Methyl Pothoscandensate, a New *ent*-18(4 → 3)-Abeokaurane from *Pothos scandens*

by **Hong-Xin Liu**[a])[b]), **Jun-Long Bi**[c]), **Yue-Hu Wang**[a]), **Wei Gu**[a])[b]), **Yao Su**[d]), **Fang Liu**[d]), **Shi-Xian Yang**[d]), **Guang-Wan Hu**[a]), **Ji-Feng Luo**[a]), **Ge-Fen Yin***[c]), and **Chun-Lin Long***[a])[e])

[a]) Key Laboratory of Economic Plants and Biotechnology, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, P. R. China (phone/fax: +86-10-68930381; e-mail: long@mail.kib.ac.cn)

[b]) Graduate University of Chinese Academy of Sciences, Beijing 100049, P. R. China

[c]) College of Animal Science and Technology, Yunnan Agricultural University, Kunming 650201, P. R. China (e-mail: yingefen383@sohu.com)

[d]) College of Horticulture and Landscape, Yunnan Agricultural University, Kunming 650201, P. R. China

[e]) College of Life and Environmental Sciences, Minzu University of China, Beijing 100081, P. R. China

Methyl pothoscandensate (**1**), a new molecular skeleton of *ent*-18(4 → 3)-abeokaurane, along with eight known compounds was isolated from the whole plant of *Pothos scandens*. The structure of the new compound was established by spectroscopic techniques and confirmed by single-crystal X-ray diffraction. The inhibitory activity of selected compounds against porcine respiratory and reproductive syndrome virus (PRRSV) was measured by the cytopathic effect (CPE) method. Compound **1** showed weak effect on PRRSV with an IC_{50} value of 40.3 ± 8.3 μM ($TI = 15.7$).

Introduction. – The genus *Pothos* is a medicinally important member in the plant family Araceae, consisting of *ca.* 75 species. There are five naturally occurring species found in China, mainly distributed in the south and southwest of Yunnan Province [1]. Some species of this genus have been used in treatment of traumatic injuries, fractures, and inflammation in Chinese traditional medicinal systems. Furthermore, boiled water decoctions of *P. scandens* leaves are used as tea by the Dai people [2]. Though this genus includes many individuals used as medicinal herbs, there have been very few chemical investigations on *Pothos*. As a result, we conducted the investigation of the chemical constituents of *P. scandens*. Repeated column chromatography and recrystallization of the AcOEt extract of the whole plant yielded a novel diterpenoid **1** (*Fig. 1*), as well as eight known compounds, *N-trans*-cinnamoyltyramine (=(2*E*)-*N*-[2-(4-hydroxyphenyl)ethyl]-3-phenylprop-2-enamide) [3], *N-trans*-feruloyltyramine (=(2*E*)-3-(4-hydroxy-3-methoxyphenyl)-*N*-[2-(4-hydroxyphenyl)ethyl]prop-2-enamide) [4], *N-trans-p*-cumaroyltyramine (=(2*E*)-3-(4-hydroxyphenyl)-*N*-[2-(4-hydroxyphenyl)ethyl]prop-2-enamide) [5], (−)-serotobenine (=*rel*-(2*R*,2a*R*)-2,2a,4,5,6,8-hexahydro-2-(4-hydroxy-3-methoxyphenyl)-3*H*-furo[2,3,4-*kl*]pyrrolo[4,3,2-*fg*][3]bentatocin-3-one) [6], (3*β*)-*ent*-kaurane-3,16,17-triol (**2**) [7], (+)-syringaresinol (=4,4′-(tetrahydro-1*H*,3*H*-furo[3,4-*c*]furan-1,4-diyl)bis[2,6-dimethoxyphenyl]) [8], (3*β*)-*ent*-kaurane-3,16,17-triol 3-*β*-D-glucopyranoside) [9], and (2*R*)-2-hydroxy-2-phenylacetonitrile 2-[*O*-*β*-D-xylopyranosyl-(1 → 6)-*β*-D-glucopyranoside] [10]. Herein we report the isolation and structure elucidation of **1**, and the results of the bioassay.

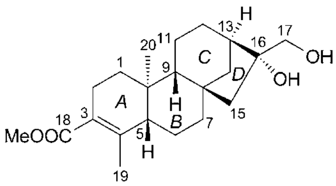


Fig. 1. *Methyl pothoscandensate* (**1**), *isolated from* Pothos scandens

Results and Discussion. – Compound **1** was isolated as colorless needle crystals and its molecular formula was established as $C_{21}H_{32}O_4$ based on the HR-ESI-MS (*m*/*z* 349.2381 ($[M+H]^+$, calc. 349.2378)), which requires 6 degrees of unsaturation. The ^{13}C-NMR and DEPT spectrum of **1** (*Table*) showed 21 C-atom signals including those of three Me, nine CH_2, and three CH groups and six quaternary C-atoms, of which the signals of an ester C-atom (δ(C) 170.2), two olefine C-atoms (δ(C) 145.4 and 123.7), two O-bearing C-atoms (δ(C) 81.8 and 66.3), and one MeO group (δ(C) 51.2) were detected. By comparing the NMR data with those of (3β)-*ent*-kaurane-3,16,17-triol (**2**; see *Scheme* below) [7], **1** was determined to be an *ent*-kaurane-type compound. Analysis of ^{1}H,^{1}H-COSY data of **1** (*Fig. 2*) established the two segments CH_2(1)CH_2(2) (**a**) and CH_2(12)CH(13)CH_2(14) (**b**)(*Fig. 2*). In the HMBC spectrum, the correlations CH_2(14)/C(9) (δ(C) 53.1), C(12) (δ(C) 25.9), C(15) (δ(C) 53.0), and C(16) (δ(C) 81.8), CH_2(11)/C(8) and C(13), and H–C(13)/(11), revealed the presence of a bicyclo[3.2.1]octane system (rings *C* and *D*). Based on the HMBCs CH_2(1)/C(20) and C(3), CH_2(2)/C(10), H–C(5)/C(1), CH_2(6)/C(8) and C(10), CH_2(7)/C(15), H–C(9) to C(1) and C(5), Me(19)/C(3), C(4), and C(5), and Me(20)/C(1), C(5), C(9), and C(10), rings *A* and *B* were assigned. The two OH groups were located at

Table. *^{1}H- and ^{13}C-NMR Data* (400 und100 MHz; $CDCl_3$) *of Compound* **1**. δ in ppm, *J* in Hz.

Position	δ(H)	δ(C)	Position	δ(H)	δ(C)
CH_2(1)	1.86–1.89, 1.05–1.08 (2*m*)	35.6 (CH_2)	CH_2(12)	1.62–1.65, 1.54–1.57 (2*m*)	25.9 (CH_2)
CH_2(2)	2.32–2.36 (*m*)	24.2 (CH_2)	H–C(13)	2.07 (br. *s*)	45.3 (CH)
C(3)		123.7 (C)	H_α–C(14)	2.01 (*d*, *J* = 11.9)	37.6 (CH_2)
			H_β–C(14)	1.63–1.66 (*m*)	
C(4)		145.4 (C)	CH_2(15)	1.58–1.61, 1.48–1.51 (2*m*)	53.0 (CH_2)
H–C(5)	1.86–1.88 (*m*)	50.2 (CH)	C(16)		81.8 (C)
CH_2(6)	1.86–1.89, 1.39–1.42 (2*m*)	22.0 (CH_2)	CH_2(17)	3.81, 3.69 (2*d*, *J* = 10.9)	66.3 (CH_2)
CH_2(7)	1.67–1.69, 1.54–1.57 (2*m*)	41.0 (CH_2)	C(18)		170.2 (C)
C(8)		44.1 (C)	Me(19)	1.92 (*s*)	18.1 (Me)
H–C(9)	1.05–1.08 (*m*)	53.1 (CH)	Me(20)	0.89 (*s*)	14.3 (Me)
C(10)		37.1 (C)	(MeO)	3.71 (*s*)	51.2 (Me)
CH_2(11)	1.66–1.69, 1.56–1.59 (2*m*)	18.5 (CH_2)			

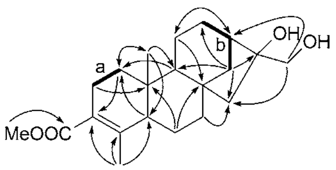


Fig. 2. *^{1}H,^{1}H-COSY* (**—**) *and key HMBC* (H → C) *features of* **1**

C(16) and C(17), respectively, by the correlations CH_2(14)/C(16), H–C(13)/C(11) and CH_2(17)/C(13) and C(15). Although the correlations between CH_2(2) and C(18) were not observed in the HMBC spectrum, the methoxycarbonyl group should be at the quaternary C-atom (C(3)). At last, the planar structure of **1** was deduced as shown in *Fig. 2*. The partial relative configuration of **1** was deduced by the analysis of ROESY correlations (*Fig. 3*). The key ROESY correlations H–C(5)/H–C(9) showed that the two H-atoms were cofacial, and were arbitrarily assigned to be β-oriented, while Me(20) was assigned the α-orientation. The correlation of CH_2(14)/Me(20) and H–C(13)/CH_2(14) confirmed H–C(13) to be α-oriented. The entire structure of **1** was finally determined by a single-crystal X-ray diffraction analysis (*Fig. 4*) [11]. Thus, **1** was elucidated as 16,17-dihydroxy-18(4 → 3)-abeo-*ent*-kaur-3-en-18-oic acid methyl ester and given the trivial name methyl pothoscandensate.

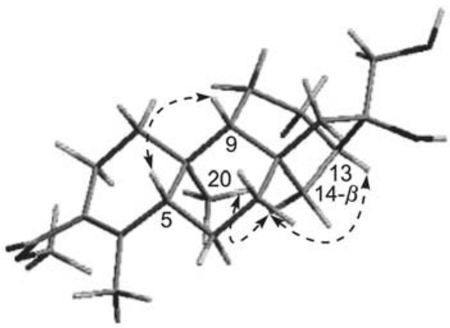


Fig. 3. *Key ROESY* (H ↔ H) *correlations of* **1**

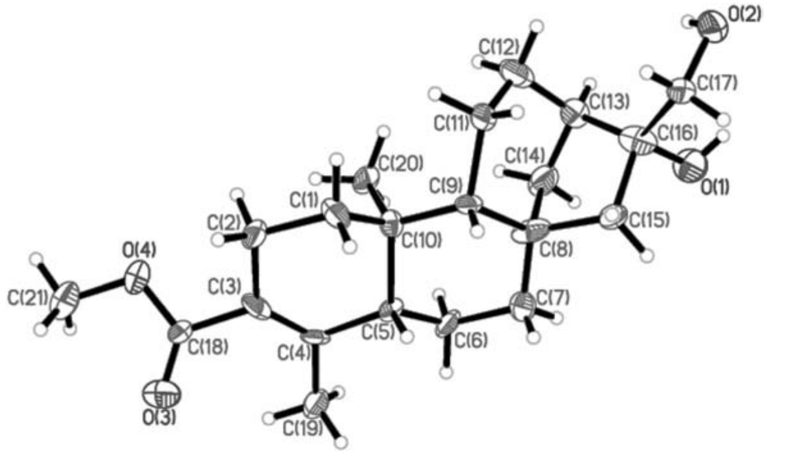
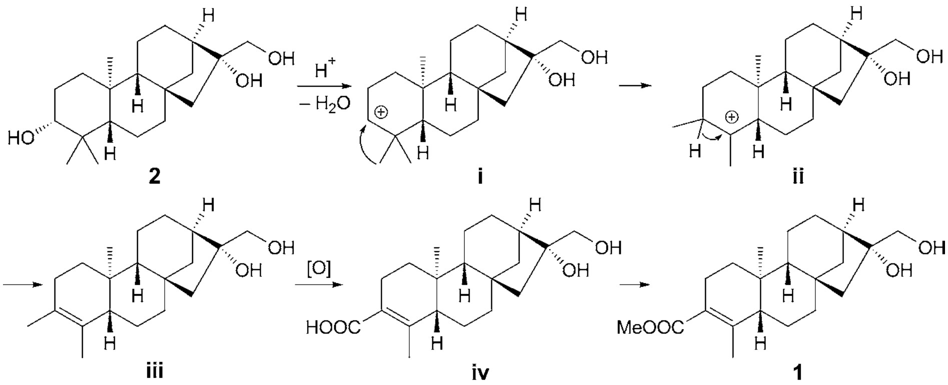


Fig. 4. *ORTEP Drawing of the X-ray structure of* **1**

A possible biogenetic pathway of **1** is proposed as shown in the *Scheme*. Compound **2** would be transformed into **iii** by *Wagner–Meerwein* rearrangements. Firstly, the OH group would leave from **2** to generate the cation intermediate **i** under acidic conditions.

Afterwards, one of the Me groups at C(4) would migrate to C(3) forming the intermediate **ii**, followed by H-atom elimination to give the key intermediate **iii**. Then, **iii** could be finally transformed to **1** by selectively oxidizing the allylic Me group to the acid followed by esterification.

Scheme. *Proposed Biogenetic Pathway of* **1**



The inhibitory activity of methyl pothoscandensate (**1**), *N-trans*-feruloyltyramine, *N-trans-p*-cumaroyltyramine, (–)-serotobenine, (3β)-*ent*-kaurane-3,16,17-triol (**2**), (+)-syringaresinol, and (2*R*)-2-hydroxy-2-phenylacetonitrile 2-[O-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside] against porcine respiratory and reproductive syndrome virus (PRRSV) was measured by the cytopathic effect (CPE) method [12]. Compound **1** showed a weak inhibitory effect on PRRSV with an IC_{50} value of 40.3 ± 8.3 μM (*TI* (therapeutic index) = 15.7) compared with tilmicosin phosphate (IC_{50} = 225.1 ± 27.4 μM, *TI* = 3.8). The other tested compounds were inactive (IC_{50} > 200 μM). Furthermore, by the real-time fluorescent quantitative reverse transcription-polymerase chain reaction (FQ RT-PCR) [13–15], the relative expression ratio of PRRSV ORF7 and NSP9 genes was tested. ORF7 and NSP9 mRNA relative expression level was significantly reduced by compound **1** at the concentration of 100 μM or more (P < 0.001; *Fig. 5*).

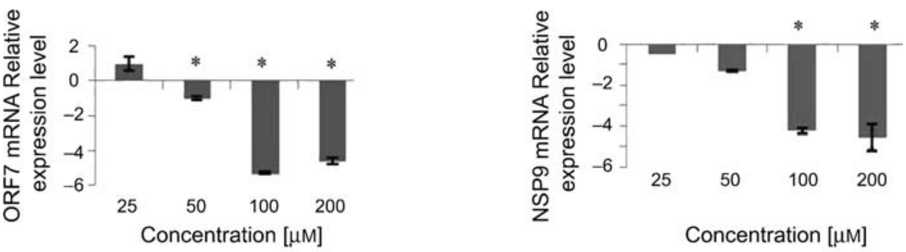


Fig. 5. *ORF7* (left) *and NSP9* (right) *mRNA relative expression level reduced by compound* **1** (*P < 0.001)

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Experimental Part

General. Column chromatography (CC): *MCI* gel (70–150 μm; *Mitsubishi Chemical Corporation*), *C₁₈* silica gel (SiO_2, 40–75 μm; *Fuji Silysia Chemical Ltd.*), *Sephadex LH-20* gel (*GE Healthcare Bio-Sciences AB*), and silica gel (SiO_2, 80–100, 20–300, and 300–400 mesh; *Qingdao Meigao Chemical Co.*). TLC: pre-coated SiO_2 F_{254} plates (*Qingdao Meigao Chemical Co.*); spots were detected under UV light (254 and 365 nm), and by spraying with 5% aq. H_2SO_4 in EtOH, followed by heating. M.p.: *X-4* melting-point apparatus (*Yingyu Yuhua Apparatus Factory*, Gongyi, P. R. China). Optical rotations: *Jasco-DIP-370* automatic digital polarimeter. UV Spectra: *Shimadzu-210A* double-beam spectrometer; λ_{max} (log ε) in nm. IR Spectra: *Bio-Rad-FTS-135* spectrophotometer; KBr pellets; ν in cm^{-1}. NMR Spectra: *Bruker-AM-400* and-*DRX-500* spectrometers; δ in ppm rel. to Me_4Si as internal standard, J in Hz. ESI-MS and HR-ESI-MS: *API-Qstar-Pulsar-1* instrument; in m/z.

Plant Material. The whole plant of *Pothos scandens* (8.3 kg) was collected from Xishuangbanna of Yunnan Province, P. R. China, in October 2010, and identified by Dr. *Guang-Wan Hu*, Kunming Institute of Botany. A voucher specimen (No. LHX-0091) was deposited with the Key Laboratory of Economic Plants and Biotechnology, Kunming Institute of Botany.

Extraction and Isolation. The air-dried powder of the plant material (8.3 kg) was exhaustively extracted with MeOH a total of 3 times, and the extract (0.79 kg) was suspended in H_2O and partitioned into three fractions with petroleum ether (*A*, 100 g), AcOEt (*B*, 165 g), and H_2O (*C*, 200 g). Subsequently, *Fr. B* was subjected to CC (SiO_2 $CHCl_3$/MeOH 1:0 → 0:1): *Frs. 1–6*. *Fr. 2* ($CHCl_3$/MeOH 15:1) was separated by CC (*RP-18*, MeOH/H_2O 2:8 → 9:1): *Frs. 2.1–2.3*. *Fr. 2.1* was fractionated by CC (*Sephadex LH-20*, MeOH; SiO_2, $CHCl_3$/MeOH 10:1): **2** (40.5 mg). *Fr. 2.2* was fractionated by CC (*Sephadex LH-20*, MeOH; SiO_2, $CHCl_3$/MeOH 15:1): (+)-syringaresinol (13.4 mg). *Fr. 3* ($CHCl_3$/MeOH 10:1) was separated by CC (*RP-18*, MeOH/H_2O 3:7 → 9:1): *Frs. 3.1–3.6*. *Fr. 3.1* was fractionated by CC (*Sephadex LH-20*, MeOH; silica gel, $CHCl_3$/MeOH 10:1): (−)-serotobenine (11.0 mg). *Fr. 3.2* was fractionated by CC (*Sephadex LH-20*, MeOH; SiO_2, petroleum ether/Me_2CO 3:1, $CHCl_3$/MeOH 10:1): N-trans-*feruloyltyramine* (29.0mg) and N-trans-p-*cumaroyltyramine* (113.0 mg). *Fr. 3.3* was fractionated by CC (*Sephadex LH-20*, MeOH; SiO_2, $CHCl_3$/MeOH 10:1): N-trans-*cinnamoyltyramine* (4.2 mg). *Fr. 3.4* was fractionated by CC (*Sephadex LH-20*, MeOH; SiO_2, petroleum ether/Me_2CO 5:1): **1** (64.5 mg). *Fr. 5* ($CHCl_3$/MeOH 3:1) was separated by CC (*RP-18*, MeOH/H_2O 6:4 → 9:1): *Frs. 5.1–5.3*. *Fr.* 5.2 was fractionated by CC (*Sephadex LH-20*, MeOH; SiO_2, $CHCl_3$/MeOH 5:1): (2*R*)-2-hydroxy-2-phenylacetonitrile 2-[*O*-β-D-xylopyranosyl-(1 → 6)-β-D-glucopyranoside] (15.0 mg). *Fr. 5.3* was fractionated by CC (*Sephadex LH-20*, MeOH; SiO_2, $CHCl_3$/MeOH 4:1): (3β)-*ent*-kaurane-3,16,17-triol 3-(β-D-glucopyranoside) (32.0 mg).

X-Ray Crystallographic Analysis of **1**. $C_{42}H_{64}O_8$ ($2 \times C_{21}H_{32}O_4$), M_r 696.93, colorless needle crystal, size $0.03 \times 0.12 \times 0.78$ mm³, monoclinic, space group $P2_1$; $a = 9.319(7)$, $b = 7.234(6)$, $c = 27.87(2)$ Å, $\alpha = \gamma = 90.00$, $\beta = 90.180(12)°$, $V = 1878(3)$ Å³, T 296(2) K, $Z = 2$, $\rho_{calc.} = 1.232$ g/cm³; $F(000) = 760$, 18137 reflections in $-12 \leq h \leq -12$, $-9 \leq k \leq 9$, $-37 \leq l \leq 7$, measured in the range $0.73° \leq \theta \leq 28.97°$, completeness $\theta_{max} = 94.7\%$, g.o.f. = 0.832. Final R indices: $R_1 = 0.1257$ and $wR_2 = 0.2601$, Flack parameter −2(4), largest difference peak and hole = 0.347 and −0.297 e Å^{-3}. The intensity data for **1** were collected on a *Bruker APEX DUO* diffractometer with graphite-monochromated MoK_α radiation. The structure of **1** was solved by direct methods (SHELXS97 [11]), expanded with difference *Fourier* techniques, and refined by the program and full-matrix least-squares calculations. The non-H-atoms were refined anisotropically, and H-atoms were fixed at calculated positions. CCDC-848942 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge *via* http://www.ccdc.cam.ac.uk./data request/cif.

Methyl Pothoscandensate (=*16,17-Dihydroxy-18(4 → 3)-abeo-*ent*-kaur-3-en-18-oic Acid Methyl Ester* = *Methyl* ent-*16,17-Dihydroxy-18-norkaur-3-ene-3-carboxylate*; **1**): Colorless needles (MeOH).

M.p. 102 – 103°. $[\alpha]_D^{23.4} = -103.5$ ($c = 0.20$, $CHCl_3$). UV ($CHCl_3$): 241.4 (3.12). IR: 3424, 1712, 1620. ^{1}H- and ^{13}C-NMR: *Table*. ESI-MS (pos.): 371 ($[M+Na]^+$). HR-ESI-MS (pos.): 349.2381 ($[M+H]^+$, $C_{21}H_{33}O_4^+$; calc.349.2378).

Cytopathic Effect Inhibition Assay. The 50% cytotoxic concentration (CC_{50}) of compounds **1**, **2** *N*-trans-feruloyltyramine, *N*-*trans*-*p*-cumaroyltyramine, (−)-serotobenine, (+)-syringaresinol, (2*R*)-2-hydroxy-2-phenylacetonitrile 2-[*O*-β-D-xylopyranosyl-(1 → 6)-β-D-glucopyranoside] and tilmicosin phosphate (positive control; *Hubei Hengshuo Chemical Co., Ltd.*, China) against Marc-145 cells (Cell Bank of the Chinese Academy of Sciences, Shanghai) was measured according to previously described protocols [16 – 18]. The antiviral activity of tested compounds against PRRSV was evaluated by the cytopathic effect (CPE) inhibition assay [12]. The YN-1 strain of PRRSV was isolated from local pigs in Yunnan Province, P. R. China [19]. The tissue culture medium infective dose ($TCID_{50}$) of 500 viral particles with twofold serial dilutions of the compounds were added to each test well, and the plates were re-incubated for 4 d to allow development of a cytopathologic effect (CPE) if any. A noninfection control was made in the absence of natural products, and tilmicosin phosphate was used for drug control. The concentration reducing CPE by 50% with respect to virus control was estimated from graphic plots and was defined as 50% inhibited concentration (IC_{50}). The therapeutic index (*TI*) was calculated from the ratio CC_{50}/IC_{50}.

PRRSV mRNA Expression Inhibition Assay. The mRNA expression of PRRSV ORF7 and NSP9 genes was determined by real-time RT-PCR [13 – 15]. Briefly, after 4 d of incubation, total virus RNA of both administration and control groups was isolated by means of *RNAiso^{TM} Plus* (*TaKaRa Biotechnology*, Dalian, P. R. China), dissolved in 30 µl of RNase-free H_2O (*TaKaRa*), and then stored at −80°. According to the GenBank data base, accession No. PRU87392, primers were selected and designed from conserved regions based on the ORF7 and NSP9 sequences by using Primer5.0 and Oligo6.0 software. A 330 base pair fragment of the PRRSV ORF7 gene was amplified by using the following primers: forward primer was 5′-AATGGCCAGCCAGTCAATCA-3′ and reverse primer was 5′-TCATGCTGAGGGTGATGCTG-3′. A 162 base pair fragment of the PRRSV NSP9 gene was amplified by using the following primers: forward primer was 5′-CACTAAAGAGGAAGTCGCACT-CA-3′ and reverse primer was 5′-GGTATGTCTCCAAACCTTGTATTCTG-3′. A 130 base pair fragment of the beta-actin gene was amplified by using the following primers: forward primer was 5′-ATCCAGGCTGTGCTGTCC-3′ and reverse primer was 5′-GAGGATCTTCATGAGGTAGTCG-3′.

cDNAs were synthesized with the *PrimeScript* ®RT reagent kit (*TaKaRa*) with 10 µl of reaction mixtures containing 4.5 µl of RNase free dH_2O, 2 µl of 5 × *PrimeScript*® buffer, 0.5 µl of *PrimeScript*® RT enzyme mix I, 0.5 µl of random 6 mers (100 µM), 0.5 µl of oligo dT primer (50 µM), and 2 µl of total RNA. The reaction programme was as follows: 37° for 15 min and 85° for 5 s. The PCR reaction mixture (25 µl) contained 12.5 µl of SYBR® *Premix Ex Taq^{TM}II* (*TaKaRa*), 0.5 µl of PCR forward primer (10 µM), 0.5 µl of PCR reverse primer (10 µM), 9.5 µl of dH_2O, and 2 µl of cDNA. The reactions were carried out in an *iQ5* real-time PCR system (*Bio-Rad Co., Ltd.*). The reaction programme was as follows: one cycle at 95° for 30 s, followed by 40 cycles at 95° for 5 s, 60° for 30 s.

Statistical Analyses. All experiments were performed in three replications. Continuous variables, expressed as mean ± s.d., were compared by using one-way ANOVA. Statistical analyses were conducted with SPSS 17.0.

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