Sesquiterpene Coumarins from *Ferula fukanensis* and Nitric Oxide Production Inhibitory Effects (2)[1,2)]

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Four new sesquiterpene coumarin derivatives, fukanemarin B (1), fukanefuromarin E (2), fukanefuromarin F (3) and fukanefuromarin G (5) were isolated from a 80% aqueous methanol extract of the roots of *Ferula fukanensis*. The structures were elucidated based on spectral evidence, especially heteronuclear multiple-bond connectivity (HMBC) and high-resolution MS. The 80% aqueous methanol extract of the roots of *Ferula fukanensis* (FFE) and the sesquiterpene coumarin derivatives inhibited nitric oxide (NO) production and inducible NO synthase (iNOS) gene expression by a murine macrophage-like cell line (RAW 264.7), which was activated by lipopolysaccharide (LPS) and recombinant mouse interferon-γ (IFN-γ).

Key words *Ferula fukanensis*; sesquiterpene coumarin; macrophage; nitric oxide

Ferula fukanensis grows on arid land in Central Asia. Previous studies have analyzed the polysulfanes in this plant by GC-MS (CI/EI),[3)] and the chemical constituents of plants in the genus *Ferula* (Umbelliferae) have been studied by many groups. Compounds commonly found in this genus are sesquiterpenes[4—10)] (especially daucanes, humulanes, and guaianes), sesquiterpene coumarins, and sesquiterpene chromones.[11—15)] *F. fukanensis* has been used as a traditional medicine for treatment of rheumatoid arthritis and bronchitis. In our investigation of *in vitro* anti-inflammatory screening of medicinal herbal extracts, the 80% aqueous methanol extract of the roots of *F. fukanensis* was observed to inhibit nitric oxide (NO) production in a lipopolysaccharide (LPS) activated murine macrophage-like cell line.

Macrophages play major roles in the immunity and inflammatory responses involved in host defense. Once activated, they initiate the production of cytokines, oxygen and nitrogen species, and eicosanoids. In macrophages, bacterial lipopolysaccharide (LPS), alone or in combination with recombinant mouse interferon-γ (IFN-γ), is the stimuli best characterized to induce the transcription of gene encoding pro-inflammatory proteins. Such stimulation results in cytokine release and the synthesis of enzymes such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). The nitric oxide (NO) radical is known to play a central role in inflammatory and immune reactions. However, excessive production of NO may cause tissue damage. In inflammatory diseases such as rheumatoid arthritis, excessive NO production by activated macrophages has been observed.

We recently discovered that 80% aqueous methanol extract of the roots of *Ferula fukanensis* (FFE) inhibited activated macrophage NO production.[2)] Therefore, the analysis of FFE was undertaken to identify the active compounds in the extract. Through bioactivity-guided fractionation, active compounds that inhibited NO production were isolated from the chloroform fraction. FFE and these compounds also inhibited iNOS mRNA expression in RAW 264.7 cells treated with LPS and IFN-γ.

Results and Discussion

FFE was partitioned by successive extraction with chloroform, ethyl acetate, and water. The chloroform soluble fraction inhibited 60% of NO production at 30 μg/ml. Therefore, the chloroform soluble fraction was separated by silica gel column chromatography to yield eleven fractions. Fraction 3 inhibited 100% of NO production at 30 μg/ml. Fraction 3 was purified by column chromatography and normal phase and reverse phase HPLC to give **1**—**6**.

Fukanemarin B (**1**) was obtained as a yellow oil, with a molecular weight of 408 based on fast atom bombardment mass spectrometry (FAB-MS) data that showed a protonated molecular ion peak at *m/z* 409 $(M+H)^+$ and a deprotonated molecular ion at *m/z* 407 $(M-H)^-$ in the negative mode. These data, together with the ^{1}H- and ^{13}C-NMR spectral data (Table 1), suggest a molecular formula of $C_{25}H_{28}O_5$, which was supported by HR-FAB-MS in the positive mode ($C_{25}H_{29}O_5$, *m/z* 409.20147).

The ^{1}H-NMR spectral data of **1** showed the presence of a 1,2,4-trisubstituted benzene ring at δ_H 7.62 (1H, d, J=8.8 Hz, H-5), δ_H 6.81 (1H, dd, J=8.8, 2.6 Hz, H-6), δ_H 6.76 (1H, d, J=2.6 Hz, H-8), a methoxy group at δ_H 3.85, and other signals characteristic of a sesquiterpene unit, determined on the basis of correlations of ^{1}H–^{1}H COSY, HMQC and HMBC spectra. The remaining ^{1}H- and ^{13}C-NMR data of **1**, except for those of the sesquiterpene unit, indicated a 7-oxygen-substituted coumarin compound. The correlations of δ_H 3.85 (OMe) with δ_H 6.81 (H-6) and δ_H 6.76 (H-8) in the differ-

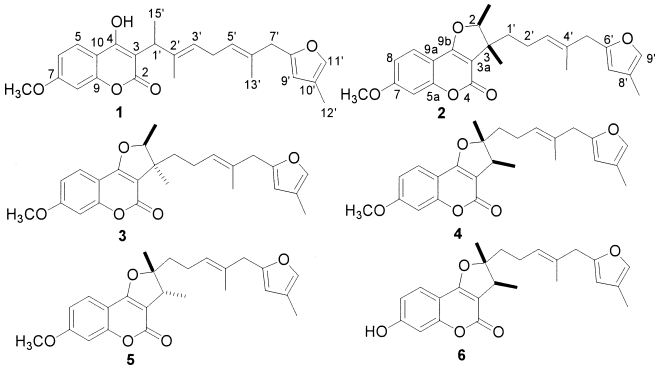

Fig. 1. Sesquiterpene Coumarins from *F. fukanensis*

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Table 1. NMR Spectral Data for Compound **1** (300 MHz for ^{1}H-NMR, 75 MHz for ^{13}C-NMR, $CDCl_3$, TMS, δ (ppm), J=Hz)[a]

Position	**1**	
	H	C
2		163.4
3		103.8
4		161.0
5	7.62 d (8.8)	123.5
6	6.81 dd (2.6, 8.8)	111.7
7		162.2
8	6.76 d (2.6)	99.8
9		153.7
10		108.9
1′	3.79 br q	37.9
2′		139.8
3′	5.74 t (7.0)	124.7
4′	2.91 m	26.9
5′	5.27 t (7.5)	123.0
6′		133.1
7′	3.29 s	38.3
8′		153.3
9′	5.91 s	108.6
10′		120.1
11′	7.07 br s	137.3
12′	2.0 s	9.8
13′	1.68 s	16.0
14′	1.75 s	17.0
15′	1.36 d (7.0)	16.2
4-OH	7.71 s	
OMe	3.85 s	55.5

a) Assignments confirmed by decoupling, ^{1}H–^{1}H COSY, HMQC, HMBC and different NOE spectra.

ences in NOE experiments, suggested the methoxy group was connected to C-7.

In the HMBC spectrum of **1**, the correlations of δ_H 3.79 (H-1′) with C-2, C-3, C-4, C-2′, and C-3′; δ_H 1.36 (H-15′) with C-3, C-1′, and C-2′; and δ_H 7.71 (OH) with C-3, C-4, and C-10 confirmed that the sesquiterpene unit is connected to C-3 of the coumarin unit and a hydroxyl group is attached to C-4. The chemical shifts of C-3 and C-4 are very close to those reported for similar compounds from *F. pallida*.[16] Furthermore, the HMBC correlations of δ_H 3.29 (H-7′) with C-5′, C-6′, C-8′ and C-9′; δ_H 5.91 (H-9′) with C-8′, C-10′ and C-11′; δ_H 7.07 (H-11′) with C-8′, C-9′ and C-10′; δ_H 9.8 (H-12′) with C-9′, C-10′ and C-11′ confirmed that **1** has a 7-substituted-4-methyl-2-furyl system. The configurations of the two double bonds of the sesquiterpene unit were determined on the basis of the differences in NOE experiments. Compound **1** showed significant NOE correlation between H-4′ and H-13′ and H-14′, H-3′ and H-5′, H-5′ and H-7′, indicating that the two double bonds of sesquiterpene unit of **1** has *E* configulation. Thus, **1** is 4-hydroxy-7-methoxy-3-[1,2,6-trimethyl-7-(4-methyl-2-furyl)-hepta-2(*E*),5(*E*)-dienyl]-coumarin.

Fukanefuromarin E (**2**) was obtained as a colorless oil. The mass spectral data for **2**—**5** showed the same molecular ions (see Experimental), which indicated the presence of isomeric compounds.

The ^{1}H- and ^{13}C-NMR spectra of **2** were similar to those of 2,3-dihydro-7-hydroxy-2*S**,3*R**-dimethyl-3-[4-methyl-5-(4-methyl-2-furyl)-3(*E*)-pentenyl]-furo[3,2-*c*]coumarin,[13] except for presence of a methoxy group (δ_H 3.86; δ_C 55.5) at

Table 2. ^{1}H-NMR Data for Compounds **2**, **3** and **5** (300 MHz), ($CDCl_3$, TMS, δ (ppm), J=Hz)[a]

H	**2**	**3**	**5**
2	4.86 q (7.0)	4.60 q (7.0)	
3			3.19 q (7.0)
6	6.82 m[b]	6.82 m[b]	6.81 m[b]
8	6.82 m[b]	6.82 m[b]	6.81 m[b]
9	7.52 d (9.0)	7.52 d (8.8)	7.51 br d
1′	1.73 m	1.66 m	1.71 m
			1.92 m
2′	2.07 m	2.03 m	2.26 m
3′	5.18 t (6.7)	5.13 t (7.0)	5.25 t (7.0)
5′	3.18 s	3.15 s	3.24 s
7′	5.84 s	5.82 s	5.88 s
9′	7.03 br s	7.02 br s	7.06 br s
2-Me	1.44 d (7.0)	1.53 d (7.0)	1.48 s
3-Me	1.28 s	1.44 s	1.29 d (7.0)
4′-Me	1.56 s	1.51 s	1.64 s
8′-Me	1.97 s	1.96 s	1.98 s
OMe	3.86 s	3.86 s	3.86 s

a) Assignments confirmed by decoupling, ^{1}H–^{1}H COSY, HMQC, HMBC and different NOE spectra. *b*) Overlapped signal.

Table 3. ^{13}C-NMR Data for Compounds **2**, **3** and **5** (75 MHz, $CDCl_3$, TMS, δ (ppm))[a]

C	**2**	**3**	**5**
2	89.3	92.7	95.8
3	46.8	46.5	44.2
3a	105.7[b]	105.7	103.5
4	159.9	160.1	160.7
5	156.3	156.4	156.3
6	100.2	100.2	100.3
7	162.9	162.6	162.5
8	111.8	111.8	111.8
9	123.2	123.3	123.3
9a	105.7[b]	106.1	106.0
9b	164.9	165.6	164.3
1′	37.9	34.4	34.9
2′	23.4	23.8	22.8
3′	125.4	125.9	125.3
4′	131.9	131.5	132.2
5′	38.2	38.2	38.3
6′	153.6	153.6	153.5
7′	108.4	108.4	108.5
8′	120.0	120.0	120.1
9′	137.2	137.2	137.3
2-Me	15.7	13.9	25.3
3-Me	19.1	23.3	13.5
4′-Me	15.9	15.9	15.9
8′-Me	9.8	9.8	9.8
OMe	55.5	55.5	55.5

a) Assignments confirmed by decoupling, ^{1}H–^{1}H COSY, HMQC, HMBC and different NOE spectra. *b*) Overlapped signal.

C-7. The correlations of δ_H 3.86 (OMe) with δ_H 6.82 (H-6, H-8) in the differences in NOE experiments, suggested the methoxy group was connected to C-7.

In the HMBC spectrum of **2**, the correlations of δ_H 1.44 (2-Me) with C-2 and C-3, and δ_H 1.28 (3-Me) with C-1′, C-2, C-3 and C-3a suggested that C-1′ is connected to C-3. That C-2 is connected to C-9b by an ether bond was deduced according to the unsaturation value and the chemical shifts of C-2 (δ_C 89.3) and C-9b (δ_C 164.9).[13] The relative configuration of the dimethyldihydrofuran moiety at C-2 and C-3 was determined on the basis of the difference NOE experiments.

Compound **2** showed significant NOE correlations between 2-Me and 3-Me, between H-2 and H-1′, indicating a *cis* relationship between 2- and 3-Me. The configuration of the double bond of the sesquiterpene unit was assigned as 4′*E* on the basis of the chemical shift of 4′-Me, which is shifted relatively upfield (δ_C 15.9).[17] Thus, **2** was established as 2,3-dihydro-7-methoxy-2*S**,3*R**-dimethyl-3-[4-methyl-5-(4-methyl-2-furyl)-3(*E*)-pentenyl]-furo[3,2-*c*]coumarin.

Fukanefuromarin F (**3**) was obtained as a colorless oil. The HMBC experiments suggested that the structure of **3** was similar to that of **2**. However, the NMR spectra of **3** differed slightly from those of **2**, especially at 3-Me (δ_C 23.3 for **3**, δ_C 19.1 for **2**) and C-2 (δ_C 92.7 for **3**, δ_C 89.3 for **2**), suggesting that **3** may be a diastereomer of **2** at chiral centers C-2 and C-3. The relative configuration of the dimethyldihydrofuran moiety at C-2 and C-3 was determined on the basis of the difference NOE experiments. Compound **3** showed significant NOE correlations between 2-Me and H-1′, between 3-Me and H-2, indicating a *trans* relationship between 2- and 3-Me. Thus, **3** is 2,3-dihydro-7-methoxy-2*R**,3*R**-dimethyl-3-[4-methyl-5-(4-methyl-2-furyl)-3(*E*)-pentenyl]-furo[3,2-*c*]coumarin.

Fukanefuromarin G (**5**) was obtained as a colorless oil. The HMBC experiment suggested that the structure of **5** was similar to that of **4**.[14] However, the NMR spectra of **5** differed slightly from those of **4**, especially C-1′ and 2-Me, indicating **5** may be a diastereomer of **4** at the chiral centers C-2 and C-3. The relative configuration of the dimethyldihydrofuran moiety at C-2 and C-3 was determined on the basis of the difference NOE experiments. Compound **5** showed significant NOE correlations between 2-Me and H-1′, between 3-Me and H-2, indicating a *trans* relationship between 2- and 3-Me. Thus, **5** is 2,3-dihydro-7-methoxy-2*R**,3*R**-dimethyl-2-[4-methyl-5-(4-methyl-2-furyl)-3(*E*)-pentenyl]-furo[3,2-*c*]coumarin.

Compounds **4** and **6** are known compounds, whose structures were elucidated by comparison with literature reports.[14]

When the RAW 264.7 were incubated with LPS and IFN-γ, NO production increased sharply. These compounds showed inhibitory activities (**1**: $IC_{50}=30.2\pm1.7\,\mu$M; **2**: $IC_{50}=29.0\pm1.0\,\mu$M; **3**: $IC_{50}=30.7\pm0.9\,\mu$M; **4**: $IC_{50}=27.8\pm4.6\,\mu$M; **5**: $IC_{50}=27.3\pm2.3\,\mu$M; **6**: $IC_{50}=87.5\pm11.7\,\mu$M, $n=3$). The cytotoxic effects of these compounds were measured using the MTT assay. These compounds (3—100 μM) did not demonstrate any significant cytotoxicity upon LPS/IFN-γ treatment for 24 h. By comparing the inhibitory activities of these compounds, we identified interesting features that may affect the activity level. Inhibitory activities of **4** were stronger than **6**, suggesting that methoxy group enhances sesquiterpene coumarin inhibitory activity. NO is synthesized by a family of enzymes termed NOS, which utilize arginine as a substrate in the generation of NO. Of the three NOS isoforms, the isoform expressed in the macrophage is termed iNOS. Its activity is regulated at the transcription level by cytokines as well as through cell exposure to immune and inflammatory stimuli. Compounds **1**, **2** and **4** inhibited iNOS mRNA expression in a dose-dependent manner (Fig. 2). The RT-PCR analysis in the present study indicated that LPS/IFN-γ treatment increased the level of iNOS mRNA expression, and the sesquiterpene coumarin derivatives inhibited this increase. Therefore, inhibition of iNOS induction by the sesquiterpene coumarin derivatives may be mediated through the expression of these transcription-activating factors, thereby inhibiting iNOS transcription.

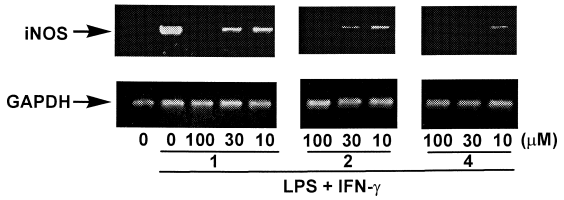

Fig. 2. Inhibitory Effect of Compounds **1**, **2** and **4** on Gene Expression on Stimulated by LPS and IFN-γ

Experimental

General Experiment Procedures UV spectra were obtained in MeOH on a Shimadzu UV-160 spectrophotometer, and IR spectra were recorded on a JASCO IR A-2 spectrophotometer. The NMR spectra were taken on a Mercury-300BB Varian spectrometer, with TMS as an internal standard. The mass spectra (MS) were obtained on a JOEL GCmate spectrometer. Column chromatography was carried out silica gel (Wako gel C-300, WAKO Pure Chemical Industry Ltd.). TLC was performed on Merck TLC plates (0.25 mm thickness), with compounds visualized by spraying with 5 % (v/v) H_2SO_4 in EtOH and then heating on a hot plate. HPLC was performed on a JASCO PU-2089 apparatus equipped with a JASCO UV-2075 detector. YMC-Pak SIL-06 (20×150 mm i.d.) columns and YMC-PAK Pro-C_{18} (20×150 mm i.d.) were used for preparative purposes.

Plant Materials The dried roots of *F. fukanensis* was collected in the Urumqi, Xinjiang, People's Republic of China, in October 2002.

Extraction and Isolation The dried roots of *F. fukanensis* (5.9 kg) were extracted with 80% MeOH. The solvent was evaporated under reduced pressure from the combined extract (448.8 g). The extract was suspended in water (3.0 l), and partitioned with chloroform (3×3 l) and ethyl acetate (3×3 l), successively. Evaporation of the solvent yielded a $CHCl_3$ fraction (272.4 g), EtOAc fraction (142.0 g), and the aqueous fraction (96.1 g). The $CHCl_3$ fraction was subjected to silica gel column chromatography (12×17 cm, eluted with hexane and EtOAc in increased polarity). The fractions (200 ml each) were combined according to TLC monitoring into eleven portions. Fraction 3, eluted with hexane–EtOAc (90 : 10), was isolated and further purified by column chromatography and normal phase HPLC (hexane–EtOAc, 85 : 15, flow rate 9 ml/min, UV detector set at 254 nm) to give **1** (13.7 mg, t_R 13.3 min), by reverse phase HPLC (CH_3CN–water, 87 : 13, flow rate 9 ml/min, UV detector set at 210 nm) to give **2** (13.2 mg, t_R 14.5 min), **3** (8.0 mg, t_R 15.3 min), **4** (23.0 mg, t_R 21.0 min) and **5** (9.7 mg, t_R 26.5 min), by reverse phase HPLC (CH_3CN–water, 72 : 28, flow rate 9 ml/min, UV detector set at 210 nm) to give **6** (18.3 mg, t_R 11.1 min).

Fukanemarin B (**1**): Yellow oil. $[\alpha]_D^{23}$ ±0° (*c*=0.23, MeOH). UV (MeOH) λ_{max} (log ε) nm: 310 (4.00), 245 (3.90), 212 (4.37). IR (KBr) ν_{max} cm^{-1}: 3408, 2974, 2935, 1767, 1707, 1616, 1512, 1444, 1379, 1277, 1249, 1195, 1159, 1110, 1026, 945, 838, 775 cm^{-1}. ^{1}H- and ^{13}C-NMR data, see Table 1. FAB-MS *m/z* 409 $[M+H]^+$. HR-FAB-MS *m/z* 409.20130 $[M+H]^+$ (Calcd for $C_{25}H_{29}O_5$ 409.20147).

Fukanefuromarin E (**2**): Colorless oil. $[\alpha]_D^{23}$ −2.0° (*c*=0.36, MeOH). UV (MeOH) λ_{max} (log ε) nm: 332 (3.89) 317 (4.00), 288 (3.68), 244 (3.75), 225 (4.03), 207 (4.37). IR (KBr) ν_{max} cm^{-1}: 3429, 2970, 2929, 1717, 1638, 1614, 1562, 1517, 1450, 1415, 1382, 1333, 1273, 1198, 1159, 1105, 1025, 983, 943 843, 768 cm^{-1}. ^{1}H- and ^{13}C-NMR data, see Tables 2 and 3. EI-MS *m/z* 408 $[M]^+$. HR-EI-MS *m/z* 408.19387 $[M]^+$ (Calcd for $C_{25}H_{28}O_5$ 408.19365).

Fukanefuromarin F (**3**): Colorless oil. $[\alpha]_D^{23}$ +41.7° (*c*=0.14, MeOH). UV (MeOH) λ_{max} (log ε) nm: 332 (3.98) 319 (4.09), 289 (3.77), 244 (3.86), 225 (4.15), 207 (4.45). IR (KBr) ν_{max} cm^{-1}: 3423, 2925, 1718, 1634, 1614, 1516, 1450, 1416, 1382, 1333, 1273, 1198, 1155, 1025, 984, 943, 841, 767 cm^{-1}. ^{1}H- and ^{13}C-NMR data, see Tables 2 and 3. EI-MS *m/z* 408 $[M]^+$. HR-EI-MS *m/z* 408.19365 $[M]^+$ (Calcd for $C_{25}H_{28}O_5$ 408.19365).

Fukanefuromarin G (**5**): Colorless oil. $[\alpha]_D^{23}$ −8.9° (*c*=0.18, MeOH). UV (MeOH) λ_{max} (log ε) nm: 325 (3.98) 316 (4.09), 288 (3.75), 244 (3.91), 225 (4.18), 207 (4.40). IR (KBr) ν_{max} cm^{-1}: 3418, 2973, 2938, 1768, 1726, 1638, 1614, 1561, 1517, 1449, 1413, 1378, 1331, 1273, 1194, 1156, 1112,

1026, 985, 948, 837, 762 cm^{-1}. ^1H- and ^{13}C-NMR data, see Tables 2 and 3; EI-MS *m/z* 408 $[M]^+$. HR-EI-MS *m/z* 408.19365 $[M]^+$ (Calcd for $C_{25}H_{28}O_5$ 408.19365).

Nitrite Assay[20] The cells were seeded at 1.2×10^6 cells/ml on to 96-well flat bottom plate (Sumitomo Bakelite, #8096R, Tokyo) and then incubated at 37 °C for 2 h. Then the test compound was added to the culture simultaneously with both *Escherichia coli* LPS (100 ng/ml) and recombinant mouse IFN-γ (0.33 ng/ml), and the cells were incubated at 37 °C, usually for 16 h. After incubation, the cells were chilled on ice. One 100 μl of the culture supernatant was placed in a well in duplicate 96-well flat-bottomed plates. A standard solution of $NaNO_2$ was also placed in other wells on the same plate. To quantify nitrite, Griess reagent 50 μl, 1% sulfanilamide in 5% H_3PO_4, and 0.1% *N*-1-naphthyletylenediamide dihydrochloride were added to each well. After 10 min, the reaction products were colorimetrically quantified at 550 nm with subtraction of the background absorbance at 630 nm, using a Model 3550 MICROPLATE READER (BIO-RAD).

Reverse Transcription-Polymerase Chain Reaction Analysis of iNOS mRNA The cells were cultured at 1.2×10^6 cells/ml on to 96-well flat bottom plate 37 °C for 2 h, then the test compound was added to the culture simultaneously with both LPS (100 ng/ml) and IFN-γ (0.33 ng/ml). The cells were incubated at 37 °C for approximately 8 h. Total RNA was isolated from the cell pellet using a RNA isolation kit (QIAGEN, Hilden, Germany). Total RNA (250 ng) was reverse-transcribed into cDNA by oligo$(dT)_{12-18}$ primer. The PCR sample contained 30 μl of the reaction mixture, comprised of 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM dNTP, 0.6 units of Ampli Taq GOLD (Applied Biosystems, CA, U.S.A), and 0.4 μmol of sense and antisense primers. The sense primer for iNOS was 5′-ACCTACTTCCTGGACATTACGACCC-3′ and the antisense primer was 5′-AAGGGAGCAATGCCCGTACCAG GCC-3′. The sense primer for glyceraldehydes-3-phosphatedehydrogenase (GAPDH) was 5′-ACCACAGTCCATGCCATCAC-3′, and the antisense primer was 5′-TCCACCACCCTGTTGCTGTA-3′. The PCR reaction was performed under the following conditions: 25 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1.5 min, using a thermal cycler (GeneAmp PCR Systems 9770; PE Applied Biosystems, U.S.A.). The PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining. The bands in the gel were then photographed.

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