-H_2O (4:1); flow rate, 2 mL/min; column temperature, 40°C; UV detection, 254 nm. This process yielded compounds **5** (65 mg) and **6** (20 mg). Three sub-fractions (B.10~B.12) were chromatographed on silica gel column and eluted with $CHCl_3$-MeOH-H_2O (60:30:8) to give compound **7** (15 mg).

Compound **1**

Colorless needles, mp. 228-230°C; IR (KBr) ν_{max} cm^{-1}: 3400-3500 (OH), 1693 (COOH); ^{1}H- and ^{13}C-NMR data were in accordance with published data (Jung *et al.*, 1993; Oh *et al.*, 1999).

Compound **2**

Colorless needles, mp. 220-221°C; IR (KBr) ν_{max} cm^{-1}: 3400-3500 (OH), 1690 (COOH); ^{1}H- and ^{13}C-NMR data were in accordance with published data (Jung *et al.*, 1993; Oh *et al.*, 1999).

Compound **3**

Colorless needles, mp. 265-268°C; IR (KBr) ν_{max} cm^{-1}: 3400-3500 (OH), 1734 (COOR); ^{1}H- and ^{13}C-NMR data were in accordance with published data (Jung *et al.*, 1993; Oh *et al.*, 1999).

Compound **4**

Colorless needles, mp. 235-238°C; IR (KBr) ν_{max} cm^{-1}: 3400-3500 (OH), 1730 (COOR); ^{1}H- and ^{13}C-NMR data were in accordance with published data (Jung *et al.*, 1993; Oh *et al.*, 1999).

Compound **5**

Colorless needles, mp. 234-235°C; IR (KBr) ν_{max} cm^{-1}: 3400-3500 (OH), 1730 (COOR); ^{1}H- and ^{13}C-NMR data were in accordance with published data (Kouno *et al.*, 1990; Jung *et al.*, 1993; Oh *et al.*, 1999).

Compound **6**

Colorless needles, mp. 255-257°C; molecular formula $C_{46}H_{74}O_{16}$ FABMS *m/z:* 881 $[M-H]^+$; IR (KBr) ν_{max} cm^{-1}: 3400-3500 (OH), 1691 (COOH); ^{1}H- and ^{13}C-NMR data were in accordance with published data (Lee *et al.,* 2000; Park *et al.*, 2001).

Compound **7**

Colorless needles, mp. 224-225°C; IR (KBr) ν_{max} cm^{-1}: 3400-3500 (OH), 1750 (COOR). IR (KBr) ν_{max} cm^{-1}: 3400-3500 (OH), 1695 (COOH); ^{1}H- and ^{13}C-NMR data were in accordance with published data (Jung *et al.*, 1993; Oh *et al.*, 1999).

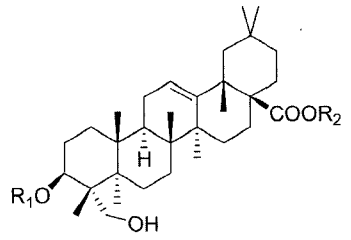
Cytotoxicity assay

Cells were maintained in RPMI 1640 which included L-glutamine (JBI) with 10% FBS (JBI) and 2% penicillin-streptomycin (GIBCO). Trypsin-EDTA was used to separate cells from the culture flask. All cell lines were cultured at 37°C in a 5% CO_2 incubator. Cytotoxicity was measured by a modified Microculture Tetrazolium (MTT) assay (Mosmann, 1983). Viable cells were seeded in the growth medium (180 μL) into 96 well microtiter plates (1×10^4 cells per each well) and incubated at 37°C, 5% CO_2. A test sample was dissolved in DMSO and adjusted to final sample concentrations ranging from 1.875 μg/mL to 30 μg/mL by diluting with the growth medium. Each sample was prepared in triplicate. The final DMSO concentration was adjusted to <0.1%. After standing for 2 h, 20 μL of the test sample was added to each well. The same volumn of DMSO was added to the control group well. Forty-eight hours after the test sample was added, 20 μL MTT was also added to the each well (final concentration, 5 μg/mL). Two hours later, the plate was centrifuged for 5 minutes at 1500 rpm, the medium was then removed and the resulting formazan crystals were dissolved with 150 μL DMSO. The optical density (O.D.) was measured at 570 nm using a Titertek microplate reader (Multiskan MCC/340, Flow). The IC_{50} value was defined as the concen-

tration of sample to reduce a 50% of absorbance relative to the vehicle-treated control.

RESULTS AND DISCUSSION

The IR spectra of all compounds showed strong hydroxyl group absorption (3400 cm^{-1}), compounds **1**, **2**, **6**, and **7** showed a carboxylic group absorption (1695-1690 cm^{-1}), and compounds **3**, **4**, and **5** showed an ester group absorption (1734, 1730, and 1730 cm^{-1}). The ^{1}H- and ^{13}C-NMR spectral data and acid hydrolysis (2M HCl in 50% MeOH) of compounds **1-7** suggested that they were triterpene glycosides with an aglycone, which was identified as hederagenin, and glucose, rhamnose, xylose and arabinose as sugar units. Hederagenin is a common aglycone, which was confirmed by direct comparison with an authentic sample, and sugar moieties were identified by Avicel TLC. Compounds **1-5** and **7**, were identified as 3-*O*-α-L-arabinopyranoside hederagenin (**1**), 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl hederagenin (**2**), 3-*O*-α-L-arabinopyranosyl hederagenin 28-*O*-β-D-glucopyranoside (**3**), hederagenin 28-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (**4**), 3-*O*-α-L-arabinopyranosyl hederagenin 28-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (**5**), 3-*O*-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl hederagenin (**7**) (Fig. 1). Compound **6** was isolated as microcrystal. Acid hydrolysis of compound **6** yielded arabinose, rhamnose and xylose. The FAB-MS spectrum of compound **6** was identical to that of compound **1**. The chemical shifts of C-3 (δ 81.7) and C-28 (δ 182.1) in the aglycone moiety indicated a 3-*O*-glycoside of hederagenin. Base on H-H COSY and HMBC data, the signals that appeared at δ 4.53 (*d*, *J* = 7.1 Hz)/104.0, 5.20 (*br s*)/101.8 and 4.43 (*d*, *J* = 7.1 Hz)/105.2 were assigned to those of the anomeric protons/carbons of α-arabinopyranosyl, α-rhamnopyranosyl and β-xylopyranosyl moieties. A comparison of the ^{1}H- and ^{13}C-NMR chemical shifts of the arabinopyranosyl moiety in compound **6** with those of compounds **1**, **2** and previous data gave evidence for glycosilation linkages through C-2 and C-3. Thus, the structure of compound **6** was determined to be 3-*O*-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl hederagenin (Lee *et al.*, 2000; Park *et al.*, 2001) (Fig. 1). To the best of our knowledge, this is first report pertaning to the isolation of this compound from *D. asper*.



	R_1	R_2
1	Ara	H
2	Rha(1→2)Ara	H
3	Ara	Glc
4	H	Glc(16)Glc
5	Ara	Glc(16)Glc
6	Xyl(1→3)Rha(1→2)Ara	H
7	Glc(1→3)Rha(1→2)Ara	H

Fig. 1. Chemical structures of saponins isolated from *D. asper*

The cytotoxic activities of the isolated compounds (**1-7**) were evaluated against L1210 (murine leukemia cancer), HL-60 (human promyelocytic leukemia), and SK-OV-3 (human ovarian tumor) according to the MTT assay described previously (Mosmann, 1983). The results were expressed as 50% inhibition concentration and summarized in Table I. Compounds **1**, **3**, **4**, and **5** did not exhibit any cytotoxic activity (IC_{50} > 30 μg/mL). Compounds **2** and **6** exhibited the most potent cytotoxicity against all three tumor cell lines with IC_{50} values ranging from 5.2 to 8.7 μg/mL when compared with adriamycin, which was used as a positive control (IC_{50} from 0.8 to 2.5 μg/mL). Compound **7** also exhibited potent cytotoxicity against L1210 (IC_{50} 4.7 μg/mL) and HL-60 (IC_{50} 5.5 μg/mL), but it showed only moderate cytotoxicity against SK-OV-3 (IC_{50} 22.5 μg/mL). Base on the results and the chemical structures of the isolated saponins, there were significant differences in the relationship between activities and chemical structures. Hederagenin was reported to be a potent cytotoxic compound against various tumor cell lines (Lee *et al.*, 2000; Park *et al.*, 2001). In this study, compound **1**, which was inactive, has a structure of hederagenin 3-*O*-α-L-arabinopyranoside and compound **2**, which was active, has a disaccharide moiety of

Table I. Cytotoxicity of compounds **1-7** from *D. asper*

Compounds	IC_{50} (μg/mL)[a]		
	L1210	HL-60	SK-OV-3
1	>30	> 30	> 30
2	7.2 ± 0.7	8.7 ± 0.5	6.3 ± 1.2
3	>30	> 30	> 30
4	> 0	> 30	> 30
5	>30	> 30	> 30
6	5.3 ± 0.7	6.7 ± 0.8	5.2 ± 0.7
7	4.7 ± 0.8	5.5 ± 1.2	22.5 ± 0.9
AM[b]	0.8 ± 0.4	2.8 ±0.2	2.5 ± 0.2

[a] IC_{50} values mean the 50% inhibition concentration and were calculated from regression lines using five different concentrations in triplicate experiments.

[b] Adriamycin was used as a positive control.

hederagenin 3-*O*-α-L-rhamnopyranosyl (1→2) α-L-arabinopyranoside. It seems that the arabinosyl moiety at C-3 blocks the activity of the aglycon, when a second sugar such as rhamnosyl is linked, the role and the position linkage are important for cytotoxic effects. Saponins, which have no substituent at C-2 of the arabinosyl moiety attached to the aglycon, exhibited no cytotoxyc activity (*Mimaki et al., 1999*). Glycoside linkage at C-2 of the first sugar could induce more electron deficiency near C-3 of the aglycone than other sites of the first sugar (Park *et al.*, 2001). This electron deficiency near C-3 due to several types of functional groups seems to correlate with their cytotoxicity. It is interesting that the rhamnose of the second sugar moiety is very common in those isolated saponins (**2**, **6**, and **7**). There are a number of saponins derived from natural sources that have a (1→2) glycoside linkage between the first and second sugar (Bhandari *et al.*, 1987). However, sugar attachments at both C-3 (-α-L-arabinopyranosyl), C-28 (β-D-glucopyranoside) in compound **3** and C-3 (-α-L-arabinopyranosyl), C-28 (β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside) in compound **5**, which have no second sugar linkage at C-3 of the aglycon, completely blocked the cytotoxicity in all cell lines. Compound **4**, which has sugars attachment at C-28 (β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside), showed no cytotoxicity. This evidence showed that ester formation and sugar moiety linkage at C-28 of the aglycon has no influence on the cytotoxic activity. In contract, both compounds having sugar attachments at C-3 (β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L arabinopyranosyl (**6**) and (β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl (**7**) inhibited cytotoxic activity entirely.

The cytotoxic results of compounds **1-7** indicate that the structure of hederagenin with 3-O-α-L-rhamnopyranosyl (1→2) α-L-arabinopyranoside plays an important role in cytotoxicity. Moreover, the presence or absence of sugar in these triterpenoids may determine their cytotoxicity and cytotoxic potency. Some of the reported significant bioactivities of saponins were their anti-inflamatory (Jung, 1995; Jung *et al.*, 2004), and anticomplementary effects (Oh *et al.*, 1999), together with this study, suggest that saponins isolated from *D. asper* can be developed as anti-disease agents.

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REFERENCES

Bhandari, P., Gray, A. I., and Rastogi, R. P., Triterpenoid saponins from *Caltha palustris*. *Planta Med.*, 53, 98-100 (1987).

Jung, H. J., Lee, C. O., Lee, K. T., Choi, J. W., and Park, H. J., Structure-activity relationship of oleanane disaccharides isolated from *Akebia quinata* versus cytotoxicity against cancer cells and NO inhibition. *Biol. Pharm. Bull.*, 27, 744-747 (2004).

Jung, K. Y., Do, J. C., and Son, K. H., Triterpene glycosides from the roots of *Dipsacus asper*. *J. Nat. Prod.*, 56, 1912-1916 (1993).

Jung, K. Y., Studies on the terpenoid constituents from the roots of *Dipsacus asper* Wall. Ph.D. Thesis, Yeungnam University (1995).

Kouno, I., Tsuboi, A., Nanri, M., and Kawano, M., Aylated triterpene glycoside from the roots of *Dipsacus asper*. *Phytochemistry*, 29, 338-339 (1990).

Lee, K. T., Sohn, I. C., Park, H. J., Kim, D. W., Jung, G. W., and Park, K. Y., Essential moiety antimutagenic and cytotoxic activity of hederagenin monodesmosides and bisdesmosides isolated from the stem bark of *Kalopanax pictus*. *Planta Med.*, 66, 329-332 (2000).

Mimaki, Y., Kuroda, M., Asano, T., and Sashida, Y., Triterpene saponins and lignans from the roots of *Pulsatilla chinensis* and their cytotoxic activity against HL-60 cells. *J. Nat. Prod.*, 62, 1279-1283 (1999).

Mosmann, T., Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, 65, 55-63 (1983).

Namba, T., The crude drugs in Japan, China and neighboring countries. Hoikusha Publishing, Osaka, (1986).

Oh, S. R., Jung, K. Y., Son, K. H., Park, S. H., Lee, I. S., Ahn, K. S., and Lee, H. K., *In vitro* anticomplementry activity of hederagenin saponins isolated from root of *Dipsacus asper*. *Arch. Pharm. Res.*, 22, 317-319 (1999).

Park, H. J., Kwon, S. H., Lee, J. H., Lee, K. H., Miyamoto, K. I., and Lee, K. T., Kalopanaxsaponin A is a basic saponin structure for the anti-tumor activity of hederagenin monodesmosides. *Planta Med.*, 67, 118-121 (2001).

Suh, H. W., Song, D. K., Son, K. H., Wie, M. B., Lee, K. H., Jung, K. Y., Do, J. C., and Kim, Y. H., Antinociceptive mechanisms of Dipsacus Saponin *C* administered intrecerebroventricularly in the mouse. *Gen. Pharmac.*, 27, 1167-1172 (1996).

Suh, H. W., Song, D. K., Huh, S. O., Son, K. H., and Kim, Y. H., Antinociceptive mechanisms of Dipsacus Saponin C administered intrathecally in mice. *J. Ethnopharmacol.*, 71, 211-218 (2000).

Tomita, H. and Mouri, Y., An iridoid glucoside from *Dipsacus asperoides*. *Phytochemistry*, 42, 239-240 (1996).

Zhang, Z. J., Qian, Y. H., Hu, H. T., Yang, J., and Yang, G. D., The herbal medicine *Dipsacus asper* Wall. extract reduces the cognitive deficits and overexpression of β-amyloid protein induced by aluminum exposure. *Life Sci.*, 73, 2443-2454 (2003).