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Recovery effect of lignans and fermented extracts from *Forsythia koreana* flowers on pancreatic islets damaged by alloxan in zebrafish (*Danio rerio*)

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Abstract

Repeated column separation yielded four enterolactone type lignans from *Forsythia koreana* flowers (FKF), whose chemical structures were identified using several spectral technics. FKF MeOH extract (FKFM) and four lignans significantly recovered aloxan induced pancreatic islet in zebrafish. Especially, aglycones, **1** and **3**, exhibited relatively higher activity than the lignan glycosides, **2** and **4**. Therefore, FKFM was fermented using a *Microbacterium esteraromaticum*, BGP1, to yield the fermented FKFM (FKFM-BGP1). FKFM and FKFM-BGP1 were extracted using *n*-butanol to give *n*-BuOH fraction of each, FKFM-nB and FKFM-BGP1-nB, respectively. FKFM-BGP1-nB showed higher activity than FKFM-nB, as well the content of the aglycones, **1** and **3**, in FKFM-BGP1-nB, 2.42 ± 0.01% and 1.15 ± 0.01%, was revealed to be much higher than that in FKFM-nB, 0.01 ± 0.01% and 0.01 ± 0.01%, respectively. In conclusion, the lignan aglycones **1** and **3** as well FKFM-BGP1-nB from *F. koreana* flowers were proved to be potential anti-diabetic agents. Furthermore, we suggest that antidiabetic efficacy of FKFM-BGP1-nB might be related to lignan aglycones **1** and **3**.

Keywords: Arctigenin, Diabetes, Fermentation, *Forsythia koreana*, Matairesinol, Zebrafish

Introduction

Diabetes mellitus (DM), which is characterized by high blood glucose levels (hyperglycemia), is originated in disorders of insulin secretion or decrease of insulin sensitivity [1, 2], which is synthesized in pancreatic islet (PI) β-cells [3]. DM is developed by decrease or dysfunction of β-cells in PI [4, 5]. Thus, protecting as well restoring PI capability effectively fulfilled DM treatments. Lots of researches have been executed for searching anti-diabetes materials to enhance β-cells in PI with safety from natural source [6, 7]. Traditionally, the discovery of active materials has been largely based on in vitro, in vivo, and ex vivo screening techniques. Among these methods, zebrafish (ZF) has emerged as a powerful experiment methods for various illness such as chronic disease over the past several years [8, 9]. The ZF is a tropical, shoaling freshwater cyprinid fish [10]. Because of its tiny size, numerous progeny, transparent embryos, amenability to chemical and genetic screening, and manageability in laboratories, ZF has been used as a various disease model for in vivo experiments [8]. Alloxan (AL), which damages the pancreas by β-cells preventing from producing insulin, has been used as diabetogenic agent on in vivo experiments [11]. Previously, AL has reported to induce diabetes and diabetic complications on the ZF model by morphological observation [12, 13]. Our preliminary study revealed *Forsythia koreana* flowers MeOH extract (FKFM) increased PI size damaged by AL in ZF. Therefore, the search for anti-diabetic compound from FKFM can be very valuable.

Forsythia koreana (FK, Oleaceae), a perennial shrub, is widely distributed in Korea and China. It grows up to

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1–3 m high and has oblong and ovate-lanceolate leaves. Flowers bloom in April with four yellow petals, while fruits ripen from September to October and are 1.5–2 cm diameter [14]. The fruits of FK (Fructus Forsythiae, Korean name, "Yeon-kyo") have been used for removal of fever and detoxification in Korean and Chinese medicine [15]. Fructus Forsythiae is also reported to contain several active components [15–17], which displayed anti-inflammatory, anti-oxidant, and anti-asthmatic activity [7, 16–18]. However, only few phytochemical and biological studies for *F. koreana* flowers (FKF) have been conducted.

This article states the isolation process of metabolites from FKF and fermented materials of FKFM (FKFM-BGP1). And the isolated lignans and fractions were evaluated for recovery effect on injured PIs in ZF model.

Materials and methods

Plant and enzyme

The samples are same as those used in the previous studies [19, 20]. BGP1 (GenBank accession number 603820) *Escherichia coli* cloning was obtained from KyungHee University, Ginseng Resource Bank, Yong-In, Korea.

General experimental procedures

General experimental procedures were performed as previously described method [19–21].

Isolation of metabolites from solvent fraction (FR)

EtOAc FR (FKE, 45 g), *n*-butanol FR (FKB, 110 g), H_2O FR (FKW, 395 g) were obtained as reported in the previous study [19, 20]. FKE was treated with silica gel (SiO_2) column chromatography (c.c.) (ϕ 12 × 17 cm) and eluted with *n*-hexane–EtOAc (10:1 → 2:1 → 1:1, 14 L of each) → $CHCl_3$–MeOH–H_2O (30:3:1 → 20:3:1 → 10:3:1 → 65:35:10, 15 L of each) and monitored using thin layer chromatography (TLC) to provide 12 fractions (FKE-1 to FKE-12). Fraction FKE-10 [1.9 g, elution volume/total volume (Ve/Vt) 0.072–0.088] was applied by SiO_2 c.c. (ϕ 4.5 × 15 cm) using $CHCl_3$–EtOAc (10:1 → 3:1, 5.6 L of both) as eluting solution, yielding 19 fractions (FKE-10-1 to FKE-10-19). Fraction FKE-10-10 [127.8 mg, Ve/Vt 0.043–0.050] was subjected to an octadecyl SiO_2 (ODS) c.c. (ϕ 3 × 6 cm) using acetone–H_2O (2:6 → 1:1, 730 ml of both), yielding six fractions (FKE-10-10-1 to FKE-10-10-6) along with a compound **1** [FKE-10-10-2, 119.2 mg, Ve/Vt 0.082–0.110, TLC (Kieselgel 60 F_{254}) R_f 0.62, $CHCl_3$–EtOAc (1:1), TLC (RP-18 F_{254S}) R_f 0.72, acetone-H_2O (3:1)]. Fraction FKE-10-14 [220.0 mg, Ve/Vt 0.736–0.780] was subjected to ODS c.c. (ϕ 2.5 × 5 cm) and eluted with acetone–H_2O (2:1 → 1:1, 2.2 L of both), yielding eight fractions (FKE-10-14-1 to FKE-10-14-8). Fraction FKE-10-14-2 [69.0 mg, Ve/Vt 0.736–0.780] was subjected to the SiO_2 c.c. (ϕ 2 × 10 cm) and eluted with $CHCl_3$–EtOAc (5:1, 500 ml), yielding three fractions (FKE-10-14-2-1 to FKE-10-14-2-3) and a compound **3** [FKE-10-14-2-3, 12.9 mg, Ve/Vt 1.000, TLC (Kieselgel 60 F_{254}) R_f 0.50, $CHCl_3$–EtOAc (3:1), TLC (RP-18 F_{254S}) R_f 0.53, acetone-H_2O (2:1)]. FKB was chromatographed using SiO_2 resins (ϕ 11 × 15 cm) using $CHCl_3$–MeOH–H_2O (60:6:2 → 40:6:2 → 20:6:2, 43 L for each) → EtOAc-*n*-BuOH–H_2O (4:5:1, 45 L) with monitoring by TLC to yield 15 fractions (FKB-1 to FKB-15) as well a compound **2** [FKB-2, 8.0 g, Ve/Vt 0.038–0.070, TLC (Kieselgel 60 F_{254}) R_f 0.45, $CHCl_3$–MeOH–H_2O (15:3:1), TLC (RP-18 F_{254S}) R_f 0.65, acetone–H_2O (1:1)]. Fraction FKB-3 [20.8 g, Ve/Vt 0.071–0.122] was applied to SiO_2 c.c. (ϕ 2 × 10 cm) and eluted with $CHCl_3$–MeOH–H_2O (25:3:1 → 10:3:1, 3 L of each), yielding 14 fractions (FKB-3-1 to FKB-3-14) along with a compound **4** [FKB-3-5, 8.8 g, Ve/Vt 0.855–0.909, TLC (Kieselgel 60 F_{254}) R_f 0.45, $CHCl_3$–MeOH–H_2O (10:3:1), TLC (RP-18 F_{254S}) R_f 0.52, acetone–H_2O (2:3)].

Arctigenin (**1**) Colorless prisms; $[\alpha]_D^{25}$-23.0° (MeOH, *c* 0.10); IR (KBr, *ν*) 3424 (OH), 1762 (γ-lactone C=O), 1599, 1514 (aromatic) cm^{-1}; m.p. 100–101 °C; positive fast atom bombardment mass spectrometry (FAB/MS) *m/z* 373 $[M+H]^+$; ^{1}H-nuclear magnetic resonance (NMR) (400 MHz, CD_3OD, δ_H) and ^{13}C-NMR (100 MHz, CD_3OD, δc) see Tables 1 and 2.

Arctiin (**2**) Colorless crystals; $[\alpha]_D^{25}$-38.4° (EtOH, *c* 1.0); IR (KBr, *ν*) 3433 (OH), 1780 (γ-lactone C=O), 1597, 1514 (aromatic) cm^{-1}; m.p. 111–112 °C; positive FAB/MS *m/z* 535 $[M+H]^+$; ^{1}H-NMR (400 MHz, CD_3OD, δ_H) and ^{13}C-NMR (100 MHz, CD_3OD, δc) see Tables 1 and 2.

Matairesinol (**3**) Colorless needles; $[\alpha]_D^{25}$-35.7° (EtOH, *c* 0.1); IR (KBr, *ν*) 3415 (OH), 1748 (γ-lactone C=O), 1604, 1509 (aromatic) cm^{-1}; m.p. 119–120 °C; positive FAB/MS *m/z* 381 $[M+Na]^+$; ^{1}H-NMR (400 MHz, $CDCl_3$, δ_H) and ^{13}C-NMR (100 MHz, $CDCl_3$, δc) see Tables 1 and 2.

Matairesinoside (**4**) White powder; $[\alpha]_D^{25}$-48.4° (EtOH, *c* 0.5); IR (KBr, *ν*) 3450 (OH), 1760 (γ-lactone C=O), 1600, 1550 (aromatic) cm^{-1}; m.p. 95–96 °C; negative FAB/MS *m/z* 519 $[M-H]^-$; ^{1}H-NMR (400 MHz, CD_3OD, δ_H) and ^{13}C-NMR (100 MHz, CD_3OD, δ_C) see Tables 1 and 2.

Fermentation of MeOH extract from *F. koreana* flowers (FKFM)

Bacterial strain and culture condition Refer to the literature [22]. Large scaled fermentation and preparation of crude enzyme BGP1 were referred to the literature [23]. Enzyme reaction for FKFM using the BGP1 was referred to the literature [24]. Then crude BGP1 enzyme was reacted with FKFM at pH 7.0 and 37 °C.

Table 1 ^{1}H-NMR (400 MHz) data of lignans 1–4 from the flowers of *Forsythia koreana* (δ in ppm, coupling pattern, *J* in Hz)

No.	1[a]	2[a]	3[b]	4[a]
1				
2	6.54, d, 1.6	6.71, br. s	6.38, d, 1.6	6.53, d, 1.6
3				
4				
5	6.77, d, 8.4	7.04, d, 8.2	6.80, d, 8.4	6.67, d, 8.4
6	6.56, dd, 8.4, 1.6	6.53, br. d, 8.2	6.49, dd, 8.4, 1.6	6.48, dd, 8.4, 1.6
7	2.47, overlapped	2.47, overlapped	2.52, overlapped	2.49, overlapped
8	2.45, m	2.61, m	2.45, m	2.44, m
9a	4.10, dd, 8.8, 6.0	4.11, dd, 8.9, 6.3	4.13, dd, 8.8, 6.5	4.13, dd, 8.8, 6.0
9b	3.98, dd, 8.8, 8.4	3.85, dd, 8.9, 8.4	3.85, dd, 8.8, 8.7	3.88, dd, 8.8, 8.3
1′				
2′	6.65, d, 2.0	6.55, br. s	6.59, d, 2.0	6.71, br. s
3′				
4′				
5′	6.69, d, 8.0	6.78, d, 8.7	6.81, d, 8.0	7.01, d, 8.0
6′	6.57, dd, 8.0, 2.0	6.52, br. d, 8.7	6.57, dd, 8.0, 2.0	6.64, br. d, 8.0
7′a	2.83, dd, 14.0, 5.2	2.87, dd, 14.8, 5.5	2.91, dd, 14.0, 5.2	2.85, dd, 14.0, 5.2
7′b	2.77, dd, 14.0, 7.2	2.74, dd, 14.8, 7.6	2.86, dd, 14.0, 7.2	2.80, dd, 14.0, 7.2
8′	2.59, m	2.42, m	2.59, m	2.63, m
9′				
1″		4.85, d, 7.8		4.85, d, 7.8
2″		3.57, overlapped		3.50, overlapped
3″		3.56, overlapped		3.49, overlapped
4″		3.99, overlapped		3.37, overlapped
5″		3.72, overlapped		3.37, overlapped
6″a		3.75, dd, 11.6, 4.4		3.86, dd, 11.6, 4.4
6″b		3.66, dd, 11.6, 1.2		3.64, dd, 11.6, 1.2
OMe	3.73, s	3.74, s	3.78, s	3.77, s
OMe	3.73, s	3.73, s	3.77, s	3.74, s
OMe	3.73, s	3.70, s		

[a] CD_3OD, [b] $CDCl_3$

Preparation of *n*-BuOH fractions of FKFM and FKFM-BGP1 from *F. koreana* flowers

FKFM and FKFM-BGP1 were extracted with *n*-BuOH to give their *n*-BuOH fractions, FKFM-nB and FKFM-BGP1-nB, respectively.

The quantitative analysis of lignans in FKFM-nB and FKFM-BGP1-nB through liquid chromatography/mass spectrometry (LC/MS) experiment

One milligram of each compound was accurately weighed and dissolved in MeOH to obtain stock solutions with 1.0 mg/mL concentration. Calibration curves were made for each standard with four different concentrations (125, 50, 25, 12.5 μg/mL). For *n*-BuOH layers of FKFM (FKFM-nB) and FKFM-BGP1 (FKFM-BGP1-nB) the high-performance liquid chromatography (HPLC) experiment was carried out as the followings. The extracts were filtered through a 0.22 μm membrane filter (Woongki Science, Seoul, Korea) and evaporated in a vacuum. A 10 μL aliquot of the fraction solution (1.0 mg/mL) was injected into the HPLC system. Analysis was achieved using a Agilent technology 1200 series (Tokyo, Japan) with a Agilent G1314B UV detector (280 nm). The column was a YMC-triart C18 (100 mm × 2.0 mm; particle size 3 μm). The mobile phase: 0.1% FA (H_2O, A), 0.1% (AcN, B); flow rate 0.4 mL/min; Elution of B; 5% (0.01 min) → 13% (5 min) → 13% (15 min) → 17% (18 min) → 17% (20 min) → 25% (25 min) → 100% (37 min) → 100% (40 min). The detection was carried out by liquid chromatography-electrospray ionization-mass spectrometry (LC–ESI–MS). Mass detector settings were as follows: gas temperature: 350^{O}C, gas flow: 10 L/min, nebulizer pressure: 45 psi, capillary voltage: 4000 V. $^{*}[M-H+HCOO^{-}]^{-}$. Quantitative analysis was replicated three times.

Evalution for recovery effect of FKFM-nB and FKFM-BGP1-nB on AL-induced PI in ZF larvae

The activity test and statistical analysis for obtained data were accomplished by the same methods as the previously used one [12, 13].

Evaluation for toxicity of lignans 1–4, FKFM-nB, and FKFM-BGP1-nB on zerbrafish embryo

Embryos were placed in 6-well plate, and incubated at 28.5 °C and a cycle of 14 h light: 10 h dark photoperiod. The treatment was as the following; normal, lignans **1–4** at the concentration 10, 25, 50, 75, 100, 250 μM, FKFM-nB and FKFM-BGP1-nB at the concentration 10, 25, 50, 75, 100, 250 μg/mL, respectively. The embryos were observed under the microscope after 72 h treatment and evaluated for hatching rate.

Results and discussion

FKFM was fractionated into FKE, FKB, and FKW by solvent fractionation using polarity according to Ref. [25]. Repeated SiO_2 as well ODS c.c. for FKE and FKB

Table 2 ^{13}C-NMR (100 MHz) data of lignans 1–4 from the flowers of *Forsythia koreana* (δ in ppm)

No.	1[a]	2[a]	3[b]	4[a]
1	132.8	132.9	129.5	134.2
2	113.5	113.8	110.9	114.8
3	150.4	151.1	146.6	150.5
4	149.0	149.3	144.5	146.1
5	113.0	113.2	114.4	117.9
6	122.0	122.3	121.3	122.2
7	38.8	39.1	38.2	38.8
8	42.4	42.6	40.9	42.5
9	72.9	73.1	71.3	72.9
1′	130.7	134.4	129.7	131.3
2′	113.8	115.0	111.4	113.3
3′	149.1	150.8	146.7	149.0
4′	146.4	147.0	144.3	146.2
5′	116.1	118.0	114.0	116.2
6′	123.0	123.1	122.0	123.0
7′	35.4	35.6	34.5	35.4
8′	47.7	47.8	46.5	47.7
9′	181.5	181.5	179.1	181.3
1″	–	103.1	–	102.8
2″	–	75.1	–	74.8
3″	–	78.0	–	77.8
4″	–	71.5	–	71.3
5″	–	78.3	–	78.1
6″	–	62.7	–	62.4
OMe	56.4	56.9	55.8	56.7
OMe	56.3	56.8	55.7	56.6
OMe	56.3	56.7	–	–

[a] CD_3OD, [b] $CDCl_3$

yielded lignans **1–4**. The lignans' molecular structures were revealed based on spectroscopic analyses.

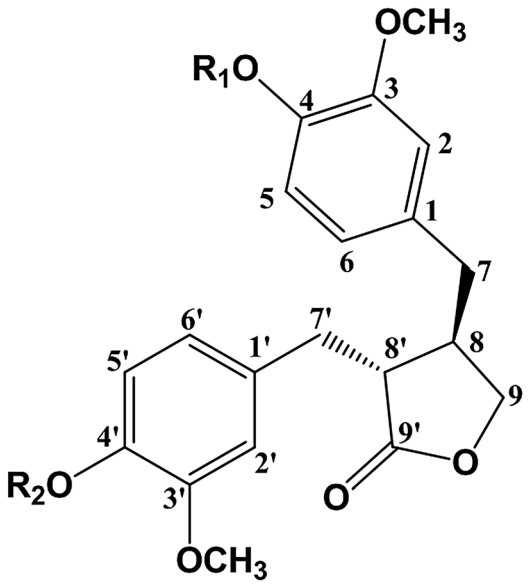
1, colorless prisms, m.p. 100–101 °C, $[\alpha]_D^{25}$-23.0°, molecular weight (MW) 372 (*m/z* 373 $[M+H]^+$, positive FAB/MS). IR, hydroxyl (3424 cm^{-1}), γ-lactone C=O (1762 cm^{-1}), aromatic (1599, 1514 cm^{-1}). ^{1}H-NMR spectrum (PMR, chemical shift, coupling pattern, *J* in Hz, proton number) showed six aromatic methine signals [δ_H 6.57 (dd, 8.0, 2.0, H-6′), δ_H 6.65 (d, 2.0, H-2′), and δ_H 6.69 (d, 8.0, H-5′)] and [δ_H 6.54 (d, 1.6, H-2), δ_H 6.56 (dd, 8.4, 1.6, H-6), and δ_H 6.77 (d, 8.4, H-5)] responsible for two 1,2,4-trisubstituted benzene rings. Four signals due to one oxygenated methylene and one methylene showing germinal coupling [δ_H 2.77 (dd, 14.0, 7.2, H-7′b), δ_H 2.83 (dd, 14.0, 5.2, H-7′a), δ_H 3.98 (dd, 8.8, 8.4, H-9b), and δ_H 4.10 (dd, 8.8, 6.0, H-9a)], and four signals due to one methylene δ_H 2.47 (2H, overlapped, H-7) and two methines [δ_H 2.45 (m, H-8), δ_H 2.59 (m, H-8′)] were detected owing to two propyl moieties of enterolactone lignan. Therefore, **1** was proposed to be a enterolactone type lignan. In addition, three methoxy proton signals δ_H 3.73 (9H, s, H-$OCH_3 \times 3$) were also detected. 21 carbon signals involving three methoxies [δ_C 56.3, δ_C 56.3, and δ_C 56.4] in the ^{13}C-NMR spectrum (CMR) confirmed **1** as be a lignan. The carbon signals of a γ-lactone δ_C 181.5 (C-9′), four oxygenated olefin quaternaries [δ_C 146.4 (C-4′), δ_C 149.1 (C-3′), δ_C 149.0 (C-4), and δ_C 150.4 (C-3)], two olefin quaternaries [δ_C 130.7 (C-1′) and δ_C 132.8 (C-1)], six olefin methines [δ_C 113.5 (C-2), δ_C 113.8 (C-2′), δ_C 113.0 (C-5), δ_C 116.1 (C-5′), δ_C 122.0 (C-6), and δ_C 123.0 (C-6′)], one oxygenated methylene δ_C 72.9 (C-9), two methines [δ_C 42.4 (C-8) and δ_C 47.7 (C-8′)], and two methylenes [δ_C 35.4 (C-7′) and δ_C 38.8 (C-7)] were detected. gHMBC showed correlation between three methoxy protons δ_H 3.73 (9H, s) and three oxygenated olefin quaternary carbons [δ_C 149.0 (C-4), δ_C 149.1 (C-3′), and δ_C 150.4 (C-3)], respectively. **1** was determined to have same planar structure as that of arctigenin. The stereostructure was revealed through comparing chemical shift, coupling pattern for NMR signals as well the specific rotation value $[\alpha]_D^{25}$-20.3° ((-)-arctigenin) [26]. Taken together, compound **1** was identified to be (-)-arctigenin, which was previously isolated from *Arctium lappa* [27].

2, colorless crystals, m.p. 111–112 °C, $[\alpha]_D^{25}$-38.4°, MW 534 (*m/z* 535 $[M+H]^+$, positive FAB/MS). IR, hydroxyl (3433 cm^{-1}), γ-lactone C=O (1780 cm^{-1}), aromatic (1597, 1514 cm^{-1}). PMR and CMR spectra of **2** were very similar to those of **1** with the exception for one additional sugar signal. The protons of a hemiacetal at δ_H 4.85 (d, 7.8, H-1″), four oxygenated methines [δ_H 3.56 (overlapped, H-3″), δ_H 3.57 (overlapped, H-2″), δ_H 3.72 (overlapped, H-5″), and δ_H 3.99 (overlapped, H-4″)], and one oxygenated methylene [δ_H 3.66 (dd, 11.6, 1.2, H-6″b) and δ_H 3.75 (dd, 11.6, 4.4, H-6″a)] were observed, indicating to the sugar be a hexose. The carbons of a hemiacetal at δ_C 103.1 (C-1″), four oxygenated methines [δ_C 71.5 (C-4″), δ_C 75.1 (C-2″), δ_C 78.0 (C-3″), and δ_C 78.3 (C-5″)], and one oxygenated methylene δ_C 62.7 (C-6″), revealed the sugar was a *β*-glucopyranose. The anomer proton coupling constant (7.8 Hz) affirmed the anomer hydroxy to have *β*-configuration. In gHMBC, the glucose anomer proton (δ_H 4.85, H-1″) and the oxygenated olefin quaternary carbon at δ_C 147.5 (C-4′) correlated each other. Three methoxy protons (δ_H 3.70, 3.73, 3.74) and the oxygenated olefin quaternary carbons [δ_C 149.3 (C-4), 150.8 (C-3′), 151.1 (C-3)] correlated respectively. **2** was identified as arctiin and confirmed through comparing spectroscopic data in literature [28]. It was previously isolated from Fructus Bardanae [29].

3, colorless needles, m.p. 119–120 °C, $[\alpha]_D^{25}$-35.7°. MW 358 (*m/z* 381 $[M+Na]^+$, positive FAB/MS). IR, hydroxyl (3415 cm^{-1}), γ-lactone C=O (1748 cm^{-1}), aromatic

(1604, 1509 cm^{-1}). PMR and CMR spectra of **3** were similar as those of **1** except for two methoxy groups [δ_H 3.77 (3H, s), δ_C 55.7; δ_H 3.78 (3H, s), δ_C 55.8]. MW of **3**, 358 Da, was 14 amu less than that of **1** (372 Da) confirming the above mention. In gHMBC, two methoxy protons (δ_H 3.77, 3.78) and two oxygenated olefin quaternary carbons [δ_C 146.6 (C-3), 146.7 (C-3′)] correlated respectively, indicating two methoxies existed in C-3 and C-3′. Therefore, **3** was identified as (-)-matairesinol, which was affirmed through comparing spectroscopic data in literature [30]. It was previously isolated from *Podocarpus spicatus* [31].

4, white powder, m.p. 93–94 °C, $[\alpha]_D^{25}$-48.4°, MW 520 (*m/z* 519 $[M-H]^-$, negative FAB/MS). IR, hydroxyl (3450 cm^{-1}), γ-lactone C=O (1760 cm^{-1}), aromatic (1600, 1550, 1460, 1385 cm^{-1}). PMR and CMR spectra of **4** showed similar signals as those of **2** except for an additional sugar, a hemiacetal (δ_H 4.85, d, 7.8, H-1″; δ_C 102.8, C-1″), four oxygenated methines [(δ_H 3.37, overlapped, H-4″; δ_C 71.3, C-4″), (δ_H 3.37, overlapped, H-5″, δ_C 78.1, C-5″), (δ_H 3.49, overlapped, H-3″; δ_C 77.8, C-3″), and (δ_H 3.50, overlapped, H-2″; δ_C 74.8, C-2″)], and one oxygenated methylene (δ_H 3.64, dd, 11.6, 1.2, H-6″b and δ_H 3.86, dd, 11.6, 4.4, H-6″a; δ_C 62.4, C-6″) due to a *β*-glucopyranose. Anomer proton coupling constant (7.8 Hz) affirmed the anomer hydroxy to have *β*-configuration. In gHMBC, the glucose anomer proton (δ_H 4.85, H-1″) and the oxygenated olefin quaternary carbon (δ_C 146.2, C-4′) correlated each other. Finally, **4** was identified as matairesinoside, which was affirmed through comparing spectroscopic data in literature [27]. It was previously isolated from *Trachelospermum asiaticum* var. *intermedium* [32]. This is the first report for isolation of **1–4** from FKF (Fig. 1).



	R_1	R_2
arctigenin (**1**)	CH_3	H
arctiin (**2**)	CH_3	*β*-Glc
matairesinol (**3**)	H	H
matairesinoside (**4**)	H	*β*-Glc

β-Glc : *β*-D-glucopyranosyl

Fig. 1 Molecular structures of lignans in *Forsythia koreana* flowers

Four lignans from FKF were valued for recovery effect on PI damaged by AL in ZF larvae. Alloxan, a diabetogenic chemical, was used for damaging PI of ZF, which suppresses β-cell mass in PI [12, 33]. AL treatment significantly decreased the PI size by 41.4% ($p<0.0001$) compared to normal group (Fig. 2a, c). Moreover, to observe PI we used 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose (2-NBDG) a fluorescent dye derived from glucose modified with an amino group at the C-2 position [34], widely utilized in diabetes study since allows to quantify glucose uptake without using radioactive tracers and can be easily quantified through fluorescence microscopy, and it has been previously validated using ZF for PI observation [12]. To evaluate four lignans and glimepiride, a positive control, for recovery effect on PI damaged by AL in ZF, PI size was examined after treatment of samples. The size of glimepiride-treated PI significantly increased by 46.12% ($p<0.0001$) compared to the AL-induced group, indicating recovery effect as previous studies [12]. Though glycosides, arctiin (**2**) and matairesinoside (**4**), increased a little PI size comparing with AL-induced groups, there was no statistical significance. However, aglycones, arctigenin (**1**) and matairesinol (**3**), significantly increased PI size by 38.53% ($p=0.0014$) and 45.19% ($p=0.0001$), respectively (Fig. 2a, c), showing similar recovery effects to those observed in the group treated with the positive control, glimepiride. Additionally, relative 2-NBDG uptake was assessed by analyzing the pixel intensity level of PI, the observed results show the same pattern with those obtained in the measurement of PI. AL-treated group significantly decreased relative glucose uptake comparing to normal treatment by 63% ($p=0.0002$), while glimepiride and all lignans that increased the relative 2-NBDG uptake compared to AL-induced group, glimepiride by 47.19% (0.0015), arctigenin by 51.39% ($p=0.0002$), matairesinol by 61.28% ($p=0.0003$), matairesinoside by 35.56% ($p=0.0265$)

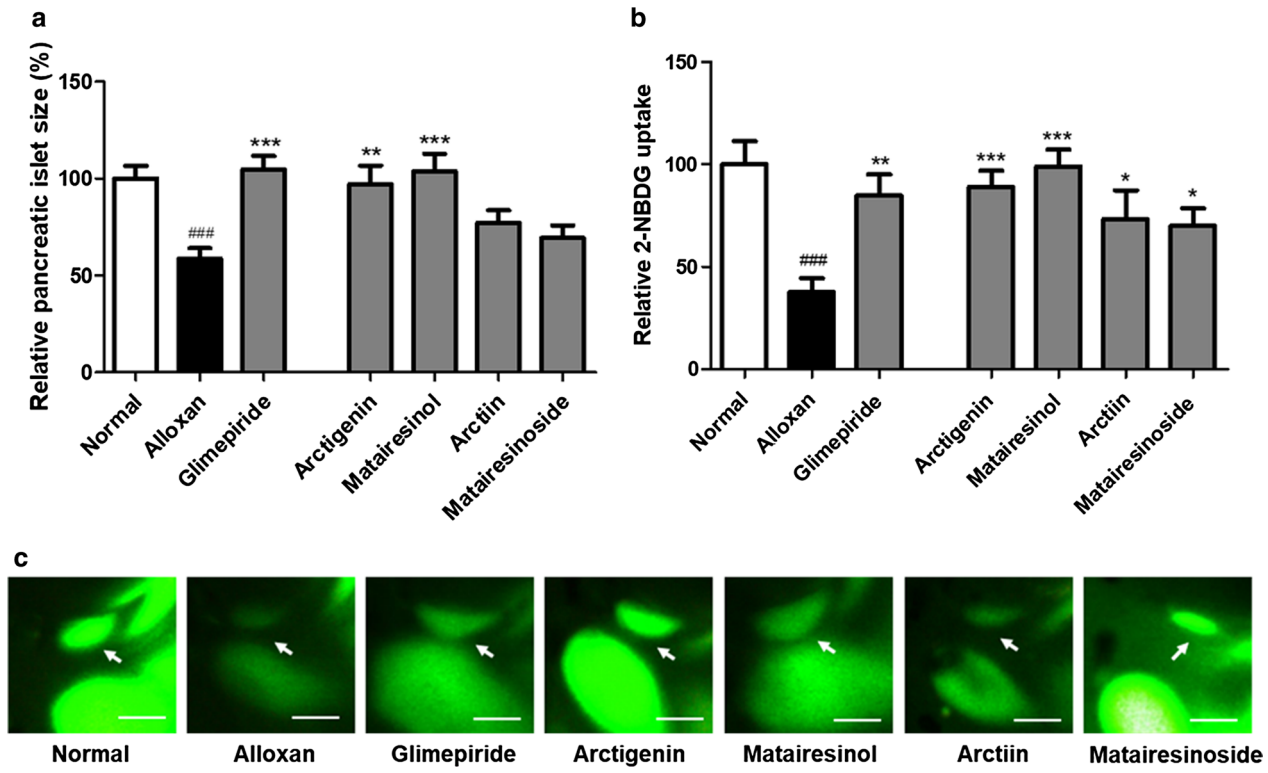


Fig. 2 Recovery effect of lignans **1–4** (10 μM) from *Forsythia koreana* flowers against AL-damaged PI in ZF. **a** Relative size of PI of each group. **b** Relative 2-NBDG uptake of PI. **c** PI images. Data are expressed as mean ± SD. ($^{###}p < 0.001$; compared to normal), ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; compared to AL). Scale bar = 100 μm

and arctiin by 32.28% ($p = 0.0203$) (Fig. 2b, c). Therefore, the efficacy of four lignans as therapeutic materials against AL-damaged PI in ZF was acknowledged.

Four lignans **1–4** and FKFM-nB and FKFM-BGP1-nB were further evaluated to confirm the absence of cytotoxic effects on zebrafish embryos. The hatching rate was checked because during embryonic development a high degree of cell differentiation and tissue organization is occurred [35]. The early life stages of zebrafish are sensitive to chemical exposure, making this one of the most accepted model for studying toxicity [35]. Thus, the hatching rate of the zebrafish exposed to both compounds and fractions was calculated. The exposure was started at the cleavage stage: 32–64 cells (2 h post fertilization), in order to check hatching rate at 72 h post fertilization. In the control group, 100% of hatching was occurred at 72 h post fertilization. Similarly, the treatment of *n*-BuOH fraction groups as well compounds **1**, **2**, and **4** showed 100% of hatching at 72 h post fertilization (Fig. 3a, b). While, the treatment of compound **3** decreased the hatching rate in high concentration without statistical difference compared to control group (Fig. 3a). Here we demonstrated that *n*-BuOH fractions and compounds **1–4** had no cytotoxic effects on zebrafish embryos.

Especially, aglycones **1** and **3** generally showed higher efficacy than those of glycosides **2** and **4**. Therefore, FKFM was fermented to gain the activity-strengthen material for recovery effect against AL-caused PI injury in ZF. FKFM was bioconverted using various hydrolyzing enzymes. TLC experiments suggested enzyme BGP1 is the most effective hydrolyzing gene, which cuts glucose in lignan glucosides (data not shown). Thereafter, FKFM-BGP1 was cloned using *E. coli*. The contents of four lignans in FKFM-nB and FKFM-BGP1-nB were quantified through LC/MS experiment. Four lignans were successfully separated at 20.892 min (**4**, matairesinoside), 25.184 min (**2**, arctiin), 27.258 min (**3**, matairesinol), and 29.174 min (**1**, arctigenin), respectively, which were identified without ambiguity based on mass analysis of each peak. In FKFM-nB, the lignan glycosides **2** (arctiin) and **4** (matairesinoside) had higher contents, 1.01 ± 0.01% and 1.42 ± 0.01%, respectively, than lignan algycones **1** (arctigenin) and **3** (matairesinol) 0.01 ± 0.01% and 0.01 ± 0.01%, respectively, (Fig. 4). In contrast, the contents of lignan aglycones **1** (2.42 ± 0.01%) and **3** (1.15 ± 0.01%) were much higher than glycosides **2**

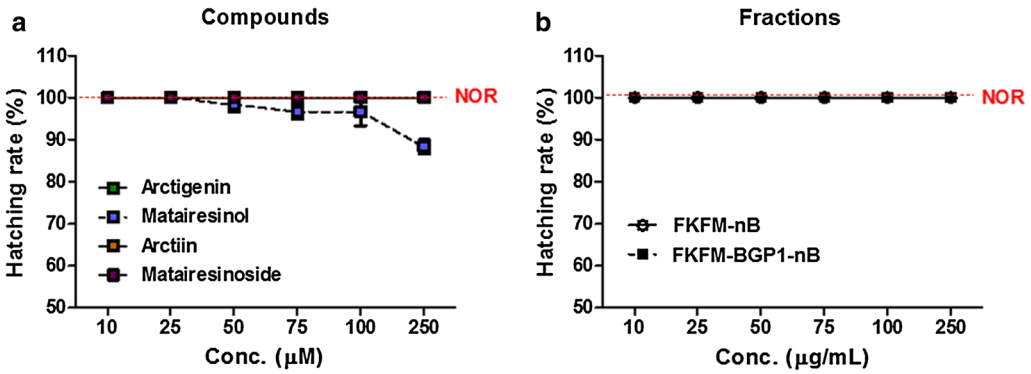
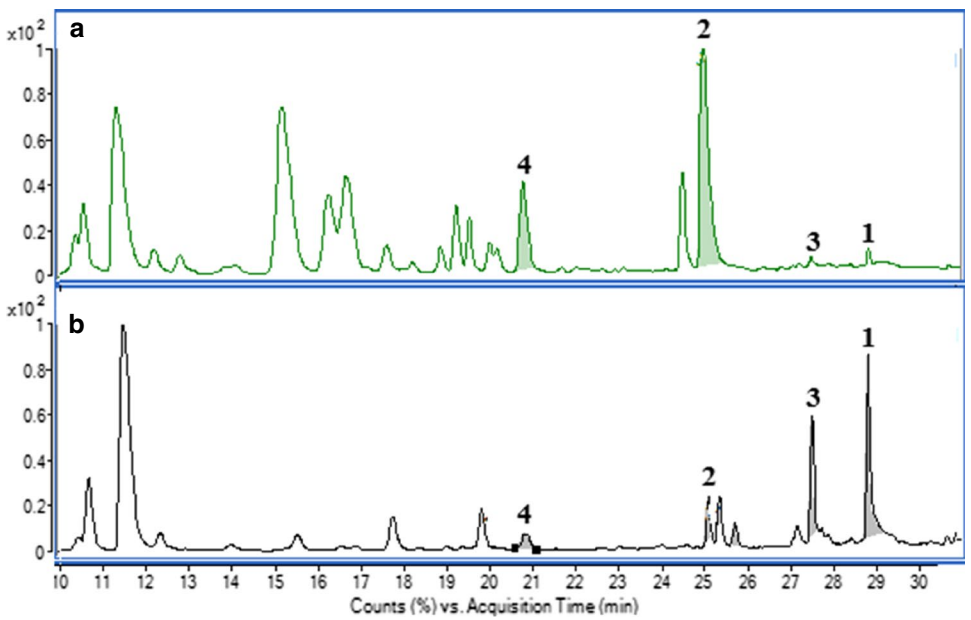


Fig. 3 Hatching rate of zebrafish embryos exposed to lignans **1–4** (**a**) and *n*-BuOH fractions (FKFM-nB and FKFM-BGP1-nB) from *Forsythia koreana* flowers for 72 h (**b**). FKFM-nB and FKFM-BGP-1-nB were obtained by extraction of both MeOH extracts using *n*-BuOH



	RT (min)	M.W.	observed molecular ion (*m/z*)	Content (%)	
				FKFM-nB	FKFM-BGP1-nB
arctigenin (**1**)	29.17	372	371 $[M-1]^-$	0.01±0.01	2.42±0.01
arctiin (**2**)	25.18	534	578 $[M+44]^-$	1.01±0.01	0.13±0.01
matairesinol (**3**)	27.25	358	357 $[M-1]^-$	0.01±0.01	1.15±0.01
matairesinoside (**4**)	20.89	520	519 $[M-1]^-$	1.42±0.01	0.32±0.01

Fig. 4 LC-ESI–MS chromatograms of compounds **1–4**, *n*-BuOH fraction of *Forsythia koreana* flowers MeOH extract (FKFM-nB), and *n*-BuOH fraction of fermented FKFM (FKFM-BGP1-nB), and mass spectra in multiple reaction monitoring (MRM) scan mode. FKFM-nB and FKFM-BGP-1-nB were obtained by extraction of both MEOH extracts using *n*-BuOH. Contents of lignans in FKFM-nB (**a**) and FKFM-BGP1-nB (**b**) from *F. koreana* flowers were determined based on LC/MS analysis

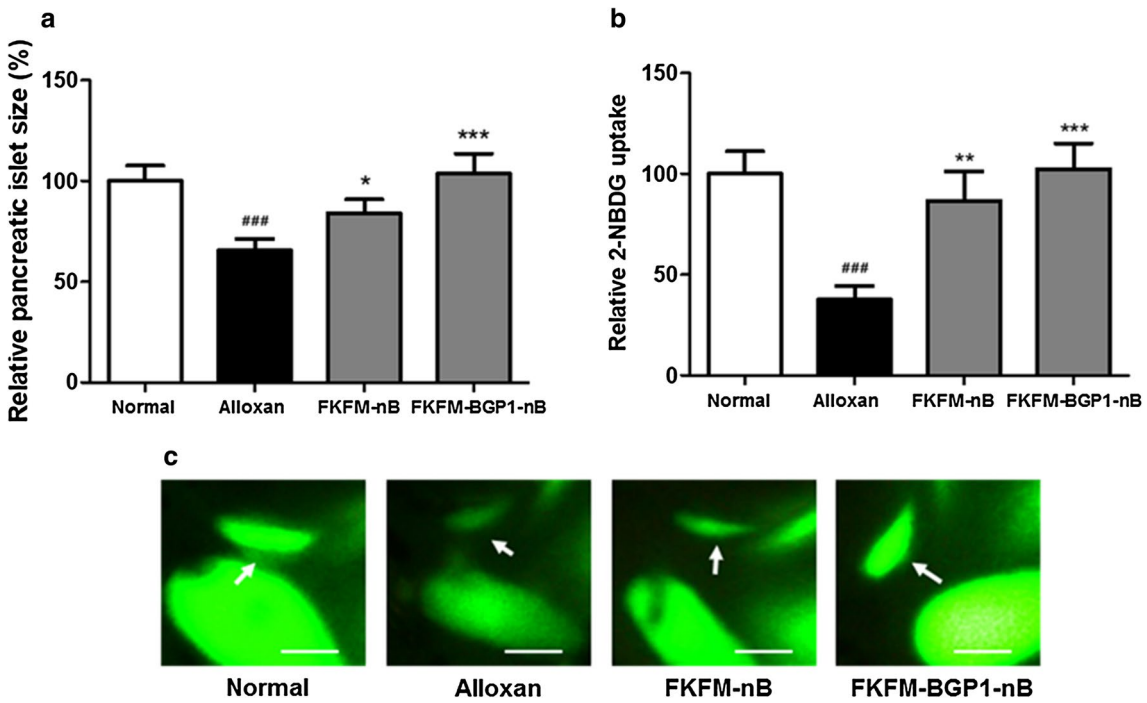


Fig. 5 Recovery effect of *n*-BuOH fractions from *Forsythia koreana* flowers MeOH extract (FKFM-nB) and fermented FKFM (FKFM-BGP1-nB) (1 μg/mL) against AL-induced PI in ZF. **a** Relative size of PI of each group. **b** Relative 2-NBDG uptake of PI. **c** PI images. Data are expressed as mean ± SD. ($^{###}p < 0.001$; compared to normal), ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; compared to AL). Scale bar = 100 μm

(0.13 ± 0.01%) and **4** (0.32 ± 0.01%) in FKFM-BGP1-nB. Accordingly, lignan glucosides, arctiin (**2**) and matairesinoside (**4**), are effectively converted into lignan aglycones, arctigenin (**1**) and matairesinol (**3**), by recombinant enzyme BGP1. On AL-caused PI injury in ZF, the islet size was also increased significantly in FKFM-nB and FKFM-BGP1-nB treated groups, 18.52%, $p = 0.0495$ and 38.30%, $p = 0.0010$, respectively, compared to AL-caused group. FKFM-BGP1-nB led to a greater increase of PI size by 19.78% than FKFM-nB (Fig. 5a, c). The relative 2-NBDG uptake results also showed a significant increase by treatment of FKFM-nB and FKFM-BGP1-nB by 49.10% ($p = 0.0047$) and 64.86% ($p = 0.0004$), severally, contrasted with AL treatment.

Consequently, this study demonstrates the pharmacological potential of lignan aglycones **1** and **3** as well FKFM-BGP1-nB obtained from FKF as anti-diabetic agents.

Authors' contributions

Y-G L, I R, TH K, and N-I B planned study and made paper. Y-G L, JE G, H-G K, and isolated lignans. Y-G L identified and quantitatively analysed all lignans. I R, YH N, SH W, BN H, and TH K performed anti-diabetic experiments. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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