

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

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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 $40.3 \pm 8.3 \mu\text{M}$ ($TI = 15.7$).

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*,2*aR*)-2,2*a*,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], (3β)-*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], (3β)-*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.

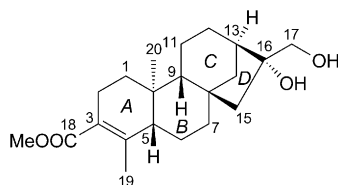


Fig. 1. *Methyl pothoscandensate* (**1**), isolated from *Pothos scandens*

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 ($\delta(C)$ 170.2), two olefine C-atoms ($\delta(C)$ 145.4 and 123.7), two O-bearing C-atoms ($\delta(C)$ 81.8 and 66.3), and one MeO group ($\delta(C)$ 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 $^1H,^1H$ -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 $CH_2(14)/C(9)$ ($\delta(C)$ 53.1), $C(12)$ ($\delta(C)$ 25.9), $C(15)$ ($\delta(C)$ 53.0), and $C(16)$ ($\delta(C)$ 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 HMBs $CH_2(1)/C(20)$ 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. 1H - and ^{13}C -NMR Data (400 und 100 MHz; $CDCl_3$) of Compound **1**. δ in ppm, J in Hz.

Position	$\delta(H)$	$\delta(C)$	Position	$\delta(H)$	$\delta(C)$
$CH_2(1)$	1.86–1.89, 1.05–1.08 (2m)	35.6 (CH_2)	$CH_2(12)$	1.62–1.65, 1.54–1.57 (2m)	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)	$H_\alpha-C(14)$	2.01 (d, $J=11.9$)	37.6 (CH_2)
			$H_\beta-C(14)$	1.63–1.66 (m)	
$C(4)$		145.4 (C)	$CH_2(15)$	1.58–1.61, 1.48–1.51 (2m)	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 (2m)	22.0 (CH_2)	$CH_2(17)$	3.81, 3.69 (2d, $J=10.9$)	66.3 (CH_2)
$CH_2(7)$	1.67–1.69, 1.54–1.57 (2m)	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 (2m)	18.5 (CH_2)			

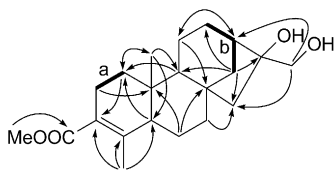


Fig. 2. $^1H,^1H$ -COSY (—) and key HMBC ($H \rightarrow C$) features of **1**

C(16) and C(17), respectively, by the correlations CH₂(14)/C(16), H–C(13)/C(11) and CH₂(17)/C(13) and C(15). Although the correlations between CH₂(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 CH₂(14)/Me(20) and H–C(13)/CH₂(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 \rightarrow 3)-abeo-*ent*-kaur-3-en-18-oic acid methyl ester and given the trivial name methyl ponthoscandensate.

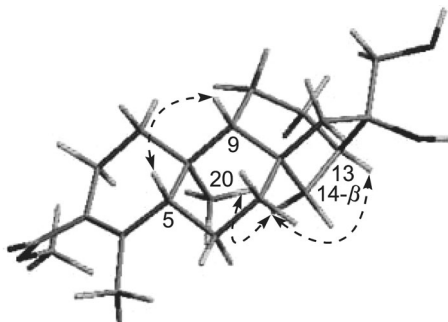


Fig. 3. Key ROESY (H \leftrightarrow H) correlations of **1**

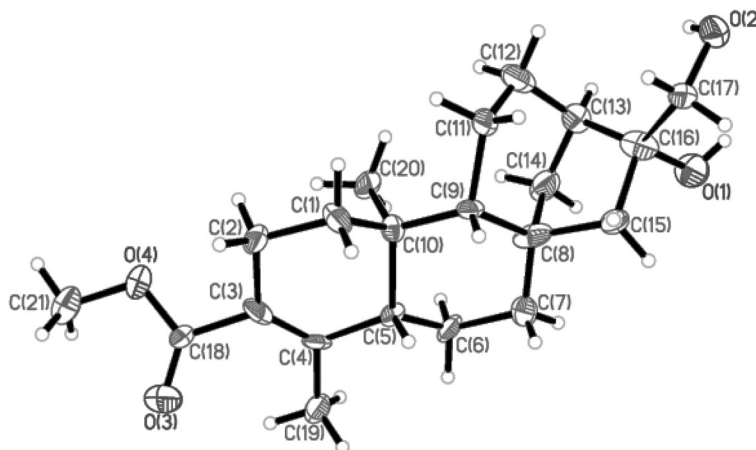
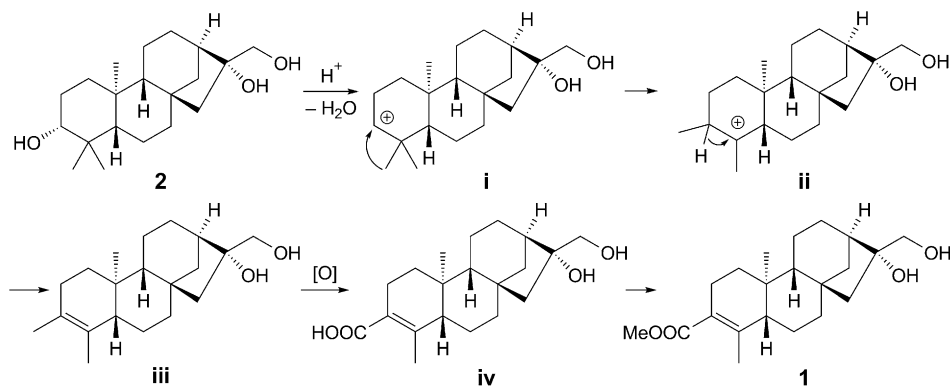


Fig. 4. ORTEP Drawing of the X-ray structure of **1**

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, (3 β)-*ent*-kaurane-3,16,17-triol (**2**), (+)-syringaresinol, and (2*R*)-2-hydroxy-2-phenylacetonitrile 2-[O- β -D-xylopyranosyl-(1 \rightarrow 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 $40.3 \pm 8.3 \mu\text{M}$ (TI (therapeutic index) = 15.7) compared with tilmicosin phosphate ($IC_{50} = 225.1 \pm 27.4 \mu\text{M}$, $TI = 3.8$). The other tested compounds were inactive ($IC_{50} > 200 \mu\text{M}$). 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).

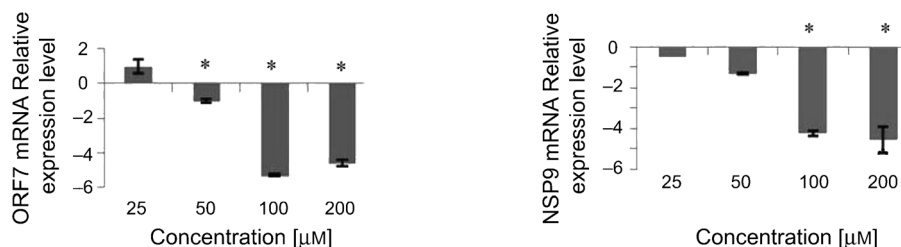


Fig. 5. *ORF7* (left) and *NSP9* (right) mRNA relative expression level reduced by compound **1** (* $P < 0.001$)

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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 *F₂₅₄* 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 $\text{CHCl}_3/\text{MeOH}$ 1:0 \rightarrow 0:1): *Frs. 1–6*. *Fr. 2* ($\text{CHCl}_3/\text{MeOH}$ 15:1) was separated by CC (*RP-18*, $\text{MeOH}/\text{H}_2\text{O}$ 2:8 \rightarrow 9:1): *Frs. 2.1–2.3*. *Fr. 2.1* was fractionated by CC (*Sephadex LH-20*, MeOH; SiO_2 , $\text{CHCl}_3/\text{MeOH}$ 10:1): **2** (40.5 mg). *Fr. 2.2* was fractionated by CC (*Sephadex LH-20*, MeOH; SiO_2 , $\text{CHCl}_3/\text{MeOH}$ 15:1): (+)-syringaresinol (13.4 mg). *Fr. 3* ($\text{CHCl}_3/\text{MeOH}$ 10:1) was separated by CC (*RP-18*, $\text{MeOH}/\text{H}_2\text{O}$ 3:7 \rightarrow 9:1): *Frs. 3.1–3.6*. *Fr. 3.1* was fractionated by CC (*Sephadex LH-20*, MeOH; silica gel, $\text{CHCl}_3/\text{MeOH}$ 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, $\text{CHCl}_3/\text{MeOH}$ 10:1): N-trans-feruloyltyramine (29.0 mg) and N-trans-p-cumaroyltyramine (113.0 mg). *Fr. 3.3* was fractionated by CC (*Sephadex LH-20*, MeOH; SiO_2 , $\text{CHCl}_3/\text{MeOH}$ 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* ($\text{CHCl}_3/\text{MeOH}$ 3:1) was separated by CC (*RP-18*, $\text{MeOH}/\text{H}_2\text{O}$ 6:4 \rightarrow 9:1): *Frs. 5.1–5.3*. *Fr. 5.2* was fractionated by CC (*Sephadex LH-20*, MeOH; SiO_2 , $\text{CHCl}_3/\text{MeOH}$ 5:1): (2*R*)-2-hydroxy-2-phenylacetone nitrile 2-[*O*- β -D-xylopyranosyl-(1 \rightarrow 6)- β -D-glucopyranoside] (15.0 mg). *Fr. 5.3* was fractionated by CC (*Sephadex LH-20*, MeOH; SiO_2 , $\text{CHCl}_3/\text{MeOH}$ 4:1): (3 β)-ent-kaurane-3,16,17-triol 3-(β -D-glucopyranoside) (32.0 mg).

X-Ray Crystallographic Analysis of **1**. $\text{C}_{42}\text{H}_{64}\text{O}_8$ ($2 \times \text{C}_{21}\text{H}_{32}\text{O}_4$), M_r 696.93, colorless needle crystal, size $0.03 \times 0.12 \times 0.78$ mm³, monoclinic, space group *P2₁*; $a = 9.319(7)$, $b = 7.234(6)$, $c = 27.87(2)$ Å, $\alpha = \gamma = 90.00$, $\beta = 90.180(12)^\circ$, $V = 1878(3)$ Å³, T 296(2) K, $Z = 2$, $\rho_{\text{calc.}} = 1.232$ g/cm³; $F(000) = 760$, 18137 reflections in $-12 \leq h \leq 12$, $-9 \leq k \leq 9$, $-37 \leq l \leq 37$, measured in the range $0.73^\circ \leq \theta \leq 28.97^\circ$, completeness $\theta_{\text{max}} = 94.7\%$, 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 \rightarrow 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°. $[\alpha]_{\text{D}}^{23.4} = -103.5$ ($c = 0.20$, CHCl_3). UV (CHCl_3): 241.4 (3.12). IR: 3424, 1712, 1620. ^1H - and ^{13}C -NMR: Table. ESI-MS (pos.): 371 ($[\text{M} + \text{Na}]^+$). HR-ESI-MS (pos.): 349.2381 ($[\text{M} + \text{H}]^+$, $\text{C}_{21}\text{H}_{33}\text{O}_4^{\ddagger}$; 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 $\text{CC}_{50}/\text{IC}_{50}$.

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 H₂O (*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'-CACTAAAGAGGAAGTCGCACTCA-3' and reverse primer was 5'-GGTATGTCTCCAAACCTGTATTCTG-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 dH₂O, 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 TaqTM II (*TaKaRa*), 0.5 μl of PCR forward primer (10 μM), 0.5 μl of PCR reverse primer (10 μM), 9.5 μl of dH₂O, 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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