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Biotransformation of Diterpenoids From *Aralia continentalis* Roots by the Genus *Fusarium*

Keumok Moon[1†], Seola Lee[2†], Eunhye Jo[2], Areum Lee[3] and Jaeho Cha[3,4*]

[1]*Laboratory Center, Pusan National University, Busan 46241, Korea*
[2]*Department of Integrated Biological Science (BK21 FOUR Program), Pusan National University, Busan 46241, Korea*
[3]*Microbiological Resource Research Institute, Pusan National University, Busan 46241, Korea*
[4]*Department of Microbiology, Pusan National University, Busan 46241, Korea*

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Aralia continentalis is widely distributed in Far East Asian countries such as Korea, China, and Japan. *A. continentalis* has traditionally been used as an herbal remedy for various conditions, including analgesia, headache, inflammation, lameness, lumbago, rheumatism, and dental diseases in Korea. Previously, *epi*-continentalic acid, continentalic acid, and kaurenoic acid as major active biological compounds belonging to the diterpenoid class were identified. To synthesize diterpenoid derivatives with enhanced bioavailability, *Fusarium fujikuroi* was employed to biotransform diterpenoids due to its known antibacterial activity. This yielded two derivatives of kaurenoic acid, namely 16α-hydroxy-ent-kauran-2-on-19-oic acid and 2β, 16α-dihydroxy-ent-kauran-19-oic acid, with their chemical structures elucidated via NMR analysis. These derivatives exhibited increased polarity compared to kaurenoic acid, as evidenced by their retention time on preparative HPLC using the ODS-A column and structural modifications. Evaluation of their antidiabetic activity targeting PTP1B, a negative regulator of the insulin signaling pathway, revealed inhibitory activities of 30.8% and 27.6%, respectively, at a concentration of 4 μg/ml. Additionally, both derivatives demonstrated low cytotoxicity, with an IC_{50} value 18 times higher than kaurenoic acid. Therefore, the augmented water solubility and reduced toxicity of 16α-hydroxy-ent-kauran-2-on-19-oic acid and 2β, 16α-dihydroxy-ent-kauran-19-oic acid, resulting from biotransformation by *F. fujikuroi*, render them promising candidates for industrial applications.

Key words : Antibacterial activity, *Aralia continentalis*, biotransformation, *Fusarium*, kaurenoic acid

Introduction

Aralia continentalis, known as “Dokwhal” in Korea, is widely distributed in Far East Asian countries such as Korea, China, and Japan. *A. continentalis* has been traditionally used as an herbal remedy for various conditions, including analgesia, headache, inflammation, lameness, lumbago, rheumatism, and dental diseases in Korea [15, 16, 21]. Previous studies have identified *epi*-continentalic acid, continentalic acid, and kaurenoic acid as major active biological compounds, belonging to the diterpenoid class [6, 22, 23, 38]. In particular, continentalic acid and kaurenoic acid are recognized for their diverse biological activities, including antibacterial, anti-cancer, anti-inflammatory, and antidiabetic properties [16, 18, 19, 29]. Despite their therapeutic potential, the poor aqueous solubility of these diterpenoids hinders their widespread use in the pharmacological industry, leading to limitations in bioavailability and pharmacological efficacy [10].

To address the issue of low solubility, researchers have explored two main synthetic strategies: chemical methods and biocatalytic processes. Chemical methods, such as solid dispersion, particle engineering, and nanotechnology, are known for improving the solubility of natural materials but often come with environmental concerns and the production of unwanted byproducts [14]. On the other hand, biocatalytic processes, involving bioconversion using enzymes or cells, offer advantages such as high selectivity, easy product separation, and fewer environmental issues [14, 27, 30, 33].

†**These authors contributed equally to this work.**
***Corresponding author**
Tel : +82-51-510-2196, Fax : +82-51-514-1778
E-mail : jhcha@pusan.ac.kr

Whole cell biotransformation, a type of biocatalytic process, offers key benefits by utilizing cost-effective raw materials and enabling the synthesis of various novel compounds through multiple reactions [25]. Therefore, it is widely applied in various fields, such as pharmaceutical, food, cosmetics, and fragrance industries [13]. However, a potential limitation lies in the toxicity of the raw materials, which can impact cell growth. Therefore, the selection of microorganisms and culture conditions is critical to obtaining desirable products [24].

Previous studies have explored the use of various microorganisms for the bioconversion of diterpenoids [13]. For instance, Çorbacı (2020) reported the conversion of terpene and terpenoid derivatives to more polar compounds by *Aspergillus niger* NRRL 326, exhibiting slightly higher biological activities [5]. Rocha et al. (2017) synthesized *ent*-2α,15α-dihydroxy-kaur-16-en-19-oic acid from the biotransformation of *ent*-15α-hydroxy-kaur-16-en-19-oic acid with *Fusarium proliferatum*, resulting in higher allelopathic activity [32]. Tronina et al. (2013) found that the fungi *Mortierella mutabilis* could convert xanthohumol to derivatives with added sugar moiety, demonstrating improved antioxidant and stronger antiproliferative activities against cancer cell lines [36].

In this study, various fungi and culture media were screened to enhance the bioavailability and antidiabetic activity of diterpenoids isolated from *A. continentalis*. Successful fungal biotransformation was achieved with *Fusarium fujikuroi*, and the antidiabetic activity of the biotransformation products was evaluated. The synthesized products from biotransformation exhibited promising industrial potential compared to the originally isolated diterpenoids from *A. continentalis*.

Materials and Methods

Chemicals and reagents

The roots of *A. continentalis* were purchased from the local market in Jeollanam-do, Korea, in February 2020. All extraction solvents were Low Particulate grade and purchased from SK Chemicals (Ulsan, Korea). Dimethylsulfoxide (DMSO) was purchased from Sigma-Aldrich (St. Louis, MO, USA). TLC Silica gel 60 (particle size, 0.063–0.200 mm) was purchased from Merck (Darmstadt, Germany). Brain heart infusion (BHI), potato dextrose broth (PDB), tryptic soy broth (TSB), and yeast extract peptone dextrose (YPD), were purchased from Difco (Sparks, MD, USA).

Microorganisms and growth conditions

Rhizopus oryzae KCTC6943 and *Trichoderma longibrachiatum* KCTC16921 purchased from the Korean Collection for Type Cultures (KCTC), Daejeon, Korea, and *Aspergillus niger* 6911 and *Fusarium fujikuroi* kindly provided by Prof. Seo, Young-Su at Pusan National University were grown at 30℃ on a PDB agar plate. *Bacillus cereus* was grown on Luria Bertani (LB) agar, while *Listeria monocytogenes* ATCC 15313, and *Klebsiella pneumoniae* ATCC4352 were cultured at 37℃ on TSB agar, and *Streptococcus mutans* UA159, *Streptococcus iniae* ATCC29178, and *Streptococcus parauberis* were grown at the same temperature on BHI agar plates. Those strains were kindly provided by Prof. Kang, Ho Young at Pusan National University.

Extraction and isolation of the diterpenoids from the *Aralia continentalis* roots

The roots of *A. continentalis* were ground using a commercial blender (Waring, PA, USA), and 50 g of the ground roots were weighed in a flask. Extraction was conducted with ethanol (EtOH) using sonication for 60 min, and the supernatant was collected. The process was repeated three times, and the supernatant was combined and filtered with filter paper (No. 2, Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The total filtrate was then concentrated using a rotary vacuum evaporator (Eyela, Tokyo, Japan) under reduced pressure at 60℃ and subsequently lyophilized. The resulting dried *A. continentalis* ethanol extract (ACE) (3.2 g) was dissolved in 10% ethanol and partitioned with *n*-hexane. The *n*-hexane fraction from ACE (ACEH) (1.1 g) was loaded on a silica gel 60 column (6×40 cm) equilibrated with chloroform/ethyl acetate (19:1, v/v), resulting in eight sub-fractions (HF01–HF08). A portion of HF07 to HF08 from ACEH (ACEHS) (0.3 g) showing antibacterial activity was loaded onto an octadecylsilyl (ODS)-A column (5 μm particle size, 250 mm × 20 mm; YMC Co., Kyoto, Japan) with equipped to a preparative recycling high-performance liquid chromatography (HPLC) system (LC-918, JAI, Japan) coupled to an ultraviolet (UV) detector. The sample was eluted with an isocratic elution system of 0.1% trifluoroacetic acid/methanol/acetonitrile (5:8.5:10.5, v/v/v) and monitored at 205 nm (Fig. 1).

Biotransformation of the diterpenoids

Biotransformation was conducted by the method described by Marquina et al. (2009), with slight modifications [26]. Four fungal species, namely *R. oryzae*, *T. longibrachiatum*, *A. niger*, and *F. fujikuroi*, were employed in this study. One

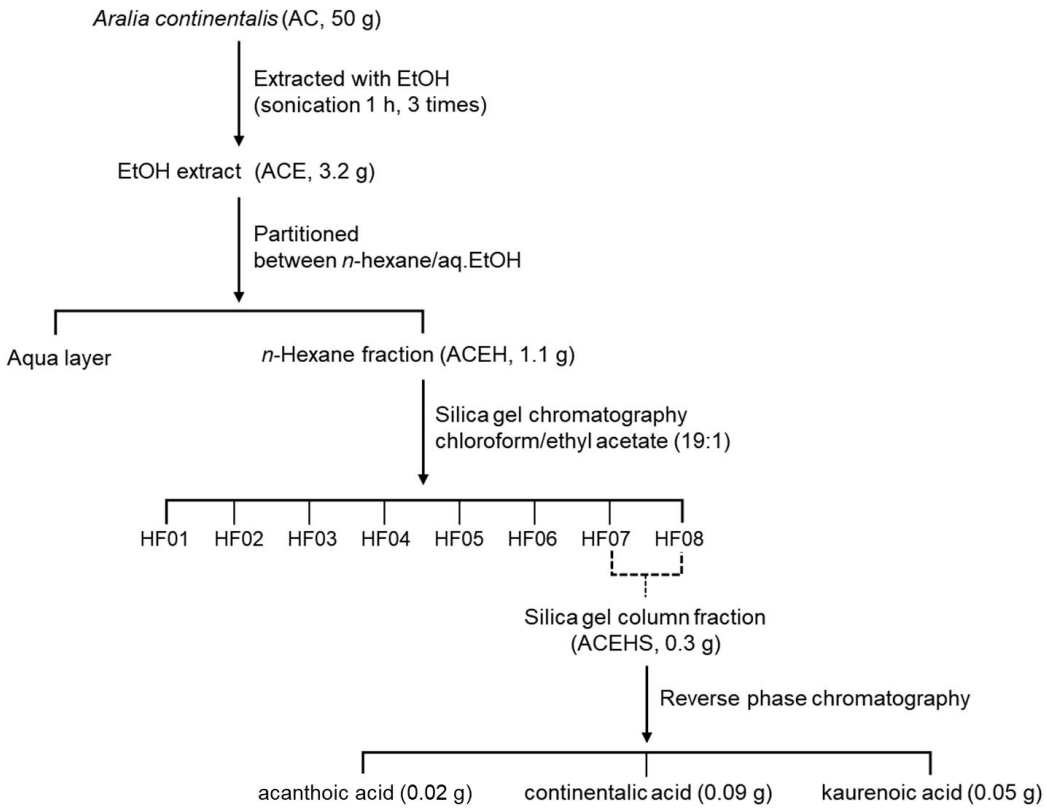

Fig. 1. Sequential extraction and isolation of acanthoic acid, continentalic acid, and kaurenoic acid from *A. continentalis*.

hundred milliliters of a medium in a 500 ml Erlenmeyer flask were inoculated with 1% fungi spores (OD_{620} = 1) and incubated at 30°C. After 24 hr of incubation, 15 mg of diterpenoids, dissolved in 500 µl of DMSO, were added to the flask and further incubated. Following a 13-day incubation period, the cultures were filtrated, and the aqueous layer was extracted with ethyl acetate. The filtered cultures were then fractionated with 100% ethyl acetate. The fraction was further purified using a preparative recycling HPLC. For analysis, 5 µl of biotransformation products, prepared at a concentration of 7 mg/ml, were injected into an ODS-A column (5 µm particle size, 250 mm × 4.6 mm id; YMC Co., Kyoto, Japan). Solvent A consisted of 0.1% trifluoroacetic acid in water (v/v), while solvent B was composed of methanol. The gradient condition was as follows: a linear gradient from 20% to 100% B over 70 min at a flow rate of 0.7 ml/min. The retention time of the sample was monitored using UV absorption at 205 nm. The culture medium containing fungi incubated without substrates served as a control and was extracted using the same procedure.

Thin-layer chromatography

Thin-layer chromatography (TLC) of biotransformation products was developed by spotting 10 µl (30 mg/ml) of the compounds onto two pre-coated silica-based gel plates with an optimized mobile phase of *n*-hexane/ethyl acetate (1:1, v/v). Chromatograms were dried with a hair dryer for complete removal of solvents. All TLC plates were run in duplicate, one of them was stained with 5%(v/v) sulfuric acid and heated for 5 min at 110℃ to visualize biotransformation products.

Bioautography

Agar overlay bioautography was employed to assess the antibacterial effects of biotransformation products, following the method described by Dewanjee et al. (2015) with minor modifications [7]. For the bioautography plate, the chromatogram was enclosed within sterile petri plates and overlaid with 20 ml of 1.5% soft agar medium seeded with 1% of the indicated bacterial cells. After solidification of the medium, the petri plates were incubated overnight at 37℃ in polyethylene boxes lined with moist chromatography paper. The bioautograms were sprayed with an aqueous solution (1 mg/ml) of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and incubated for 4 hr. Clear inhibition zones were observed against a purple background.

Antidiabetic activity assay

The inhibitory activity of diterpenoid compounds against human recombinant protein tyrosine phosphatase 1B (PTP1B) was assessed following the method described by Wu et al. [37]. In each well of a 96-well plate, 10 μl of the test compounds and 89 μl of a reaction buffer solution containing 10 mM Tris (pH 7.4), 50 mM NaCl, 2 mM dithiothreitol, 1 mM $MnCl_2$, and 10 mM para-nitrophenyl phosphate (*p*NPP) were combined and pre-warmed at 37℃ for 20 min. Subsequently, 1 μl of PTP1B solution in water (1 mg/ml) was added to each well. After incubation at 37℃ for 30 min, the reaction was terminated by adding 10 μl of a 0.1 M NaOH solution. The amount of para-nitrophenol (*p*NP) produced was determined by measuring the absorbance at 405 nm using a microplate reader. A positive control using 1 μM of sodium vanadate was included. The inhibition activity (%) was calculated using the following equation:

$$\text{Inhibition activity } (\%) = \frac{OD_{blank} - OD_{sample}}{OD_{blank}} \times 100$$

where OD_{sample} represents the mean value of the estimated optical density for each sample, and OD_{blank} represents the mean value of the estimated optical density for the blank control.

Cell viability assay

L929 cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (10,000 U/ml penicillin and 10,000 mg/ml streptomycin), and maintained at 37℃ under 5% CO_2 in a humidified culture chamber. For cytotoxicity assessment, L929 cells (2×10^4 cells/cm^2) were seeded in 96-well plates, cultured for 24 hr, and then treated with samples at various concentrations ranging from 2 to 128 μg/ml. After a 24 hr incubation, the media was replaced with fresh media (100 μl/well) containing 0.5 mg/ml of MTT reagents, and the cells were incubated at 37℃ for 1 hr. Finally, 100 μl of DMSO was added to each well to dissolve the insoluble formazan, and the absorbance was measured at 550 nm using a microplate reader. All samples were compared with the blank control to estimate the percentage of viable cells, using the following equation:

$$\text{Viability } (\%) = \frac{OD_{sample}}{OD_{blank}} \times 100$$

where OD_{sample} represents the mean value of the estimated optical density for each sample, and OD_{blank} represents the mean value of the estimated optical density for the blank control.

Nuclear magnetic resonance (NMR) analysis

The ^{1}H-and ^{13}C-NMR spectra of purified biotransformation products were obtained using a Varian Inova AS 600 MHz NMR spectrometer (Varian, Palo Alto, CA). The sample was dissolved in DMSO-d_6 at 24°C with tetramethylsilane as a chemical shift reference.

Statistical analysis

All experiments were performed in triplicate. Data were analyzed by employing the one-way analysis of variance test using SigmaPlot software (version 12.5, Systat, San Jose, CA) and expressed as the mean ± standard deviation. Also, the mean value was considered to be significantly different at $p<0.05$, $p<0.005$, and $p<0.001$.

Results

Isolation of three major diterpenoids in *Aralia continentalis* roots

Three major diterpenoids were isolated from *A. continentalis* through a multi-step purification process involving ethanol extraction, *n*-hexane fractionation, silica gel chromatography, and reverse-phase preparative HPLC. The extraction yield, determined from the weight of dried *A. continentalis* roots, resulted in yields of 6.44% for ACE, 4.56% for ACEH, and 1.33% for ACEHS. In the reverse-phase preparative HPLC, three compounds previously identified as acanthoic acid, continentalic acid, and kaurenoic acid by Moon et al. (2022) were successfully isolated, with yields of 0.09%, 0.41%, and 0.22%, respectively [28].

Biotransformation of three diterpenoids by four different fungal strains

The isolated samples from *A. continentalis* were dissolved in DMSO and evaluated for antibacterial activity using bioautography. In agar overlay TLC bioautography, the colored background of formazan results from the dehydrogenase activity of microorganisms, which converts vital dyes into a chromogenic product through the reduction process. A colorless zone indicates the inhibition of test pathogens due to the presence of fractionated active metabolites on the chromatogram. The agar overlay TLC bioautography confirmed the *in situ* detection of ACEHS, acanthoic acid, continentalic acid, and kaurenoic acid, showing the presence of these four compounds and a clear zone of inhibition against gram-pos-

itive bacteria such as *B. cereus, L. monocytogenes, S. aureus, S. mutans, S. parauberis*, and *S. iniae*. This result suggests that the three major diterpenoids isolated from *A. continentalis* exhibit potent inhibitory effects on the growth of gram-positive pathogens.

Among microorganisms employed in the biotransformation of diterpenoids, fungal species have demonstrated noteworthy capabilities in conducting diverse chemical reactions [1, 3, 8]. Four fungal strains and three types of media were investigated to assess the production of major diterpenoid derivatives with potent antibacterial activity. The synthesis of diterpenoid derivatives exhibiting antibacterial activity against *S. mutans* was examined through TLC and bioautography. Among the three media tested, the YPD medium proved to be the most effective for the synthesis of diterpenoid derivatives, while the use of PDB and SMB media resulted in the degradation of diterpenoids (Table 1). Among the three diterpenoids examined, kaurenoic acid emerged as a promising candidate for derivative production. All four fungal species tested demonstrated the ability to produce diterpenoid derivatives, contingent on the medium used. In the case of *T. longibrachiatum*, it was observed that diterpenoid derivatives were synthesized when SMB medium was used. Upon examining the fungal strains and media, it was determined that *F. fujikuroi*, when cultured in the YPD medium, successfully synthesized kaurenoic acid derivatives with antibacterial activity (Fig. 2, Table 1).

In Fig. 2D, the spot corresponding to the antibacterial activity of kaurenoic acid disappeared, and a new spot with an R_f value of 0.28, exhibiting antibacterial activity, emerged. This R_f value is lower than that of kaurenoic acid (R_f is 0.90), suggesting the production of a more polar compound through the transformation with *F. fujikuroi*. This observation implies the complete conversion of the added kaurenoic acid into new derivatives with enhanced bioavailability. Consequently, *F. fujikuroi* was chosen as the fungus for the biotransformation of diterpenoids into novel antibacterial agents among the tested fungal strains and was subsequently utilized for further investigations.

Effect of biotransformation products of kaurenoic acid on antibacterial activity against various gram-positive bacterial pathogens

Diterpenoid compounds are known to exhibit high antimicrobial activity against gram-positive bacteria [12]. To investigate the antimicrobial activity of bioconversion products of kaurenoic acid using *F. fujikuroi* against various pathogens, including *B. cereus, L. monocytogenes, S. aureus, S. parauberis*, and *S. iniae*, an overlay TLC bioautography was conducted. *B. cereus, S. aureus,* and *L. monocytogenes* are known as foodborne pathogens, while *S. parauberis* and *S. iniae* are considered fish pathogens. As depicted in Fig. 3, both the kaurenoic acid before bioconversion and the newly synthesized kaurenoic acid derivatives exhibited antimicrobial activity against *B. cereus, L. monocytogenes, S. parauberis*, and *S. iniae*. Notably, for *S. aureus*, the compound before bioconversion showed no activity, while the biotransformation products after bioconversion demonstrated antimicrobial activity (Fig. 3E). These findings indicate that the bioconversion of kaurenoic acid using *F. fujikuroi* not only pre-

Table 1. Screening of fungi and media for biotransformation of diterpenoids with antibacterial activity

Fungi	Medium	Diterpenoids			Antibacterial activity
		AA	CA	KA	
Aspergillus niger	SMB	−	−	−	−
	YPD	−	−	+	−
	PDB	−	−	−	−
Rhizopus oryzae	SMB	−	−	−	−
	YPD	−	−	+	−
	PDB	−	−	−	−
Trichoderma longibrachiatum	SMB	−	−	+	−
	YPD	−	−	−	−
	PDB	−	−	−	−
Fusarium fujikuroi	SMB	−	−	−	−
	YPD	−	−	+	+
	PDB	−	−	−	−

AA, acanthoic acid; CA, continentalic acid; KA, kaurenoic acid; SMB, Sabouraud maltose broth; YPD, yeast extract peptone dextrose; PDB, potato dextrose broth, (−) represents negative while (+) represents positive, respectively.

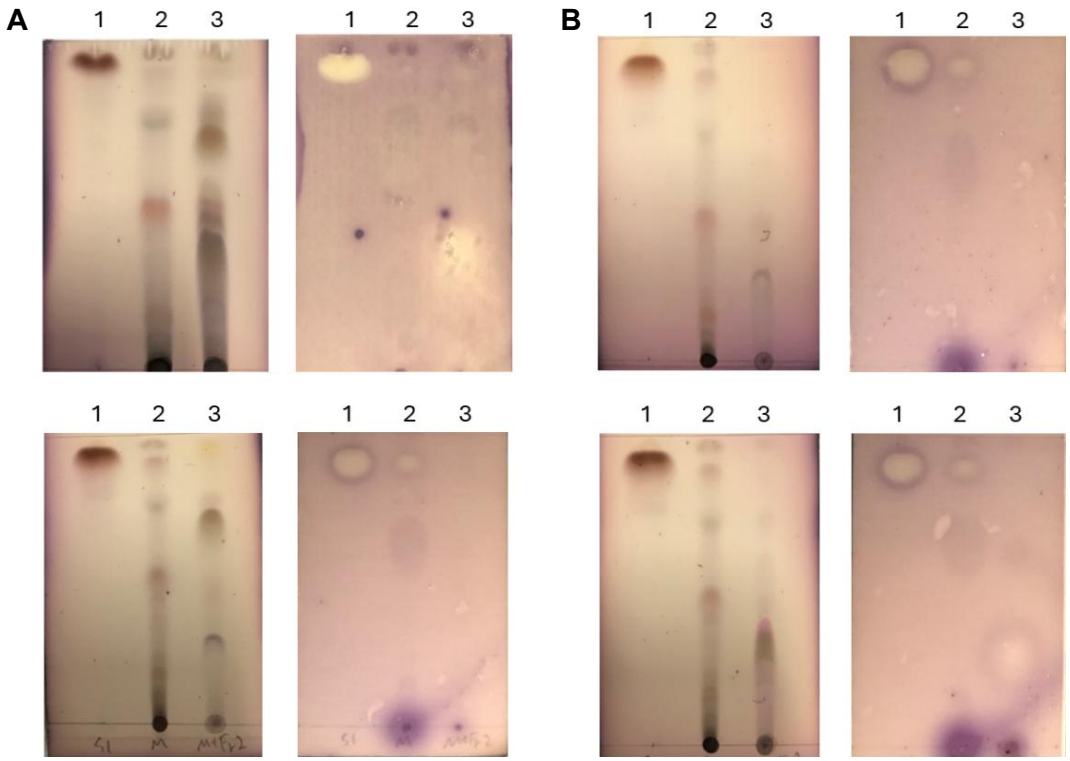

Fig. 2. TLC and bioautogram showing the biotransformation products derived from (A) ACEHS, (B) acanthoic acid, (C) continentalic acid, and (D) kaurenoic acid by *F. fujikuroi* cultured in YPD medium. Lane 1, diterpenoid compound; lane 2, culture supernatant of *F. fujikuroi* without diterpenoid compound; lane 3, culture supernatant of *F. fujikuroi* with diterpenoid compound. For biotransformation, 300 μg of each compound was employed, while as controls before the biotransformation, each compound at a concentration of 30 μg was used. The halo zone in bioautogram indicates the antibacterial activity against *S. mutans*.

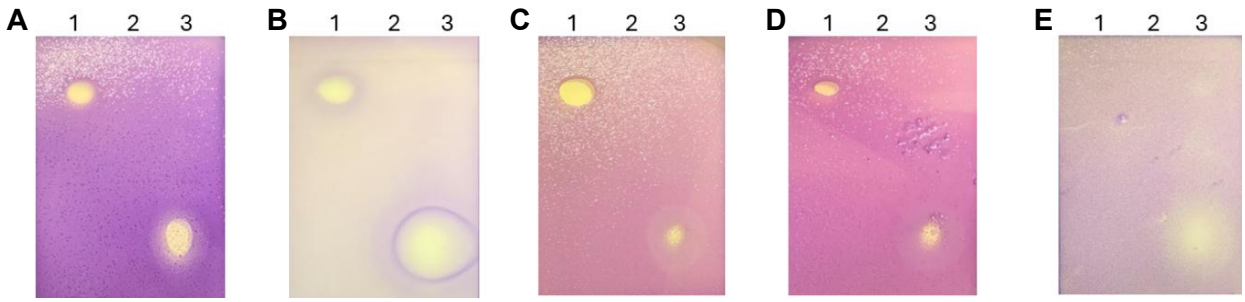

Fig. 3. Bioautography of biotransformation products of kaurenoic acid against various bacterial pathogens. (A) *B. cereus*; (B) *L. monocytogenes*; (C) *S. parauberis*; (D) *S. iniae*; (E) *S. aureus*. Lane 1, kaurenoic acid; lane 2, culture supernatant of *F. fujikuroi* without kaurenoic acid; lane 3, culture supernatant of *F. fujikuroi* with kaurenoic acid. For biotransformation, 300 μg of each compound was employed, while as controls before the biotransformation, each compound at a concentration of 30 μg was used. The halo zone in bioautogram indicates the antibacterial activity.

serves the current antimicrobial activity with enhanced bioavailability but also holds the potential to modify antimicrobial efficacy.

Effect of biotransformation products of kaurenoic acid on antidiabetic activity

Kaurenoic acid is recognized for its antidiabetic properties through the inhibition of PTP1B, a key player in the negative regulation of insulin signaling [17]. In a prior study by Jung et al. (2012), kaurenoic acid isolated from *A. continentalis* demonstrated a PTP1B inhibition effect with an IC_{50} value of 1.4 μg/ml [18]. Therefore, we aimed to compare the

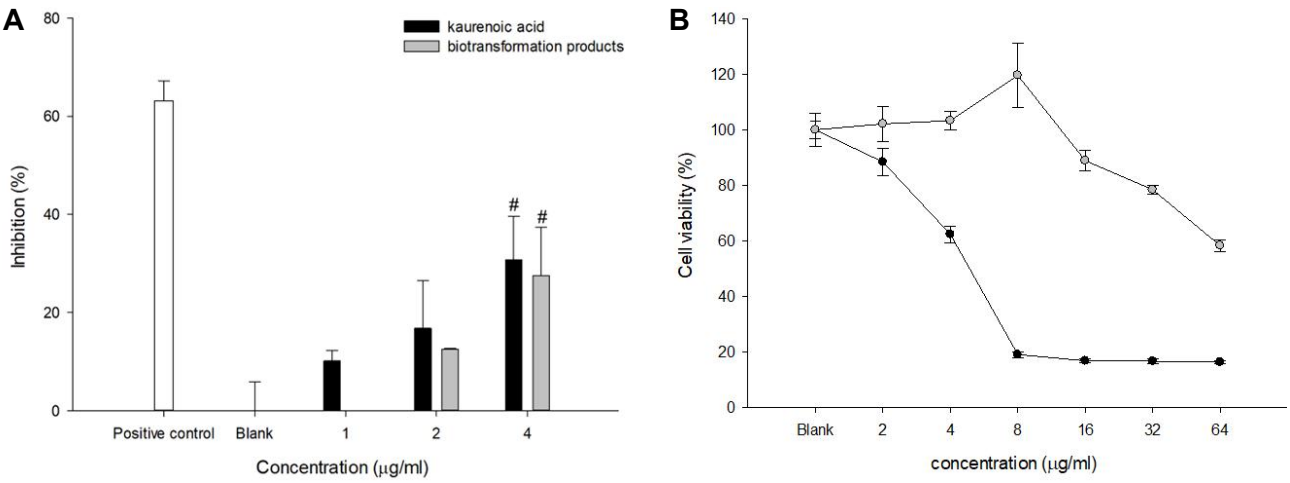

Fig. 4. PTP1B inhibitory activity (A) and L292 cell viability (B) of kaurenoic acid (black color) and biotransformation products (gray color). Sodium orthovanadate (1 mM) was used as a standard inhibitor of PTP1B. Data were derived from three independent experiments and expressed as mean ± SE ($^{\#}p<0.05$). Cells were cultured with the indicated concentrations of compounds at 37℃ in a 96-well plate for 24 hr. Cell viability was evaluated as described in "Materials and Methods". Data were derived from three independent experiments and expressed as mean ± SE.

PTP1B inhibition activity between kaurenoic acid and biotransformation products. Both compounds exhibited increased antidiabetic activity in a concentration-dependent manner. At a concentration of 4 μg/ml, kaurenoic acid, and biotransformed products displayed similar PTP1B inhibitory activities of 30.8% and 27.6%, respectively (Fig. 4A). The result indicates that the biotransformation of kaurenoic acid did not impact its antidiabetic activity.

Effect of the biotransformation products of kaurenoic acid on cell viability

Murine fibroblast L929 cells are known to be more sensitive to cytotoxicity compared to other cell lines, making them suitable for measuring cytotoxic effects [11]. To compare the cytotoxicity of kaurenoic acid and its biotransformation products, MTT analysis was conducted using non-malignant L929 cells. Cells were cultured with compounds at concentrations ranging from 1 to 64 μg/ml for 24 hr. As shown in Fig. 4B, kaurenoic acid did not exhibit significant cytotoxicity ($p<0.05$) up to a concentration of 2 μg/ml in L929 cells but exhibited pronounced cytotoxicity at 4 μg/ml and above. Conversely, the biotransformation products showed no cytotoxicity up to a concentration of 64 μg/ml ($p<0.005$). The IC_{50} value for kaurenoic acid is 3.9 μg/ml, while its biotransformation products have an IC_{50} value of 72.8 μg/ml. Given that biotransformation products have an IC_{50} value 18 times higher than that of kaurenoic acid, these findings suggest that biotransformation products display lower toxicity in normal cells compared to kaurenoic acid. This reduced cytotoxicity implies that the transformed compounds may exhibit enhanced biocompatibility compared to the original compounds.

Isolation and identification of the two biotransformation products of kaurenoic acid

Kaurenoic acid derivatives biotransformed by *F. fujikuroi* was extracted with ethyl acetate and purified by a reverse phase HPLC with an analytical ODS-A column. Two kaurenoic acid derivatives were isolated and their retention time was 39.2 (compound 1) and 40.2 min (compound 2), respectively while the retention time of kaurenoic acid was 51.6 min (Fig. 5A, 5B). Compared to the retention time of two kaurenoic acid derivatives, the biotransformed products were judged to have more water-soluble properties than kaurenoic acid. The yield of the compounds 1 and 2 were 4.2% and 7.4%, respectively.

The molecular structure of the compounds 1 and 2 was identified by NMR analysis (Fig. 5C, 5D). The ^{13}C-NMR (150 MHz) and ^{1}H-NMR (600 MHz) spectra of the two compounds were compared with those of kaurenoic acid (Table 2, 3). The ^{13}C-NMR spectra of compound 1 showed a ketone carbon signal (δ_C = 212.79), carboxyl carbon signal (δ_C = 180.14), and oxygenated-quaternary carbon signal (δ_C = 80.03). The ^{1}H-NMR spectra of compound 1 showed a singlet methyl proton signal at H-18 and H-20. These results indicated that the ketone group was formed at C-2. Heteronu-

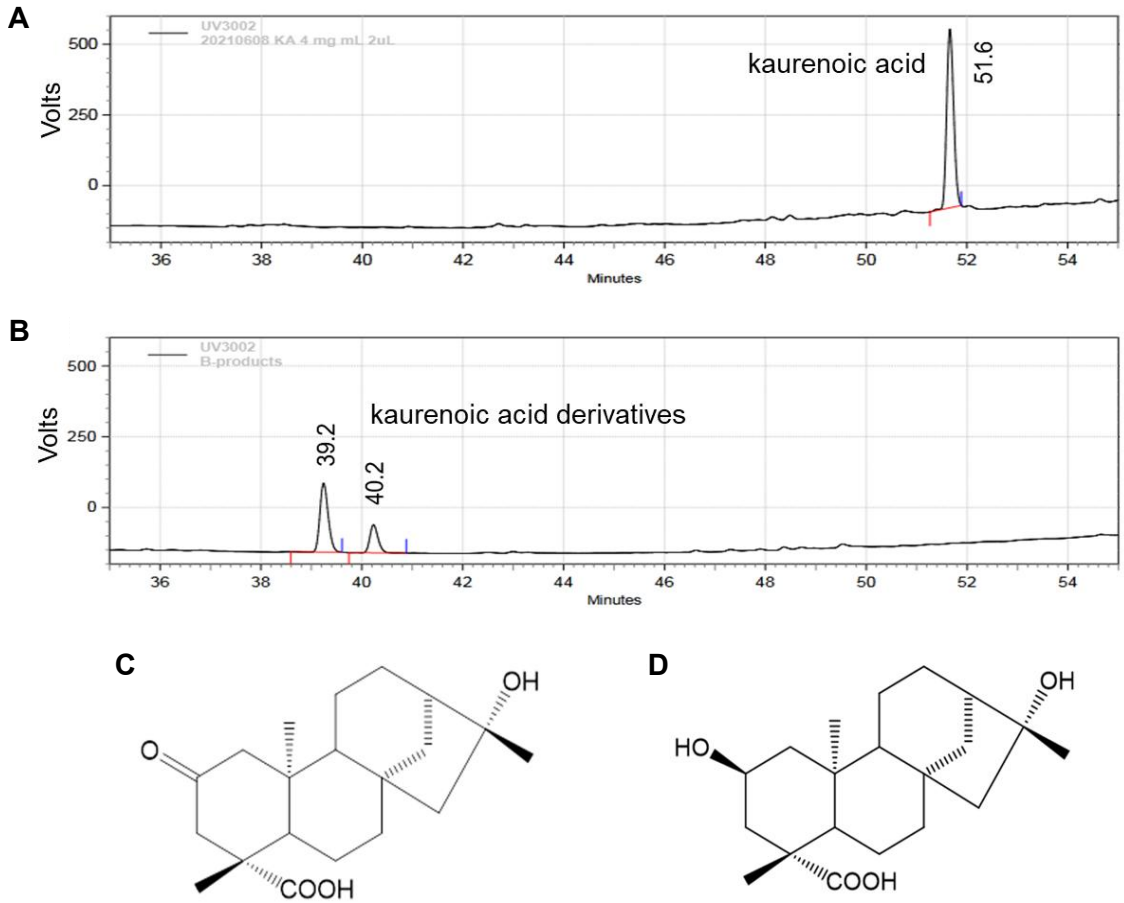

Fig. 5. HPLC analysis (A and B) and chemical structure (C and D) of kaurenoic acid derivatives biotransformed by *F. fujikuroi*. For analysis, 5 μl of biotransformation products, prepared at a concentration of 7 mg/ml, were injected into a YMC ODS-A column (250 mm × 4.6 mm id, 5 μm particle size). Solvent A consisted of 0.1% trifluoroacetic acid in water (v/v), while solvent B was composed of methanol. The gradient condition was as follows: a linear gradient from 20% to 100% B over 70 min at a flow rate of 0.7 ml/min. The retention time of the sample was monitored using UV absorption at 205 nm. Two kaurenoic acid derivatives are 16α-hydroxy-ent-kauran-2-on-19-oic acid (C) and 2β, 16α-dihydroxy-ent-kauran-19-oic acid (D), respectively.

clear Multiple Bond Correlation (HMBC) experiment was used to establish the ketone group and hydroxyl group at C-2 and C-16, respectively. Correlation between H-1 and H-3 with C-2 indicated that a ketone group was formed at C-2. The correlation of H-14, H-15, and H-17 with C-16 indicated that the hydroxyl group was determined at C-16 by hydroxylation. Taken together, compound 1 was identified to be a new compound 16α-hydroxy-ent-kauran-2-on-19-oic acid.

The ^{1}H-NMR spectra of compound 2 showed oxygenated methine protons signal at C-2 and methyl groups at C-17, C-18, and C-20. HMBC experiment was used to establish hydroxyl group formation at C-2 and C-16. In the HMBC spectrum, correlations were observed between H-1 and H-3 with C-2 and of H-14, H-15, and H-17 with C-16. Moreover, in the Nuclear Overhauser Effect Spectroscopy (NOESY) spectrum, a correlation was observed between H-2 and H-20 indicating β-configuration of the hydroxyl group at C-2. A NOESY correlation between H-17 and H-13 indicated α-configuration of the hydroxyl group. Taken together, the compound 2 was identified to be a new compound 2β, 16α-dihydroxy-ent-kauran-19-oic acid.

Discussion

The valuable pharmaceutical and medicinal properties of various plant-derived secondary metabolites have encouraged research to modify their structures to produce more effective derivatives using microbial biotransformation [20]. Kaurenoic acid, readily obtained from natural sources, serves as a promising starting material for creating new derivatives through

Table 2. ^{1}H-NMR (δ in ppm downfield from TMS, in CD_3OD) data for compounds 1 and 2

H	compound 1		compound 2	
	δ H_α	δ H_β	δ H_α	δ H_β
1	2.55, 1H, d, J = 12.6 Hz	2.04, 1H, d, J = 12.6 Hz	2.22, 1H, dd, J = 4.2, 11.4 Hz	0.72, 1H, dd, J = 11.4, 11.4 Hz
2			4.16-4.11, 1H, m	
3	2.82 1H, d, J = 13.2 Hz	2.24, 1H, d, J = 13.2 Hz	2.43, 1H, dd, J = 3.6, 12.0 Hz	0.97, 1H, dd, J = 12.0, 12.0 Hz
4				
5	1.77, 1H, dd, J = 6.0, 9.6 Hz		1.10, 1H, dd, J = 6.0, 9.6 Hz	
6	2.02-1.98, 1H, m	1.90,1H, overlapped	1.91-1.82, 2H, m	
7	1.71, 1H, overlapped	1.58, 1H, overlapped	1.66, 1H, overlapped	1.47, 1H, ddd, J = 4.2, 12.9, 12.9 Hz
8				
9	1.08, 1H, br. d, J = 12.6 Hz		1.08, 1H, dd, J = 2.4, 12.0 Hz	
10				
11	1.67, 1H, overlapped	1.57-1.51,1H, m	1.67, 2H, overlapped	
12	1.84, 1H, overlapped 1.56, 1H, overlapped	1.84, 1H, overlapped 1.56, 1H, overlapped	1.64, 1H, overlapped	1.57, 1H, overlapped
13	1.88, 1H, overlapped		1.93-1.89, 1H, m	
14	1.87, 1H, dd, J = 10.2, 10.2 Hz	1.70, 1H, overlapped	1.90, 1H, dd, J = 11.4, 11.4 Hz	1.68, 1H, overlapped
15	1.61, 2H, s		1.58, 2H, s	
16	–			
17	1.38, 3H, s		1.38, 3H, s	
18	1.39, 3H, s		1.28, 3H, s	
19	–			
20	1.00, 3H, s		1.03, 3H, s	

synthesis for biological screening. There is a particular focus on developing more hydrophilic kaurane derivatives due to the enhanced biological activity often found in highly oxygenated kaurane diterpenoids. These compounds, though limited in natural availability, tend to exhibit higher bioactivity compared to their less hydroxylated counterparts [9]. However, incorporating functional groups into the relatively inert structure of kaurenoic acid proves to be a challenging task. An alternative approach involves fungal biotransformation, offering a valuable method for partially synthesizing highly hydroxylated compounds that hold potential bioactivity [4].

Table 3. ^{13}C-NMR (δ in ppm downfield from TMS, in CD_3OD) data for compounds 1 and 2

C	compound 1	compound 2	C	compound 1	compound 2
1	57.54	50.67	11	19.59	19.73
2	212.79	65.26	12	27.82	28.07
3	52.69	47.99	13	50.04	49.72
4	45.06	46.65	14	38.19	38.79
5	56.61	57.81	15	58.27	58.68
6	23.41	23.30	16	80.03	80.10
7	42.73	43.40	17	24.67	24.72
8	46.69	46.06	18	28.87	29.67
9	56.52	57.58	19	180.14	181.29
10	40.47	42.38	20	17.91	17.59

In the present study, biotransformation using fungal strains was performed to overcome the low solubility of diterpenoids extracted from *A. continentalis* and enhance their biological activity. For exploiting desirable fungal transformation, we tested *A. niger, T. longibrachiatum, R. oryzae,* and *F. fujikuroi* under various culture conditions. While most fungi altered the chemical structure of diterpenoids, *F. fujikuroi* demonstrated the synthesis of transformed compounds that retained biological activities. Specifically, two biotransformed compounds generated from kaurenoic acid through hydration and hydroxylation displayed antibacterial and antidiabetic activity comparable to natural kaurenoic acid with anticipated improved bioavailability.

F. fujikuroi, a highly adaptable filamentous fungus, exhibited remarkable versatility in transforming various diterpenoids into hydroxylated derivatives [31]. This capability is attributed to its numerous hydroxylating enzymes with broad

specificities, leading to the endogenous production of hydroxylated diterpenoids with potential antiviral and antitumoral properties [2, 35].

Microbial biotransformation is known to enhance water solubility and reduce cytotoxicity by triggering chemical reactions like glycosylation and hydroxylation in biologically active compounds. Inspired by the observed decrease in cytotoxicity of kaurenoic acid, researchers synthesized a range of kaurenoic acid derivatives including amides and diols [34]. In our study, these derivatives showed no adverse effects on cell viability even at concentrations 32 times higher than kaurenoic acid. This transformation of kaurenoic acid, initially poorly soluble, into a more soluble form through hydroxylation represents a significant achievement. The kaurenoic acid derivatives produced through biotransformation by *F. fujikuroi* showed similar PTP1B inhibitory activity compared to kaurenoic acid itself. These derivatives not only exhibited enhanced water solubility and reduced cytotoxicity but also retained broad antibacterial and antidiabetic properties. Therefore, they hold significant promise as potential substitutes for kaurenoic acid.

In summary, through the biotransformation mediated by the filamentous fungus *F. fujikuroi*, we were able to produce derivatives of kaurenoic acid that are extracted from *A. continentalis*. These derivatives maintained the original antibacterial and antidiabetic activities while exhibiting low cytotoxicity. This study demonstrates that this specific fungal-mediated transformation method not only applies to kaurenoic acid but also aids in discovering potential novel compounds that address the shortcomings of bioactive compounds, showing various physiological activities albeit with somewhat limited biocompatibility.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 곰팡이 Fusarium 속을 이용한 독활 뿌리 추출물로부터 디테르페노이드의 생물전환

문금옥[1†] · 이설아[2†] · 조은혜[2] · 이아름[3] · 차재호[3,4*]
([1]부산대학교 공동실험실습관, [2]생명시스템학과, [3]미생물지원연구소, [4]미생물학과)

독활 뿌리 추출물은 한국을 포함한 극동아시아 국가에 널리 분포하고 있다. 이 추출물은 한국에서 두통, 염증, 허리통증 및 치과질환 등 다양한 질환에 오랫동안 사용되어져 왔다. 활성물질은 에피-콘티넨탈산, 콘티넨탈산 그리고 카우레노산으로 동정되었다. 생체이용능을 향상시킨 디테르페노이드 유도체의 합성을 위해, *Fusarium fujikuroi*가 생물전환용 균주로 선발되었다. 생물전환을 통하여 16α-hydroxy-ent-kauran-2-on-19-oic acid 와 2β, 16α-dihydroxy-ent-kauran-19-oic acid의 두 가지 유도체가 얻어졌으며, 이들의 화학구조는 HPLC, MS 및 NMR 분석을 통해 확인되었다. 이러한 유도체들은 HPLC에서의 산물 유출시간과 구조적 특성을 통해 카우레노산에 비해 증가된 극성을 나타내었다. 인슐린 신호전달경로의 음성조절자인 PTP1B를 대상으로 한 항당뇨 활성을 평가한 결과, 4 μg/ml 농도에서 각각 30.8% 및 27.6%의 억제율을 나타냈으며, 두 유도체는 기존의 카우레노산 보다 18배 높은 IC_{50} 값을 갖는 낮은 세포독성을 보여주었다. 따라서, *F. fujikuroi*에 의한 생물전환으로 인해 증가된 수용성과 감소된 독성을 갖는 두 카우레노산 유도체는 산업적 응용을 위한 유망한 후보물질로 평가된다.