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Novel CRF1-receptor Antagonists from *Pulsatilla koreana* Root

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Abstract – In this study, twenty-one oleanane-type triterpenoid saponins were isolated from a methanol extract of the roots of *Pulsatilla koreana.* Antagonistic activities were measured in these compounds by the aequorin based cellular functional assay system for the corticotropin releasing factor receptor (CRF1). Of them, compounds **7** - **10** showed the highest degree of CRF1 inhibition further at the concentration of 10 μM. Moreover, by the analysis based on the structure-activity relationship of isolated saponins, a sugar chain at C-3 and a carboxyl group at C-28, as well as a methyl group at C-23 seems to be key functional elements. To our knowledge, this is the first report on CRF1 inhibition of saponins from *P. koreana*.
Keywords – *Pulsatilla koreana*, Ranuculaceae, Corticotropin releasing factor receptor, Saponin

Introduction

Corticotropin-releasing factor (CRF), a 41 amino acid peptide, is a main trigger of the hypothalamic-pituitary-adrenal axis (HPA) and plays a central role in the activation of hormonal and neuronal signaling cascade as a response to stress.[1,2] The actions of CRF are mediated via CRF receptors, CRF1 receptor and CRF2 receptor, that belong to the G protein coupled receptors.[3,4] Recently, there are several evidences that CRF inhibited hair shaft elongation and promoted the apoptosis of hair matrix keratinocytes in cultured human hair follicles.[5] CRF receptor antagonist overcame the alopecia associated with chronic stress in a CRF over-expressing mouse model.[6]

Pulsatilla koreana Nakai (Ranuculaceae), a hairy, tufted, perennial herb that grows in Korea, is a traditional medicine that has been used to treat various maladies such as amoebic dysentery and malaria.[7] Phytochemical studies on *P. koreana* roots have demonstrated the presence of protoanemonin, deoxypodophyllotoxin, oleanane, and lupane-type triterpenoid saponins.[7-12] Root extracts of *P. koreana* possess antitumor, antibiotic, and anti-inflammatory activities.[9,13,14] In this report, twenty-one oleanane-type triterpenoid saponins were isolated from *P. koreana* root.[15,16] CRF1 antagonistic activities were determined against twenty-one saponins, and structure-activity relationships in these compounds were investigated for the development of cosmetic materials in the purpose of the hair loss protection.

Experimental

General experimental procedures – The NMR spectra were recorded using a JEOL ECA 600 spectrometer (^{1}H, 600 MHz; ^{13}C, 150 MHz), with tetramethylsilane (TMS) as an internal standard. Heteronuclear multiple quantum correlation (HMQC), heteronuclear multiple bond correlation (HMBC), and ^{1}H-^{1}H correlation spectroscopy (COSY) spectra were recorded using a pulsed field gradient. EI-MS spectra were obtained using A Hewlett Packard HP 5985B spectrometer, ESI-MS using an Aglient 1200 LC-MSD Trap spectrometer. Melting points were determined using an Electro thermal IA-9200 system. Column chromatography was performed using a silica gel (Kieselgel 60, 70-230, and 230-400 mesh, Merck, Darmstadt, Germany), YMC RP-18 resins, and thin layer chromatography (TLC) was performed using pre-coated silica-gel 60 F_{254} and RP-18 F_{254S} plates (both 0.25 mm, Merck, Darmstadt, Germany), the spots were detected under UV light and using 10% H_2SO_4.

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Plant material – Dried roots of *P. koreana* were purchased from herbal market, Kumsan, Chungnam, Korea in March 2009 and identified by one of the authors (Prof. Young Ho Kim). A voucher specimen (CNU 09106) was deposited at the Herbarium of College of Pharmacy, Chungnam National University, Daejeon, Korea.

Extraction and isolation – Dried roots of *P. koreana* (2.0 kg) were extracted with MeOH under reflux for 10 h (7 L × 3 times) to yield 500.0 g of extract. This extract was suspended in water and partitioned with ethyl acetate to yield 37.0 g ethyl acetate extract and 463.0 g water extract. The water extract was partitioned with *n*-BuOH to yield 130.0 g BuOH extract. The ethyl acetate extract was subjected to various column chromatography to give compounds **10** (19.0 mg) and **21** (75.0 mg). Compounds **7** (78.0 mg), **8** (2.1 g), **9** (25.0 mg), **17** (30.0 mg), **18** (120.0 mg), and **20** (180.0 mg) were isolated from *n*-BuOH extract. Compounds **1** (44.0 mg), **2** (50.0 mg), **3** (44.0 mg), **4** (78.0 mg), **5** (300.0 mg), **6** (36.0 mg), **11** (110.0 mg), **12** (17.0 mg), **13** (77.0 mg), **14** (38.0 mg), **15** (12.0 mg), **16** (460.0 mg), and **19** (196.0 mg) were isolated from water extract.[15,16]

Evaluation of CRF1 receptor antagonists – To establish the aequorin based cellular functional assay system for CRF1 receptor, the cDNA for CRF1 in pcDNA3.1$^+$ was transfected to aequorin parental cells (ES-000-A30, PerkinElmer Life and Analytical Sciences) with 4D Nucleofector electroporation system (AAF-1001X, LONZA) according to the manufacturer's instructions. Through the calcium assay protocol,[17,18] clones with the highest positive response were carefully selected and kept in culture to grow in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml zeocin and 400 μg/ml G418. Functional assays based on the luminescence of mitochondrial aequorin following intracellular calcium release was performed with established stable cells. Cells were grown at mid-log phase without antibiotics 18 hours before the day of experiment and washed with PBS/5mM EDTA followed by gently detaching with DPBS/5 mM EDTA (pH 7.4). Collected cells from culture plates were centrifuged and resuspended in assay buffer (DMEM/HAM's F12 without phenol red, with L-Glutamine, 15 mM HEPES, pH 7.0 and 0.1% BSA) at a density of 1×10^6 cells/ml. Cells were incubated for 4 hours at room temperature in the dark with 5 μM of coelenterazine h in a constant agitation. After the coelenterazine h loading, cells were diluted in assay buffer at a concentration of 1×10^5 cells/ml and incubated for 60 min. For the measurement of antagonist activity, cells were pre-injected on the antagonist at the indicated dilutions and were incubated for 15 min. Samples were tested at a concentration of 500, 10, and 1 μM with minimized dimethyl sulfoxide (DMSO) concentration. 50 μL of the reference agonist at a concentration equal to 2 × the EC_{80} was then injected and the light emission was recorded for the determination of antagonistic effects. The light emission was recorded for the determination of cell activation using EnVision excite model integrated with injectors (PerkinElmer life and analytical sciences, Boston, USA). The total light (AUC integrated signal) was used as a measurement of the response of the cells. The percent inhibition at the desired sample concentration was calculated with the resulted relative luminescence unit (RLU) data implementing the designated positive and negative controls.

Results and Discussion

Identification of compounds 1 - 21 – Twenty-one oleanane-type triterpenoid saponins were isolated from a methanol extract of the roots of *P. koreana.* Their structures were elucidated as cernuoside A (**1**),[19] hederacholchiside E (**2**),[14] beesioside Q (**3**),[20] 3-*O*-*β*-D-glucopyranosyl (1 → 4)-*β*-D-glucopyranosyl (1 → 3)-*α*-L-rhamnopyranosyl (1 → 2)[*β*-D-glucopyranosyl (1 → 4)]-*α*-L-arabinopyranosyl oleanolic acid 28-*O*-*α*-L-rhamnopyranosyl (1 → 4)-*β*-D-glucopyranosyl (1 → 6)-*β*-D-glucopyranoside (**4**),[21] hederacoside B (**5**),[22] raddeanoside R17 (**6**),[23] pulsatilla saponin I (**7**),[10] raddeanoside R13 (**8**),[10] 3-*O*-*β*-D-glucopyranosyl (1 → 4)-*β*-D-glucopyranosyl (1 → 3)-*α*-L-rhamnopyranosyl (1 → 2)[*β*-D-glucopyranosyl (1 → 4)]-*α*-L-arabinopyranosyl oleanolic acid (**9**),[24] 3-*O*-*β*-D-glucopyranosyl (1 → 4)-*β*-D-glucopyranosyl (1 → 3)-*α*-L-rhamnopyranosyl (12) [*β*-D-glucopyranosyl (1 → 4)]-*α*-L-arabinopyranosyl oleanolic acid (**10**),[24] hederacholchiside F (**11**),[14] fatsiaside G (**12**),[25] pulsatilla saponin F (**13**),[26] patrinia saponin H3 (**14**),[27] hederasaponin D (**15**),[26] cernuoside B (**16**),[19] scabioside C (**17**),[10] hederoside C (**18**),[25] pulsatilla saponin D (**19**),[28] kalopanaxsaponin H (**20**),[29] and scabioside A (**21**)[14] (Fig. 1). Their structures were elucidated on the basis of spectroscopic data and comparison of 1D- and 2D-NMR and mass spectral data with reported values.

CRF1 antagonistic effects and structure-function relationships of compounds 1 - 21 – To identify novel antagonist of CRF_1 among 21 compounds isolated from the roots of *P. koreana*. Antagonistic activities were measured in isolated compounds by the aequorin based cellular functional assay system for the CRF1. Antalarmine was used as a positive control. Of them, compounds

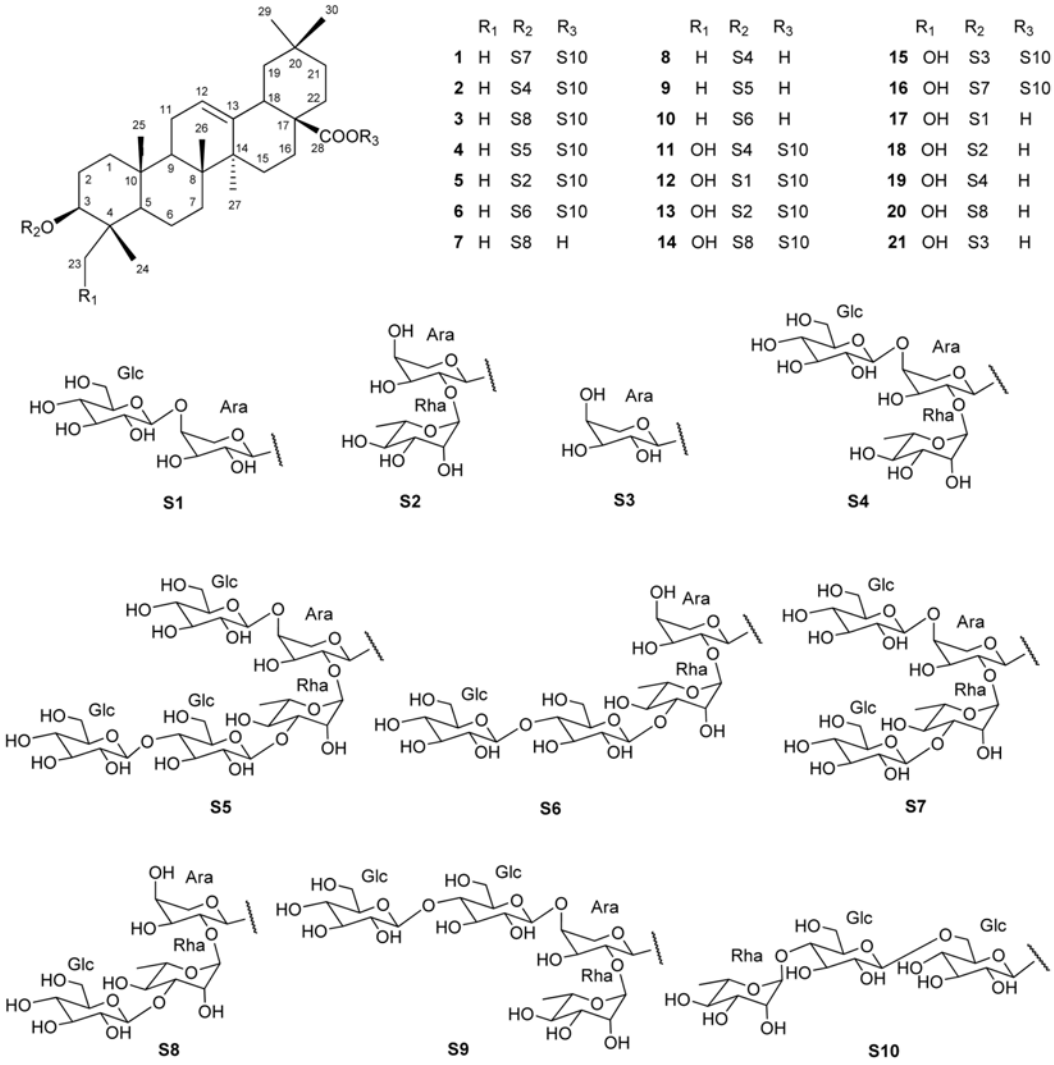

Fig. 1. Structures of compounds **1** - **21** from the roots of *P. koreana*.

7 - **10** showed the highest degree of CRF1 inhibition further at the concentration of 10 μM. In particular, compound **8** exhibited the strongest inhibition (100%) at the concentration of 1 μM (Fig. 2). Moreover, these results allowed us to determine information regarding the structure-function relationship of isolated saponins. Compounds **7** - **10** showed the strongest activity, and they have common structures. The effects increased significantly when C-3 of the aglycone was linked to a sugar chain and C-28 was linked to a carboxyl group. Compounds **1** - **6** and **11** - **16**, which linked to two sugar chains at C-3 and C-28, showed negligible effects (0 - 43.0%) at 500 μM. This suggests that bisdesmosidic saponins lacked activity in this bioassay system. Therefore, a sugar chain at C-3 and a carboxyl group at C-28 are key functional elements. Moreover, compounds **17** - **21** showed

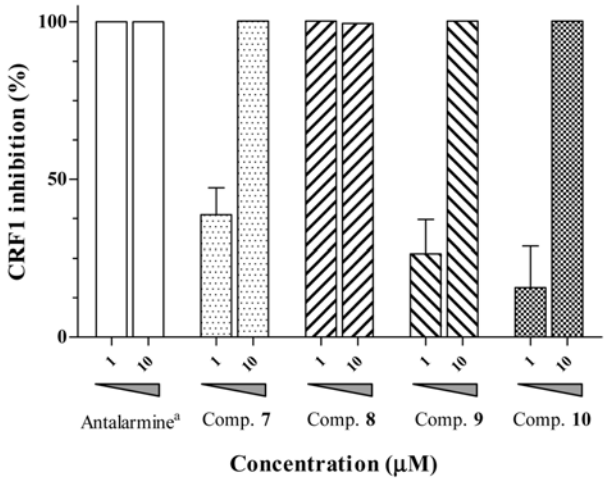

Fig. 2. CRF1 antagonistic activities of compounds **7** - **10**. [a]Antalarmine: positive control.

strongest inhibition (100%) at 500 μM. However, the inhibitory effects of compounds **17** -**20** were weaken seriously at 10 μM (0 - 16.4%), and compound **21** showed moderate effect (80.0%). By comparison with compounds **7** - **10**, compounds **17** - **21** contains a hydroxyl group at C-23 of the aglycone. This suggests that the methyl group at C-23 also plays an important role. In addition, a monosaccharide moiety (compound **21**) at C-3 may increases inhibitory activity which compared with compounds **17** - **20.** These results were similar to the structure-function relationships of anti-inflammatory and anticancer activity of these compounds.[30,31]

In conclusion, present study reported the phytochemistry investigation of *P. koreana* roots and their CRF1 antagonistic activities. To the best of our knowledge, this is the first report on CRF1 inhibition of triterpenoid saponins from *P. koreana*. These results might contributes to the development of cosmetic materials in the purpose of the hair loss protection.

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References

(1) Aguilera, G. *Trends Endocrinol. Metab.* **1998**, *9*, 329-336.
(2) Slominski, A. T.; Zmijewski, M. A.; Zbytek, B.; Tobin, D. J.; Theoharides, T. C.; Rivier, J. *Endocr. Rev.* **2013**, *34*, 827-884.
(3) Grammatopoulos, D. K.; Chrousos, G. P. *Trends Endocrinol. Metab.* **2002**, *13*, 436-444.
(4) Hillhouse, E. W.; Randeva, H.; Ladds, G.; Grammatopoulos, D. *Biochem. Soc. Trans.* **2002**, *30*, 428-432.
(5) Ito, N.; Ito, T.; Kromminga, A.; Bettermann, A.; Takigawa, M.; Kees, F.; Straub, R. H.; Paus, R. *FASEB J.* **2005**, *19*, 1332-1334.
(6) Slominski, A.; Zbytek, B.; Szczesniewski, A.; Semak, I.; Kaminski, J.; Sweatman, T.; Wortsman, J. *Am. J. Physiol. Endocrinol. Metab.* **2005**, *288*, E701-706.
(7) Kim, Y.; Bang, S. C.; Lee, J. H.; Ahn, B. Z. *Arch. Pharm. Res*. **2004**, *27*, 915-918.
(8) Kim, Y.; You, Y. J.; Nam, N. H.; Ahn, B. Z. *Bioorg. Med. Chem. Lett*. **2002**, *12*, 3435-3438.
(9) Martin, L. M.; San Román L.; Domínguez, A. *Planta Med*. **1990**, *56*, 66-69.
(10) Bang, S. C.; Kim, Y.; Lee, J. H.; Ahn, B. Z. *J. Nat. Prod*. **2005**, *68*, 268-272.
(11) Lee, K. Y.; Cho, Y. W.; Park, J.; Lee, D. Y.; Kim, S. H.; Kim, Y. C.; Sung, S. H. *Phytochem. Anal*. **2010**, *21*, 314-321.
(12) Yeom, H. S.; Suh, J. H.; Youm, J. R.; Han, S. B. *Bull. Kor. Chem. Soc*. **2010**, *31*, 1159-1164.
(13) Cuong, T. D.; Hung, T. M.; Lee, M. K.; Thao, N. T. P.; Jang, H. S.; Min, B. S. *Nat. Prod. Sci*. **2009**, *15*, 250-255.
(14) Yang, H.; Cho, Y. W.; Kim, S. H.; Kim, Y. C.; Sung, S. H. *Phytochemistry* **2010**, *71*, 1892-1899.
(15) Li, W.; Sun, Y. N.; Yan, X. T.; Yang, S. Y.; Lee, S. J.; Byun, H. J.; Moon, C. S.; Han, B. S.; Kim, Y. H. *Molecules* **2013**, *18*, 5306-5316.
(16) Li, W.; Yan, X. T.; Sun, Y. N.; Ngan, T. T.; Shim, S. H.; Kim, Y. H. *Biomol. Ther*. **2014**, *22*, 334-340.
(17) Dupriez, V. J.; Maes, K.; Le Poul, E.; Burgeon, E.; Detheux, M. *Receptor Channel* **2002**, *8*, 319-330.
(18) Noh, H.; Lee, S. *Convergence Information Tech.* **2013**, *8*, 400-406.
(19) Zhang, Q.; Ye, W.; Yan, X.; Zhu, G.; Che, C. T.; Zhao, S. *J. Nat. Prod*. **2000**, *63*, 276-278.
(20) De Tommasi, N.; Autore, G.; Bellino, A.; Pinto, A.; Pizza, C.; Sorrentino, R.; Venturella, P. *J. Nat. Prod*. **2000**, *63*, 308-314.
(21) Liu, J. Y.; Guan, Y. L.; Zou, L. B.; Gong, Y. X.; Hua, H. M.; Xu, Y. N.; Zhang, H.; Yu, Z. G.; Fan, W. H. *Molecules* **2012**, *17*, 5520-5531.
(22) Majester-Savornin, B.; Elias, R.; Diaz-Lanza, A. M.; Balansard, G.; Gasquet, M.; Delmas, F. *Planta Med*. **1991**, *57*, 260-262.
(23) Sun, Y.; Li, M.; Liu, J. *Int. Immunopharmacol*. **2008**, *8*, 1095-1102.
(24) Mimaki, Y.; Kuroda, M.; Asano, T.; Sashida, Y. *J. Nat. Prod*. **1999**, *62*, 1279-1283.
(25) Li, X. C.; Wang, D. Z.; Wu, S. G.; Yang, C. R. *Phytochemistry* **1990**, *29*, 595-599.
(26) Quang, T. H.; Ngan, N. T.; Minh, C. V.; Kiem, P. V.; Nhiem, N. X.; Tai, B. H.; Thao, N. P.; Tung, N. H.; Song, S. B.; Kim, Y. H. *J. Nat. Prod.* **2011**, *74*, 1908-1915.
(27) Kang, S. S.; Kim, J. S.; Kim, Y. H.; Choi, J. S. *J. Nat. Prod*. **1997**, *60*, 1060-1062.
(28) Shimizu, M.; Shingyouchi, K.; Morita, N.; Kizu, H.; Tomimori, T. *Chem. Pharm. Bull*. **1978**, *26*, 1666-1671.
(29) Ye, W. C.; Ji, N. N.; Zhao, S. X.; Liu, J. H.; Ye, T.; McKervey, M. A.; Stevenson, P. *Phytochemistry* **1996**, *42*, 799-802.
(30) Li, W.; Ding, Y.; Sun, Y. N.; Yan, X. T.; Yang, S. Y.; Choi, C. W.; Cha, J. Y.; Lee, Y. M.; Kim, Y. H. *Chem. Pharm. Bull.* **2013**, *61*, 471-476.
(31) Li, W.; Ding, Y.; Sun, Y. N.; Yan, X. T.; Yang, S. Y.; Choi, C. W.; Kim, E. J.; Kang, H. K.; Kim, Y. H. *Arch. Pharm. Res*. **2013**, *36*,768-774.

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