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Screening and Biotransformation of Interleukin-1β Converting Enzyme Production Inhibitors from *Arctii fructus*

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Abstract Five dibenzylbutyrolactones were isolated from a methanol extract of *Arctii fructus* (*Arctium lappa* L.) by bioassay-guided isolation, using the interleukin-1β converting enzyme (caspase-1, ICE) production inhibitory assay *in vitro*. These compounds were spectroscopically identified as lappaol E (1), lappaol A (2), matairesinol (3), arctigenin (4), and arctiin (5). Among the compounds tested, arctigenin (4) showed the strongest inhibitory activity for ICE production in IL-1β-induced proliferation of D10S cells. Western blot analysis demonstrated that the arctigenin suppressed the expression of ICE protein in a dose-dependent manner. To estimate the biotransformation of *Arctii fructus in vivo* by human intestinal bacteria, we carried out an anaerobic incubation of the *Arctii fructus* extract with a human fecal suspension. From the HPLC analysis of metabolites, Arctiin (IC_{50}=74.2 μg/ml), a major component of *Arctii fructus*, was transformed to aglycone, arctigenin (IC_{50}=12.5 μg/ml), by human intestinal bacteria. The ICE production inhibitory activity of *Arctii fructus* would be much stronger *in vivo* than *in vitro* due to the biotransformation by human intestinal bacteria.

Key words: *Arctii fructus*, interleukin-1β converting enzyme, D10S cells

IL-1β-converting enzyme (ICE, caspase-1) is the prototype of a family of cysteine proteases, termed caspases, which share the active site cysteine and aspartate binding clefts [1–5]. The biological function of ICE was originally thought to be restricted to the maturation process of IL-1β, a central mediator in the cytokine network. Proteolytic maturation of the inactive 33-kD IL-1 precursor (proIL-1) into the 17-kD, a biologically functional form, results from cleavage at the Asp^{116}-Ala^{117} site [19]. IL-1 is a proinflammatory cytokine produced by activated macrophages and monocytes [18], and IL-1 functions in the generation of systemic and local responses to infection, injury, and immunological challenges, and it is the primary cause of chronic and acute inflammation [7, 8]. ICE inhibitors have been shown to prevent inflammation in several acute models, suggesting that ICE inhibitors would be useful as anti-inflammatory drugs, thus providing inhibition of ICE as a new anti-inflammatory strategy [15]. In our previous screening studies on more than 300 kinds of Korean medicinal plants, the methanol (MeOH) extract of *Arctii fructus* showed a significant inhibitory effect on the production of ICE in T-helper type 2 D10S cells. *Arctii fructus* (*Arctium lappa* L.) is distributed in Korea, China, and Japan, and it has been used as an anti-inflammatory, detoxifying, and diuretic agent in folk remedies [6].

In this study, five known dibenzylbutyrolactone lignans were isolated from the MeOH extract of *Arctii fructus* by activity-guided fractionation, which monitored the inhibitory effect of each fraction on the production of ICE in IL-1β-induced D10S cells. Specifically, arctigenin was found to possess a significant ICE production inhibitory ability. These results suggest a pharmacological basis for arctigenin as a traditional herbal medicine for the treatment of inflammation. In addition, we examined the *in vivo* biotransformation of *Arctii fructus* by human intestinal bacteria and the inhibitory effect of its metabolites on the production of ICE in IL-1β-induced proliferation of D10S cells.

Materials and Methods

Plant Material

Arctii fructus was purchased from a drug store in Taejon, Korea as herbal medicine, which was identified by Dr.

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Sang-Myung Lee and is used for Korean traditional prescriptions. The voucher specimens (IM-00-25) are deposited in our laboratory.

Extraction and Isolation

Air-dried *Arctii fructus* (600 g) was percolated three times with MeOH at room temperature. The MeOH extract (25 g) was concentrated, suspended in H_2O, and sequentially partitioned with ethyl acetate (EtOAc) and butanol (BuOH). The EtOAc-soluble fraction (6 g) was subjected to silica gel (Merck Kieselgel 60; 0.063-0.2 mm particle size; 6× 35 cm) column chromatography with $CHCl_3$-MeOH gradient systems [0% to 50% MeOH in $CHCl_3$ (v/v), 1,000 ml each, 8 fraction]. Gel filtration on Sephadex LH-20 (3×80 cm) of active fraction (1 g) eluted with MeOH-H_2O (8:2) gave two fractions (Fractions I and II). Fraction I (240 mg) was concentrated *in vacuo* and chromatographed on a reversed-phase HPLC with an eluting solvent system of acetonitrile-water (3:7) (YMC-ODS-AM 250×6 mm, 10 μm; flow rate, 1.5 ml/min; detection, UV at 220 nm) to yield compound **1** (2.5 mg). Fraction II (90 mg) was concentrated *in vacuo* and chromatographed on a reversed-phase HPLC with an eluting solvent system of acetonitrile-water (4:6) (YMC-ODS-AM 250×6 mm, 10 μm; flow rate, 1.5 ml/min; detection, UV at 220 nm) to yield compounds **2** (4 mg), **3** (10 mg), and **4** (12 mg). BuOH-soluble fraction (1 g) was fractionated by Sep-pak C_{18} cartridge (5 g), eluting with H_2O and increasing proportion of MeOH (10:1-1:1). The active fraction (40 mg) was subjected to reversed-phase HPLC, using a gradient from 20 to 100% CH_3CN in H_2O over 40 min (YMC-ODS-AM 250×6 mm, 10 μm; flow rate, 1.5 ml/min; detection, UV at 220 nm) to yield compound **5** (6 mg).

Methylation of Arctigenin

Arctigenin was methylated with dimethylsulfate (10 mg) and potassium carbonate anhydrous (0.1 g) in acetone for 12 h at room temperature. The solvent was removed by evaporation under reduced pressure, and the residue was purified by a reversed-phase column chromatography to yield O-methylated derivative of arctigenin (**4a**).

Lappaol E (**1**): colorless needles (MeOH), ESI-MS *m/z* = 553 $[M-H]^-$, 577 $[M+Na]^+$, $[\alpha]_D^{20}$ -26.56° (*c* 1.0, MeOH).

Matairesinol (**2**): colorless needles (MeOH), ESI-MS *m/z* =357 $[M-H]^-$, 481 $[M+Na]^+$, $[\alpha]_D^{20}$ -40° (*c* 0.1, EtOH).

Lappaol A (**3**): colorless needles (MeOH), ESI-MS *m/z* =535 $[M-H]^-$, 559 $[M+Na]^+$, $[\alpha]_D^{20}$ -17.4° (*c* 1.0, MeOH).

Arctigenin (**4**): colorless needles (MeOH), ESI-MS *m/z* = 371 $[M-H]^-$, 395 $[M+Na]^+$, $[\alpha]_D^{20}$ -37.7° (EtOH).

4-Methyl-arctigenin (**4a**): white gum, ESI-MS *m/z*=385 $[M-H]^-$, 409 $[M+Na]^+$.

Arctiin (**5**): white amorphous powder, ESI-MS *m/z*=533 $[M-H]^-$, 557 $[M+Na]^+$, $[\alpha]_D^{20}$ -51.5° (*c* 2.0, EtOH).

Cell Culture

D10S cells, a subclone of the murine D10G.1.helper T-cell, were maintained in RPMI 1640 medium (Gibco/BRL, Gaithersburg, MD, U.S.A.) supplemented with 5% heat-inactivated fetal bovine serum (Gibco/BRL, Gaithersburg, MD, U.S.A.) and 10% (v/v) mouse-conditioned medium, as described [12, 20]. Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Interleukin-1β-Converting Enzyme (ICE) Production *In Vitro*

D10S cells, grown in RPMI supplemented with 5% FBS and 10% mouse conditioned medium, were harvested, washed twice with cold PBS, and cultured at the concentration of 2.5×10^4 cells/well in RPMI containing 5% FBS. The cultures were treated with various concentrations of compounds to be tested and stimulated with 2 ng/ml of IL-1β. The cells were incubated for 48 h at 37°C in a 5% CO_2 air atmosphere. After observing the cells on a microscope, the ICE activity was estimated from the cell lysate, using Ac-YVAD-AFC as a substrate. The cells were lysed with a TTE buffer (10 mM Tris-HCl, 0.5% Triton ×100, 10 mM EDTA, pH 8.0) and kept on ice for 30 min, and then centrifuged. The enzyme reactions were performed in a buffer (100 mM HEPES, 10 mM DTT, 10% sucrose, 0.1% CHAPS, 0.1% BSA), supplemented with 100 μM Ac-YVAD-AFC, at 37°C for 4 h. The released fluorescent was measured by using a spectrofluorimeter (Perkin-Elmer LS50B) [12]. The excitation and emission wavelengths were 400 nm and 505 nm, respectively.

Cytotoxicity Assay

Cytotoxicity of the test compounds was evaluated by MTS assay [10]. In the MTS assay, the cell suspension, having a concentration of 2.5×10^4 cells/well, was plated (100 μl) in 96-well microculture plates. After seeding, the test compounds at various concentrations were added to the plate and incubated for 48 h. The MTS/PMS solution was prepared by mixing 25 μl of PMS (1.53 mg/ml in PBS) for every 975 μl of MTS (1.71 mg/ml in PBS). Finally, 50 μl of MTS/PMS solution was added to each well and incubated for 1 to 3 h. Absorbance of formazan at 490 nm was measured directly from 96-well assay plates without additional processing.

Western Blot Analysis

D10S cells were incubated with various concentrations of arctigenin and 2 ng/ml of IL-1β for 48 h. Cells lysate was subjected to SDS-PAGE (8%) followed by Western blotting, using the antibody against rabbit polyclonal caspase-1 (Sigma, St. Louis, MO, U.S.A.). Protein expression was detected by using the ECL detection kit (Amersham Int., Arlington Heights, IL, U.S.A.). The expression of β-actin was used as a normalizing control.

Preparation of Human Intestinal Bacterial Mixture

Fresh feces (5 g) obtained from a healthy subject was homogenized in 100 ml of General anaerobic medium (GAM) broth (Nissui Co., Tokyo, Japan), and the sediments were removed by decantation to give a 5% human intestinal bacterial mixture [23].

Incubation of *Arctii fructus* with Human Fecal Suspension

Arctii fructus was incubated with a human fecal suspension for 15 h at 37°C in an anaerobic incubator. The mixture was then extracted with BuOH, and the extract was subjected to reversed-phase HPLC with an eluting solvent system of acetonitrile-water (4:6) (YMC-ODS-AM 250× 6 mm, 10 μm; flow rate, 1.5 ml/min; detection, UV at 220 nm).

Table 1. ICE production inhibitory effects of lignan in IL-1β-induced D10S cells.

Compound	IC_{50} (μg/ml)[a]	
	ICE production	Cell viability[b]
YVAD-CHO	80.3±0.2	>100
1	>100	>100
2	41.0±1.1	>100
3	46.1±0.3	>100
4	12.5±0.9	>100
4a	8.3±0.9	>100
5	74.2±0.4	>100

[a]IC_{50} values of the compounds represent the concentration to cause 50% inhibition.
[b]Cytotoxic effects of dibenzylbutyrolactonelignan compounds in D10S cells after 48 h of measurement by MTS assay.

Results and Discussion

Inhibitory Activities of the Isolated Compounds

In the search for an ICE production inhibitory activity from medicinal plants, we have studied the constituents of *Arctii fructus* and have isolated five compounds. The repeated column chromatography of the EtOAc-soluble fraction of *Arctii fructus* on silica gel, followed by gel filtration on Sephadex LH-20 and HPLC, led us to the isolation of four dibenzylbutyrolactones: lappaol E (**1**) [14], matairesinol (**2**) [17, 20], lappaol A (**3**) [13], and arctigenin (**4**) [9, 17, 22], whose structures were elucidated by comparing with the spectral data reported previously. Methylation of arctigenin (**4**) with dimethylsulfate and potassium carbonate anhydrous in acetone yielded 4'-methylarctigenin (**4a**). Arctiin (**5**), as arctigenin glucoside [17, 21], was isolated from the BuOH-soluble fraction of the MeOH extract of *Artii fructus*. All the compounds were tested for their ICE production inhibitory activity and for cytotoxic activity against D10S cells (Table 1). Among these isolated compounds, the compound **4** showed the most potent inhibitory activity on ICE (IC_{50} value of 12.5 μg/ml) production from D10S cells. Methyl arctigenin (**4a**) with IC_{50} value of 8.3 μg/ml against ICE, which was methylated by dimethylsulfate, showed a higher inhibitory activity than that of **4**. The compound **1** did not suppress ICE production, and the compounds **2**, **3**, and **5** showed low inhibitory activities against ICE (IC_{50} value of 41.0, 46.1, and 74.2 μg/ml, respectively) on D10S

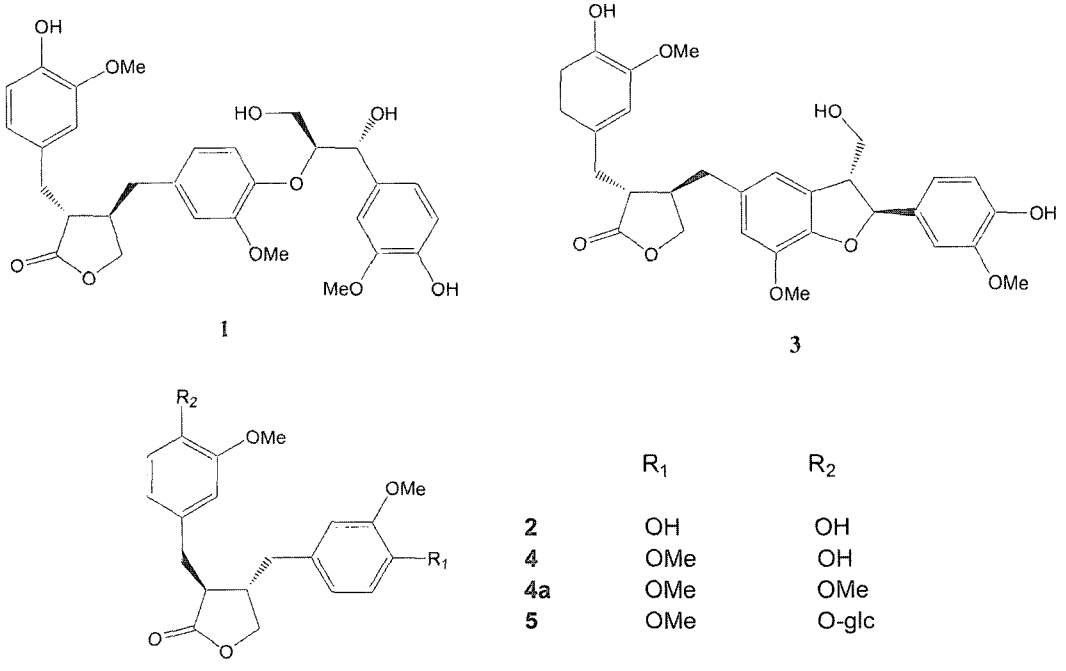

Fig. 1. Chemical structures of dibenzylbutyrolactoneignan compounds isolated from *Arctii fructus*.

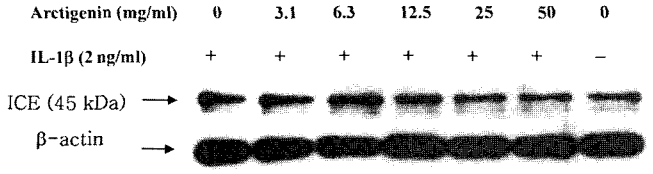

Fig. 2. Effects of arctigenin on IL-1β-induced ICE expression in D10S cells.
Cells were incubated with or without IL-1β (2 ng/ml) in the presence of indicated concentrations of arctigenin. Cellular protein was collected after 48 h of induction and then subjected to 8% SDS-PAGE. Western blot was probed with a polyclonal antibody to ICE. Equal loading was confirmed by reprobing the gel with β-actin.

cells. As shown in Table 1, no compounds showed cytotoxicity against D10S cells at the concentrations tested (10, 50, 100 μg/ml) during 48 h of incubation. We investigated the structure-activity relationships of these compounds. The compound **5**, an arctigenin glucoside with a glucose moiety at the 4' position, did not show inhibitory effects on the ICE productions. Tetra- (**1**), tri- (**3**), di- (**2**), mono- (**4**), and none- (**4a**) hydroxylated compounds showed their inhibitory activity reduced in hydroxylation order with IC_{50} values of >100, 46.1, 41.0, 12.5, and 8.3 μg/ml, respectively. Our investigation suggests that the presence of hydroxy and glucose moiety reduced their inhibitory activity. In Western blot analysis of IL-1β-induced ICE expression from D10S cells, the compound **4** suppressed the IL-1β-induced 45 kD ICE protein expression (Fig. 2) and the proliferation of D10S cells in a dose-dependent manner (Fig. 3). Therefore, these findings led us to conclude that some dibenzylbutyrolactones from *Arctii fructus* possess anti-ICE production activities, and that the major important component in the activity is arctigenin (**4**).

Biotransformation of *Arctii fructus* by Human Intestinal Mixture

To estimate the biotransformation of *Arctii fructus in vivo* by human intestinal bacteria, we carried out an anaerobic incubation of the *Arctii fructus* extract with human fecal suspension. The culture was then extracted with BuOH,

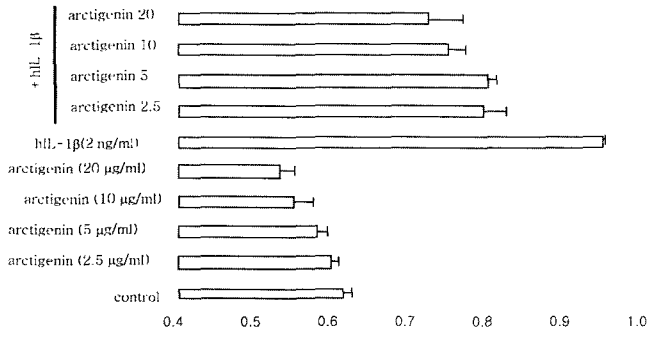

Fig. 3. Effects of arctigenin on IL-1β-induced proliferation of D10S cells.

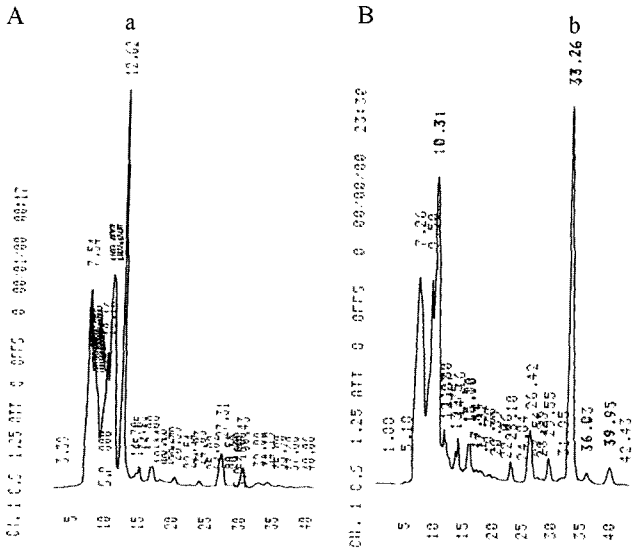

Fig. 4. HPLC profiles of constituents in *Arctii fructus* (A) and *Arctii fructus* fermented by human fecal suspension (B).
a: Arctigenin-glucoside (arctiin); b: arctigenin.

and the BuOH extract was subjected to HPLC analysis (YMC-ODS-AM 250×6 mm; 40% CH_3CN; flow rate, 1.5 ml/min; detection, UV at 220 nm). Figure 4 shows HPLC profile for the culture broth after anaerobic incubation of the *Arctii fructus* with human fecal suspension. After the incubation with fecal suspension, the content of arctiin (IC_{50}=74.2 μg/ml) in *Arctii fructus* decreased, whereas its metabolite, arctigenin (IC_{50}=12.5 μg/ml), increased (Fig. 4). The transformation of *Arctii fructus* by human intestinal bacteria includes hydrolysis of glucoside: The glucose moiety of arctiin is cleaved to form arctigenin. As shown in Fig. 5, BuOH extract obtained from the biotransformation of *Arctii fructus* showed inhibitory activity higher than that

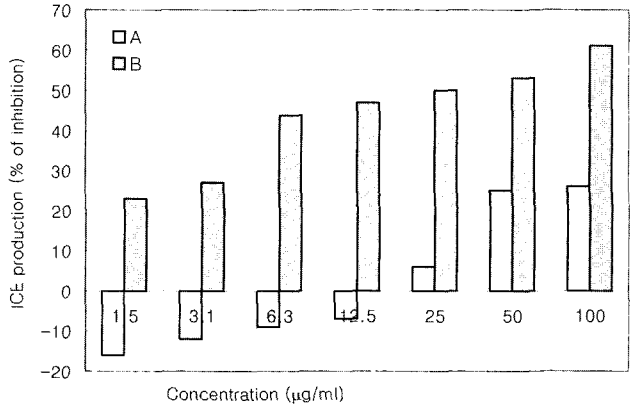

Fig. 5. ICE production inhibitory effects of *Arctii fructus*, transformed by human fecal suspension.
A: Broth containing *Arctii fructus*. B: Broth containing *Arctii fructus* fermented by human fecal suspension.

of *Arctii fructus*. These results demonstrate the role of human intestinal bacteria in the activation of dietary lignan, and suggest that, lignan glycosides could be converted to aglycones by human intestinal bacteria, following oral consumption of *Arctii fructus*.

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