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Chemical constituents of the *Ajuga multiflora* bunge and their protective effects on dexamethasone-induced muscle atrophy in C2C12 myotubes

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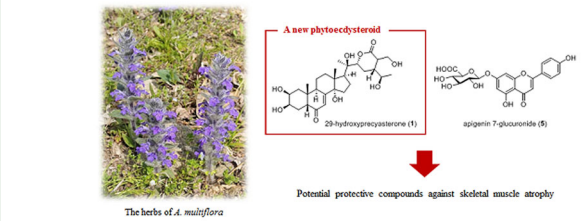
ABSTRACT

Ajuga multiflora Bunge is a perennial ornamental herb and has been used for the treatment of fever in Korean folk medicine. In the course of searching for protective agents associated with the potential of *A. multiflora* against dexamethsone (DEX)-induced muscle atrophy, a new phytoecdysteroid, 29-hydroxyprecyasterone (**1**), together with four known compounds (**2**–**5**), were isolated from *A. multiflora*. The structures of the compounds were determined by spectroscopic analyses, including 1D-, 2D-NMR and HR-MS interpretation. To elucidate the effects of obtained compounds on DEX-induced muscle atrophy, the myotubes diameter, myosin heavy chain (MyHC) positive area, and fusion index were evaluated by immunofluorescence staining. Overall, each compound treatment effectively prevented the atrophic myotubes through an increase of MyHC-positive myotubes and the number of nuclei. Particularly, the measurement of myotube diameter showed that compounds **1** and **5** treatment significantly alleviated the myotube thickness.



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1. Introduction

Skeletal muscle accounts for approximately 40–50% of the human body and has important functions in metabolism, energy consumption, physical strength, and physical performance. Maintenance of muscle mass is dependent on the relative balance between protein synthesis and degradation (Sanchez et al. 2013). Skeletal muscle atrophy characterized by protein degradation is often caused by multiple pathophysiologic conditions. It generates exercise intolerance and an inability to perform daily activity because of muscle weakness and fatigue, which leads to a poor quality of life (Powers et al. 2016).

Glucocorticoids (GC) are a vital family of regulatory molecules in the body, as well as the most widely used and most effective anti-inflammatory and immunosuppressant agents. However, sustained high-dose administration of glucocorticoids can cause a series of side effects such as hyperglycemia, weight loss, osteoporosis, depression, hypertension, and skeletal muscle atrophy (Bodine and Furlow 2015). Dexamethasone (DEX), as a commonly used synthetic glucocorticoid, induces muscle atrophy, which involves both induction of protein degradation and suppression of general protein synthesis (Schiaffino et al. 2013).

C2C12 are murine skeletal myoblasts originally derived from satellite cells, and have been used as a model system for the study of skeletal muscle development (Burattini et al. 2004). Treatment of C2C12 mytotubes with DEX is widely used to induce muscle atrophy in both in vivo and in vitro models (Sandri et al. 2004). Therefore, a DEX-induced cell atrophy model in this study is established to clarify the effects of active ingredients from medicinal herbs.

Ajuga multiflora Bunge (Korean pyramid bugle) is a Labiatae perennial herb, and distributed in Korea, China, and Japan. *A. multiflora* has been used in traditional medicine, in the treatment of fever, pneumonia, tonsillitis, bronchitis, acute cholecystitis, liver problem, and contusion. Previous phytochemical investigations on the genus *Ajuga* have been reported to the isolation of phytoecdysteroids (Yu et al. 1998; Chen et al. 2018), diterpenes (Yang et al. 2019), flavonoids (Qing et al. 2017), iridoid glycosides (Chung and Yoo 1985; Frezza et al. 2017), triterpenoids (Venditti et al. 2016; Li et al. 2020) and essential oils (Karami 2017). Among these components, *A. multiflora* is reported to contain a number of phytoecdysteroids, which have been shown to possess pesticidal activity against several insect pests (De-fu et al. 2002). The MeOH extract of *A. multiflora* has been found to exhibit cytotoxicity and antimicrobial activity against murine leukemia tumor (P388D1) cell lines (Ryu et al. 2000). However, the protective effects of components from *A. multiflora* on DEX-induced muscle atrophy have not been established.

The aim of this study was to investigate the protective molecules from *A. multiflora* against DEX-induced muscle atrophy. Five compounds, including a new phytoecdysteroid (**1**) and four known compounds (**2**–**5**) were isolated and structurally characterized from MeOH extract from *A. multiflora*. Thereafter, all isolated compounds were evaluated for their protective effects on DEX-induced muscle atrophy in cultured C2C12 myotubes.

Herein, this paper describes the structural identification of a new compounds and biological assessment of obtained compounds associated with the potential of *A. multiflora* in skeletal muscle atrophy induced by DEX.

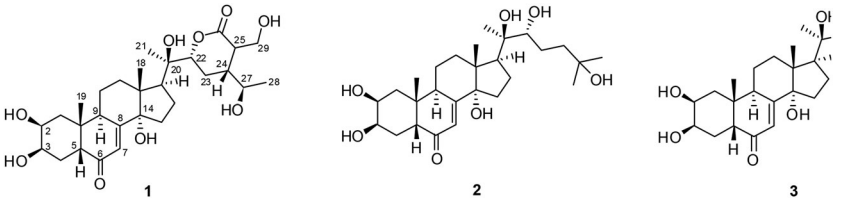

Figure 1. Chemical structures of compounds **1**–**5** isolated from *A. multiflora*.

2. Results and discussion

2.1. Isolation and identification of compounds from A. multiflora

The main observed peaks of MeOH extract from *A. multiflora* presented HPLC chromatograms were isolated and identified as four phytoecdysteroids (**1**–**4**) and a flavonoid (**5**), including a new compound (**1**) (Figures 1 and S1).

Compound **1** possesses the molecular formula as $C_{29}H_{44}O_9$ by high-resolution mass spectrometry (HR-MS) data ($m/z = 537.3065$ $[M+H]^+$ calcd for $C_{29}H_{45}O_9^+$, 537.3058) (Figure S2). According to previous papers, the characteristic fragmentation patterns of ecdysteroids yielded ions of successive losses of water molecules, partial loss of the side-chain, and cleavage of the skeleton bonds (Wang et al. 2008). In the case of compound **1**, the observed ions at *m/z* 519, 501, 483, 465 result from consecutive loss of multiple water molecules. The fragment ions with *m/z* 303, 301 corresponded to the loss of one water molecule and then side-chain cleavage between C-17 and C-20 (Figure S3). These fragmentation patterns are identical with that of other isolated ecdysteroids (**2**–**4**). Further, the partial structure from C-1 to C-22 is concluded to be identical with that of compounds **2**–**4** from the UV maximum at 247 nm and the NMR signals. The ^{1}H-NMR data showed four tertiary methyl groups appeared at δ 0.76 (3H, s, Me-18), 0.83 (3H, s, Me-19), 1.06 (3H, s, Me-21), and 1.33 (3H, d, $J = 6.1$ Hz, Me-28) and one olefinic proton at δ 5.63 (1H, s, H-7) (Figure S4). The ^{13}C-NMR spectrum indicated the presence of 29 carbon signals, including a ketone group (δ_C 202.6), double bond carbons (δ_C 120.5 and 165.1) and 2β-, 3β-, 14α-, 20*R* hydroxyl groups (δ_C 66.8, 66.6, 83.0 and 75.7) in accord with the occurrence of 7-en 6-one system strongly suggested that this compound is a C_{29} phytoecdysone (Figure S5). The skeleton from C-22 to C-29 of **1** were assigned by ^{1}H-^{13}C HSQC experiment (Figure S6). Furthermore, the connectivity of seven protons (H-22/H-23, H-23/H-24, H-24/H-25, H-25/H-29, H-24/H-27 and H-27/H-28) was revealed by the ^{1}H–^{1}H COSY experiment (Figure S7 and S8). The HMBC experiment exhibited cross-peaks from H-25 to C-26 and H-29 to C-26, confirmed the correlation between C-25 and C-26 (Figure S7 and S9). The above spectroscopic data suggested that the side-chain contains a δ-lactone ring.

The large coupling constant ($J_{24,\ 27}$) showed that the relative configuration of H-24 and H-27 is a *trans*-conformation in the ^{1}H NMR spectrum (Figure S4). Also, the relative configuration of C-25 was assigned by the NOESY correlation between H-25 and H-27 (Figure S10). The absolute stereochemistry of C-27 was determined by modified Mosher's method. Compound 1 was treated with (*S*)-(+)-and (*R*)-(-)-MTPA-Cl to afford the (*S*) and (*R*) MTPA esters of **1**. The positive $\Delta\delta$ values (δ_S–δ_R) of H-28 in the ^{1}H NMR spectrum were observed, while the negative $\Delta\delta$ values of H-24 were shown. This result

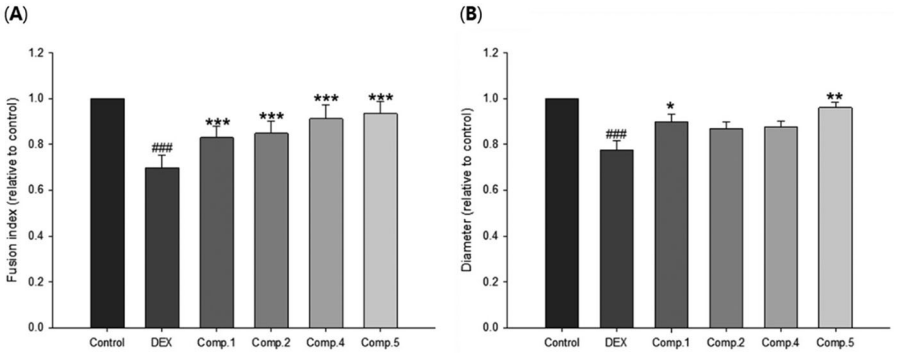

Figure 2. Effects of isolates on fusion index and myotube diameter in DEX-induced C2C12 myotubes. C2C12 myotubes were incubated with 10 μM of DEX and/or 10 μM of compounds (**1**, **2**, **4** and **5**). (A) Quantification of the fusion index in DEX-induced C2C12 myotubes. The fusion index was calculated as the ratio of the nuclei number in myotubes with two or more nuclei versus the total number of nuclei. (B) Quantification of the myotube diameter in DEX-induced C2C12 myotubes. These data are expressed as mean ± SD of three independent experiments. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. control; $^{*}p < 0.005$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. DEX.

revealed the absolute stereochemistry of C-27 to be *R* configuration, and then all the stereochemistry of this side-chain was assigned. Accordingly, these results indicated 24β, 25α, 27α- configuration. The structure of compound **1** was superimposable to those of the already reported compound, precyasterone (Hikino et al. 1970), except the presence of a hydroxy group at C-29, and was elucidated as 29-hydroxy precyasterone.

The previously reported compounds were identified to be 20-hydroxyecdysone (**2**) (Yu et al. 1998), makisterone A (**3**) (Le Duc Dat et al. 2012), cyaserone (**4**) (Sadati et al. 2012), and apigenin 7-glucuronide (**5**) (Yu et al. 1998) by comparing the UV, MS, and NMR spectroscopic data with published values.

2.2. Protective effects of isolated compounds against DEX-induced muscular atrophy in cultured C2C12 myotubes

The four compounds isolated from *A. multiflora*, except compound **3** which is not determined due to a limited amount of the sample, were evaluated for protection effects to C2C12 myotubes by DEX-induced muscle atrophy. First, the cell viability was determined to evaluate the cytotoxicity of isolates by using ELUS cell viability assay kit. C2C12 myoblasts were induced to differentiate in differentiation medium, followed by treatment with 10 μM DEX along with vehicle or isolates for an additional 24 h. Even though cell viability was decreased to 70% with 10 μM of DEX, treatment with each compound did not affect cell viability (Figure S11A). To examine the effects of isolates on atrophic myotubes, C2C12 myotubes were then subjected to immunofluorescence staining for myosin heavy chain (MyHC), is one of the muscle specific proteins, to assess myotube formation. All isolates treatment enhanced the formation of MyHC-positive myotubes (Figure S11B and S11C). As expected, DEX treatment blocked myotube formation, whereas all obtained compounds treatment in DEX-treated C2C12 cells restored myotube formation, as evidenced by the presence of large amounts of

myotubes and nuclei within MyHC-positive myotubes (Figure 2(A)). In particular, quantification of myotube diameter indicated that treatment with DEX induced a reduction in myotube diameter, which was partially recovered by treatment with compounds **1** and **5** (Figure 2(B)).

3. Experimental

3.1. General procedures

Open column chromatography (CC) was performed with Diaion HP-20 (Mitsubishi Chemical Co.). Analytical HPLC was conducted using the Agilent 1260 HPLC system with Capcell pak C18 UG120 (4.6 × 250 mm i.d., 5 μm, Shiseido, Japan). Semi-preparative HPLC was performed using a Gilson 321 pump and a Waters 2487 UV/VIS detector, with a INNO C18 column (20.0 × 250 mm, 5 μm; Youngin Biochrome, Korea). HR-MS data were acquired using an Agilent 6530 quadrupole time-of-flight (QTOF) mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). NMR experiments were conducted on a Bruker 400 MHz using TMS as an internal standard and chemical shifts are expressed as δ values. All solvents used for the chromatographic separations were distilled before use.

3.2. Plant material

The herbs of *A. multiflora* were collected from a Hanyang University Herbarium (Hanyang University, Ansan, Gyeonggi-do 426-791, Republic of Korea). The voucher specimen (HYUP-AM-001) was deposited in the laboratory.

3.3. Extraction and isolation

The dried herbs of *A. multiflora* were extracted three times with MeOH under reflux. The solution was filtered and concentrated by rotary evaporator. After freeze-drying, a 62.39 g extract was obtained. The MeOH extract was subjected to Diaion HP-20 column chromatography and eluted with increasing concentration of MeOH in water (0:1 to 1:0, *v/v*), affording 12 subfractions (F1 to F12). Subfractions F6 (1.13 g) and F7 (1.03 g) were further separated by a preparative HPLC on a INNO C18 column, using 15 or 17% aqueous acetonitrile containing 0.1% formic acid (v/v) to isolate compounds **1**–**5**.

3.3.1. 29-Hydroxyprecyasterone (1)

UV (MeOH) λ_{max} 247 nm; ESI-QTOF-MS (positive mode) m/z 537.3065 $[M+H]^+$ (calcd. for $C_{29}H_{45}O_9^+$, 537.3058); ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.76 (3H, s, Me-18), 0.83 (3H, s, Me-19), 1.06 (3H, s, Me-21), 1.33 (3H, d, J = 6.1 Hz, Me-28), 1.85 (2H, m, H-16), 2.02 (2H, td, J = 13.0, 4.6 Hz, H-12), 2.28 (2H, m, H-24), 2.46 (1H, d, J = 3.3 Hz, H-25), 3.00 (1H, m, H-9), 3.31 (1H, d, J = 11.0 Hz, H-22), 4.21 (1H, dq, J = 12.2, 6.0 Hz, H-27), 5.63 (1H, s, H-7); ^{13}C NMR (100 MHz, DMSO-d_6) δ 36.6 (C-1), 66.8 (C-2), 66.6 (C-3), 31.5 (C-4), 50.1 (C-5), 202.6 (C-6), 120.5 (C-7), 165.1 (C-8), 33.1 (C-9), 37.6 (C-10), 20.1 (C-11), 30.8 (C-12), 46.9 (C-13), 83.0 (C-14), 30.4 (C-15), 20.2 (C-16), 48.6 (C-17), 17.2 (C-18), 23.9 (C-

19), 75.7 (C-20), 20.8 (C-21), 73.6 (C-22), 34.0 (C-23), 41.0 (C-24), 49.8 (C-25), 177.0 (C-26), 79.7 (C-27), 19.7 (C-28), 58.9 (C-29).

3.4. Mosher's ester method

(*S*)- or (*R*)-MTPA esters of compound **1** were prepared using a Mosher's esterification method previously described (Kelly 1999). Dissolved compound (2 mg, 3.73 μmol) in pyridine-d_5 (550 μL) was divided equally into a NMR tubes, respectively. (*S*)-(+) or (*R*)-(-) MTPA-Cl (6 μL, 31.76 mol) were added into the each NMR tubes immediately under a N_2 gas stream. The NMR tubes reacted at room temperature for 24 h. After the chemical reaction has finished, the (*R*) and (*S*) MTPA ester derivatives of **1** were determined with 400 MHz NMR, respectively.

3.5. Cell culture and treatment

Mouse myoblasts, C2C12 cells, were purchased from American Type Culture Collection (ATCC, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Welgene, Gyeongsan, South Korea) and 1% penicillin/streptomycin (P/S) (Invitrogen Co., NY, USA) at 37 °C in a humidified 5% CO_2 atmosphere. To evaluate the anti-muscle atrophy effect, C2C12 myoblasts were plated onto 12-well plates at a density of 5×10^4 cells/well. Then, cells were grown to 80-90% confluence in DMEM supplemented with 10% FBS and 1% P/S at 37 °C for 48 h. Afterwards, the medium was replaced with DMEM containing 2% horse serum (HS) and 1% P/S to induce differentiation into myotubes for 5 days, and the medium was changed every 2 days. The fully differentiated myotubes were co-treated with 10 μM compounds (**1**, **2**, **4**, and **5**) and 10 μM DEX for 24 h.

3.6. Cell viability assay

The effect of compounds (**1**, **2**, **4**, and **5**) and DEX on C2C12 myotubes was evaluated using an ELUS cell viability assay kit (Biosesang, Seongnam, Korea) according to the manufacturer's instructions. Briefly, differentiated C2C12 myotube cells (1×10^4 cells/well) cultured onto 48-well plates and then incubated with each compound (10 μM) with DEX (10 μM) for 24 h at 37 °C in a humidified atmosphere of 5% CO_2 incubator. Subsequently, 20 μL of 2-(2-Methoxy-4-nitrophenyl)-3- (4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium Sodium Salt (WST-8) solution was added to each well followed by a 4 h incubation, and the absorbance was measured using an Enspire® Multimode Plate Reader (PerkinElmer, MA, USA) at 450 nm.

3.7. Immunofluorescence staining

C2C12 myotubes were fixed with 4% paraformaldehyde for 10 min at room temperature and washed 3 times with PBS. Subsequently, permeabilized with 0.1% Triton X-100 for 15 min in phosphate-buffered saline (PBS). After permeabilization, blocking

was performed with BSA for 1 h at room temperature. After blocking, the cells were stained with MyHC primary antibody (1:100, Santa Cruz, TX, USA) overnight at 4 °C. After washing 3 times with 0.1% PBST, cells were incubated with secondary antibody conjugated with Alexa Fluor 488 (1:500, Proteintech Group, Shanghai, China) at 37 °C for 1 h. Nuclei were counterstained with 1 μM of 4-6-diamidino-2-prenylindole (Sigma-Aldrich, MO, USA). The myotubes immunofluorescence was observed using fluorescence microscope JuLI^{TM} stage (NanoEnTek Inc., MA, USA). Myotubes diameters, MyHC positive area, and fusion index were measured using ImageJ analysis software and calculated according to previous study (Yamaguchi et al. 2010).

4. Conclusions

The new phytoecdysteroid (**1**) and four known compounds (**2**–**5**) were isolated from the MeOH extract of *A. multiflora*. The structure of the new compound (**1**) was elucidated by its physical and spectroscopic data, and its absolute configuration was determined by Mosher's ester method. The isolates were screened for protective activity against DEX-induced muscle atrophy in C2C12 myotubes. Each compound treatment led to enhance in myotube formation through an increase of MyHC-positive area and fusion index. Among the compounds, compounds **1** and **5** partially enlarged myotube diameter in DEX-induced atrophic myotubes. These findings, for the first time, provide protective effects of components from *A. multiflora* on skeletal muscle atrophy induced by DEX and suggest that compounds **1** and **5** might be potential herbal medicinal resources to intervene muscle weakness and atrophy associated with various diseases.

Disclosure statement

The authors declare no conflict of interest.

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