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Chemical Constituents of *Crinum asiaticum* L. var. *sinicum* BAKER and Their Cytotoxic Activities

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A phytochemical investigation of the bulbs of *Crinum asiaticum* L. var. *sinicum* BAKER resulted in the isolation of two new alkaloids, asiaticumines A and B (**1** and **2**, resp.), together with 21 known compounds, including nine alkaloids, four amides, five phenolic compounds, and three flavonoids. All 23 compounds were isolated for the first time from *Crinum asiaticum* L. var. *sinicum* BAKER. Their structures were elucidated by spectroscopic methods. In addition, ten alkaloids, **1**–**10**, were evaluated for their cytotoxic activities against human tumor cell lines A549, LOVO, HL-60, and 6T-CEM. Compounds **3**, **4**, and **7**–**10** selectively showed remarkable inhibition against one or more of the tested cell lines.

Introduction. – The genus *Crinum*, with *ca.* 120 to 130 species [1], belongs to the family Amaryllideae. Only two species of this genus, *Crinum latifolium* L. and *Crinum asiaticum* L. var. *sinicum* BAKER, occur in China [2]. In China, the bulbs of *C. asiaticum* L. var. *sinicum* have been used in traditional medicine for the treatment of abscesses, aching joints, and sores [3]. The alkaloids from *Crinum* species possess various bioactivities, such as antitumor, antibacterial, and antifungal, and immunostimulating activities. So far, investigation on alkaloids from the genus *Crinum* has attracted particular attention. Our investigation on the cytotoxic constituents of *C. asiaticum* L. var. *sinicum* BAKER has led to the isolation of two new alkaloids, asiaticumines A and B (**1** and **2**, resp.), together with 21 known compounds. Furthermore, we also evaluated the cytotoxicities of compounds **1**–**10** against four cell lines, A549, LOVO, HL-60, and 6T-CEM.

Results and Discussion. – The $CHCl_3$- and AcOEt-soluble fractions of an EtOH extract of the bulbs of *Crinum asiaticum* L. var. *sinicum* BAKER were subjected to column chromatography over silica gel, *RP-18*, and *Sephadex LH-20*, as well as preparative TLC, to yield two new alkaloids **1** and **2** (*Fig. 1*) and 21 known compounds. By comparison of the physical and spectroscopic data with the published data, 21 known compounds were identified as: nine alkaloids, *i.e.*, crinumaquine (**3**) [4], (−)-lycorine (**4**) [5], hippacine (**5**) [6], hippadine (**6**) [7], ungeremine (**7**) [8], 11-*O*-methylcrinamine (**8**) [9], 3-*O*-acetylhamayne (**9**) [10], crinamine (**10**) [11], criwelline [12]; four amides, *i.e.*, *N*-4-*trans*-coumaroyltyramine [13], *N*-4-*trans*-caffeoyltyramine

[14], 4-hydroxystyryolamine [15], 4-aminobenzaldehyde [16]; three flavonoids, *i.e.*, (2*S*)-3′,7-dihydroxy-4′-methoxyflavan [17], 7-hydroxyflavanone [18], 4′,7-dihydroxyflavone [6]; five phenolics, *i.e.*, *trans*-caffeic acid [19], 4-coumatic acid [20], 4-hydroxybenzonic acid [21], ethyl 4-hydroxybenzoate, 2-(3,4-dihydroxyphenyl)1,3-benzodioxole-5-carboxaldehyde [22].

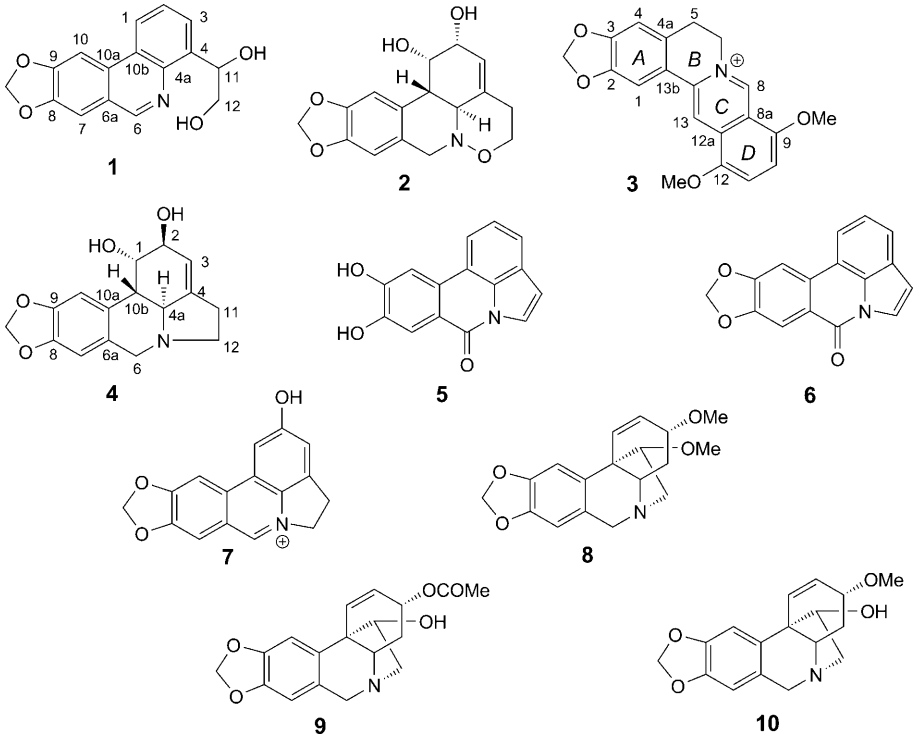

Fig. 1. *Structures of compounds* **1**–**10**

Compound **1** was obtained as a light yellow solid. The positive-ion HR-ESI-MS showed a quasimolecular-ion peak $[M+Na]^+$ at *m*/*z* 306.0740 (calc. 306.0742), in agreement with the molecular formula $C_{16}H_{13}NO_4$. The UV spectrum exhibited absorption bands at 253, 279, 310, 335, and 353 nm, typical for a phenanthridine alkaloid [1].

The ^{1}H-NMR spectrum of compound **1** (*Table 1*) displayed four *singlet*s at δ(H) 9.16, 8.27, 7.62, and 6.26, assigned to the aromatic H-atoms H−C(6), H−C(10), and H−C(7), and to a OCH_2O unit, respectively. In the aromatic region, another three signals at δ(H) 8.55 (*d*, *J* = 7.0), 7.66 (*dd*, *J* = 7.0, 8.0), and 7.82 (*d*, *J* = 8.0) were observed, indicating a 1,2,3-trisubstituted phenyl ring. The ^{13}C-NMR and DEPT spectra (*Table 1*) of **1** showed resonances for 16 C-atoms, including seven sp^2 quaternary C-atoms, seven CH (one OCH), and two CH_2 (one OCH_2O and one CH_2OH) groups. Detailed analysis of the NMR data of **1** revealed a great similarity to those of the previously reported compound trisphaeridine [23], except for an additional OCH (δ(C) 70.2) and a CH_2OH (δ(C) 67.1) group. The ^{1}H-NMR spectrum showed the mutual coupling CH

Table 1. *^{1}H- and ^{13}C-NMR Data of Compounds* **1**–**3**. δ in ppm, J in Hz.

1			**2**			**3**		
Position	δ(H)[a])	δ(C)[b])	Position	δ(H)[c])	δ(C)[d])	Position	δ(H)[e])	δ(C)[f])
H–C(1)	8.55 (*d*, *J* = 7.0)	121.4 (*d*)	H–C(1)	4.58 (br. *s*)	70.7 (*d*)	H–C(1)	7.79 (*s*)	105.3 (*d*)
H–C(2)	7.66 (*dd*, *J* = 7.0, 8.0)	126.2 (*d*)	H–C(2)	4.19 (br. *s*)	71.6 (*d*)	C(2)		149.7 (*s*)
H–C(3)	7.82 (*d*, *J* = 8.0)	125.6 (*d*)	H–C(3)	5.78 (br. *s*)	122.8 (*d*)	C(3)		147.5 (*s*)
C(4)		141.0 (*s*)	C(4)		136.6 (*s*)	H–C(4)	7.07 (*s*)	108.3 (*d*)
C(4a)		140.6 (*s*)	H–C(4a)	4.08 (*d*, *J* = 11.5)	72.8 (*d*)	C(4a)		120.3 (*s*)
H–C(6)	9.16 (*s*)	150.5 (*d*)	H_a–C(6)	4.60 (*d*, *J* = 15.0)	67.8 (*t*)	CH_2(5)	3.21 (*t*, *J* = 6.0)	26.2 (*t*)
C(6a)		123.5 (*s*)	H_b–C(6)	4.75 (*d*, *J* = 15.0)		CH_2(6)	4.95 (*t*, *J* = 6.0)	55.1 (*t*)
H–C(7)	7.62 (*s*)	105.2 (*d*)	C(6a)		127.4 (*s*)	H–C(8)	9.89 (*s*)	145.3 (*d*)
C(8)		148.0 (*s*)	H–C(7)	6.59 (*s*)	108.0 (*d*)	C(8a)		121.3 (*s*)
C(9)		151.4 (*s*)	H–C(8)		148.0 (*s*)	C(9)		150.2 (*s*)
H–C(10)	8.27 (*s*)	100.4 (*d*)	H–C(9)		148.7 (*s*)	H–C(10)	8.01 (*d*, *J* = 9.0)	123.4 (*d*)
C(10a)		129.9 (*s*)	H–C(10)	6.92 (*s*)	105.6 (*d*)	H–C(11)	8.19 (*d*, *J* = 9.0)	126.7 (*d*)
C(10b)		122.4 (*s*)	C(10a)		124.3 (*s*)	C(12)		143.6 (*s*)
H–C(11)	5.78 (*dd*, *J* = 4.0, 8.0)	70.2 (*d*)	H–C(10b)	3.31 (overlapped CD_3OD)	35.3 (*d*)	C(12a)		132.9 (*s*)
H_a–C(12)	3.77 (*dd*, *J* = 10.0, 4.0)	67.1 (*t*)	H_a–C(11)	2.82–2.86 (*m*)	26.9 (*t*)	H–C(13)	8.96 (*s*)	120.0 (*d*)
H_b–C(12)	3.50 (*dd*, *J* = 10.0, 8.0)		H_b–C(11)	2.98–3.04 (*m*)		C(13a)		137.4 (*s*)
OCH_2O	6.26 (*s*)	102.1 (*t*)	H_a–C(12)	3.79 (*ddd*, *J* = 10.5, 9.0, 1.5)	68.7 (*t*)	C(13b)		130.5 (*s*)
			H_b–C(12)	3.96–3.99 (*m*)		OCH_2O	6.16 (*s*)	101.9 (*t*)
			OCH_2O	5.96, 5.95 (2*s*, each 1 H)	102.4 (*t*)	MeO	4.07 (*s*)	57.0 (*q*)
						MeO	4.10 (*s*)	61.8 (*q*)

[a]) Recorded at 500 MHz in (D_6)DMSO. [b]) Recorded at 125 MHz in (D_6)DMSO. [c]) Recorded at 500 MHz in CD_3OD. [d]) Recorded at 125 MHz in CD_3OD. [e]) Recorded at 600 MHz in (D_6)DMSO. [f]) Recorded at 150 MHz in (D_6)DMSO.

and CH_2 due to ^1H resonances at δ(H) 5.78 (*dd*, $J = 8.0, 4.0$), 3.77 (*dd*, $J = 10.0, 4.0$), and 3.50 (*dd*, $J = 10.0, 8.0$), indicating the presence of a fragment $-CHOHCH_2OH$. The above deduction was further confirmed by the $^1H,^1H$-COSY correlation between H−C(11) and CH_2(12). The HMBC spectrum (*Fig. 2*) exhibited the correlations of H−C(11) with C(4) (δ(C) 141.0), and H−C(3) with C(11), implying that the fragment $-CHOHCH_2OH$ was located at C(4). The HMBC correlations of OCH_2O (δ(H) 6.26) with C(8) (δ(C) 148.0) and C(9) (δ(C) 151.4) indicated that OCH_2O moiety was connected to C(8) and C(9). Consequently, the structure of **1** with as yet unknown absolute configuration was determined and named asiaticumine A.

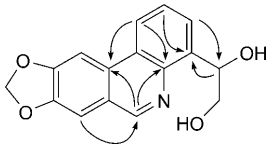

Fig. 2. *Key HMBC correlations of* **1**

Compound **2**, a yellow powder, has a molecular formula $C_{16}H_{17}NO_5$ as deduced from the negative-ion HR-ESI-MS ($[M+Cl]^-$ at *m/z* 338.0782; calc. 338.0795). The UV spectrum, similar to that of lycorine [24], showed absorption bands at 291, 236, and 217 nm.

The ^1H- and ^{13}C-NMR spectra (*Table 1*) indicated the presence of one 1,2,4,5-tetrasubstituted phenyl (δ(H) 6.59 (*s*), 6.92 (*s*); δ(C) 127.4 (*s*), 108.0 (*d*), 148.0 (*s*), 148.7 (*s*), 105.6 (*d*), 124.3 (*s*)), one OCH_2O (δ(H) 5.96 (*s*) and 5.95 (*s*), each 1 H; δ(C) 102.4 (*t*)), and one trisubstituted C=C group (δ(H) 5.78 (br. *s*); δ(C) 122.8 (*d*), 136.6 (*s*)). Moreover, the ^{13}C-NMR and DEPT spectra also displayed signals of two OCH groups at δ(C) 70.7 (*d*) and 71.6 (*d*), and of one OCH_2 group at δ(C) 68.7 (*t*). Compared to those of lycorine, the C-atom resonances of C(4), C(6a), and C(10b) of **2** were shifted upfield by 2–5 ppm, while those of C(4a), C(6), and C(12) were shifted downfield by 8–15 ppm. Considering that the molecular formula of **2** indicated an additional O-atom compared to that of lycorine, and that their structures possess the same degrees of unsaturation, it was suggested that the O-atom was located between C(12) and the N-atom. The HMBC correlations (*Fig. 3*) of H−C(10) with C(10b), H−C(7) with C(6), H−C(1) with C(4a), H−C(6) with C(4a), and H−C(12) with C(4) also supported the above deduction. The NOESY spectra of **2** showed the following correlations: H−C(1)/H−C(2), H−C(1) and H−C(2)/H−C(10b), which suggested that both H−C(1) and H−C(2) are β-configurated. The structure of **2** was thus determined, and named asiaticumine B.

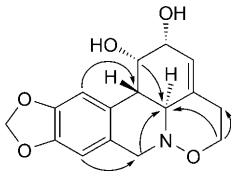

Fig. 3. *Key HMBC correlations of* **2**

Compound **3**, a yellow powder, was inferred to have the molecular formula $C_{20}H_{18}NO_4$, with twelve degrees of unsaturation, by the molecular-ion peak M^+ at m/z 336 in the positive-ion ESI-MS, in conjunction with the NMR data. The UV (MeOH) spectrum showed maximum absorption bands at 230, 267, and 347 nm, which are typical for berberine alkaloids [13].

Analysis of the ^{13}C-NMR and DEPT spectra (*Table 1*) revealed the presence of one OCH_2O, two MeO, and two sp^3-CH_2 groups, and 15 aromatic C-atoms (six CH and nine quarternary C-atoms). The ^{1}H-NMR spectrum showed four *singlet*s for aromatic H-atoms at δ(H) 7.79, 7.07, 9.89, and 8.96, two *doublet*s for aromatic H-atoms at δ(H) 8.01 (d, J = 9.0), 8.19 (d, J = 9.0), and a *singlet*, for the OCH_2O group at δ(H) 6.16 (s). The above evidence further confirmed that **3** was a berberine alkaloid. The only difference between **3** and berberine was in the location of two MeO groups. In the HMBC spectrum (*Fig. 4*), two MeO groups showed correlations with C(9) and C(10), respectively, suggesting that two MeO were groups at C(9) and C(10), respectively. Accordingly, the structure of **3** was identified and named crinumaquine. The structure of **3** has already been reported as a dye molecule [4]. We isolated this compound from the nature and assigned its NMR data, for the first time.

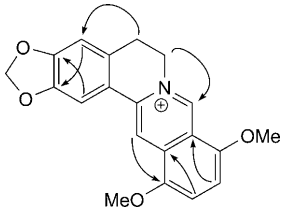

Fig. 4. *Key HMBC correlations of* **3**

Cytotoxicity. – Compounds **1**–**10** (*Fig. 1*) were tested for their cytotoxicities against human tumor cell lines A549, LOVO, HL-60, and 6T-CEM by the standard MTT method, with adriamycin as positive control. As shown in *Table 2*, compound **2** only

Table 2. *Cytotoxic Activities of Compounds* **1**–**10**. The data represent means ± S.D.

Compound	IC_{50} [μg/ml]			
	A549	LOVO	6T-CEM	HL-60
1	> 100	> 100	> 100	> 100
2	16.3 ± 6.8	31.4 ± 9.8	11.7 ± 3.5	13.7 ± 4.4
3	1.13 ± 0.67	42.8 ± 11.8	0.65 ± 0.25	2.28 ± 1.62
4	0.65 ± 0.36	0.59 ± 0.35	1.42 ± 1.56	0.17 ± 0.06
5	40.0 ± 13.2	18.2 ± 7.2	29.8 ± 16.8	27.2 ± 11.2
6	> 100	> 100	> 100	> 100
7	0.43 ± 0.34	13.1 ± 5.8	3.89 ± 2.45	0.63 ± 0.35
8	9.15 ± 4.80	81.0 ± 13.1	14.4 ± 5.5	6.16 ± 2.94
9	15.8 ± 9.8	5.49 ± 3.19	3.45 ± 1.67	4.27 ± 2.15
10	15.9 ± 5.5	4.30 ± 2.11	2.82 ± 1.89	1.70 ± 1.14
Adriamycin[a])	0.01 ± 0.007	0.32 ± 0.16	0.11 ± 0.08	0.0015 ± 0.0004

[a]) Positive control.

exhibited weak cytotoxicity against A549, HL-60, and 6T-CEM with IC_{50} values of 16.3, 13.7, 11.7 μg/ml, respectively, while compound **3** demonstrated significant inhibition against A549, 6T-CEM, and HL-60 cell lines with IC_{50} values of 1.13, 0.65, and 2.28 μg/ml, respectively. Compound **4** showed strong cytotoxicity against A549, LOVO, 6T-CEM, and HL-60 with IC_{50} values of 0.65, 0.59, 1.42, and 0.169 μg/ml, respectively. Compound **7** also strongly inhibited the proliferation of cell lines A549, LOVO, 6T-CEM, and HL-60 with IC_{50} values of 0.43, 13.1, 3.89, and 0.63 μg/ml, respectively. Compound **8** exhibited cytotoxicity only against 6T-CEM and HL-60 with IC_{50} values of 14.4 and 6.16 μg/ml, respectively. Compound **9** demonstrated cytotoxicity against A549, LOVO, 6T-CEM, and HL-60 with IC_{50} values of 15.8, 5.49, 3.45, and 4.27 μg/ml, respectively. Compound **10** exhibited remarkable cytotoxicity against A549, LOVO, 6T-CEM, and HL-60 with IC_{50} values of 15.9, 4.30, 2.82, and 1.70 μg/ml, respectively.

Experimental Part

General. Column chromatography (CC): silica gel (200–300 mesh), *H60* (*Qingdao Marine Chemical Plant*, Qingdao, P. R. China); *Sephadex LH-20* (*Pharmacia Fine Chemicals*, Piscataway, NJ, USA). TLC: pre-coated silica gel GF_{254} plates (*Qingdao Marine Chemical Plant*, Qingdao, P. R. China). Optical rotations: *Perkin-Elmer 341* digital polarimeter (*Perkin-Elmer*, Norwalk, CT, USA) at 589 nm. UV: *Shimadzu UV-2550*; λ_{max} in nm (log ε). IR Spectra: *Bruker Vector-22* spectrophotometer; KBr pellets, in cm^{-1}. ^{1}H-, ^{13}C-, and 2D-NMR spectra: *Bruker DRX-500* spectrometer; in $CDCl_3$; δ in ppm rel. to Me_4Si, *J* in Hz. MS: *Agilent-1100-LC/MSD-Trap* (ESI-MS) and *Agilent Micro-Q-Tof* (HR-ESI-MS) spectrometer; in *m*/*z*.

Plant Material. The bulbs of *C. asiaticum* L. var. *sinicum* BAKER were collected in September 2005, in Fugong county, Yunnan, P. R. China, and authenticated by Prof. *Yuanchuan Zhou*. A voucher specimen (No. 2005071509) is deposited with the School of Pharmacy, Second Military Medical University.

Extraction and Isolation. The dried and powdered bulbs of *C. asiaticum* L. var. *sinicum* BAKER (30 kg) were extracted with 95% EtOH by infiltration. The solvent was evaporated under vacuum to afford 1010 g of crude extract, which was suspended in H_2O and partitioned with petroleum ether (PE), $CHCl_3$, AcOEt, and H_2O-sat. BuOH successively. The $CHCl_3$ extract (150 g) was subjected to CC (silica gel (200–300 mesh, 900 g); $CHCl_3$/MeOH 100 : 1 → 1 : 1) to give ten fractions (*A*–*J*), which were further purified by repeated CC (silica gel, *Sephadex LH-20*; MeOH), and recrystallization to afford **1** (12 mg), **2** (10 mg), **3** (30 mg), **4** (15 mg), and **5** (16 mg). Similarly, the AcOEt extraction (40 g) yielded **6** (17 mg), **7** (19 mg), **8** (13 mg), **9** (14 mg), and **10** (12 mg), together with 13 other compounds (see *Results and Discussion*).

Asiaticumine A (= *1-([1,3]Dioxolo[4,5-*j*]phenanthridin-4-yl)ethane-1,2-diol*; **1**). Light yellow powder. $[\alpha]_D^{20} = +6$ (*c* = 0.29, MeOH). UV (MeOH): 253 (4.01), 279 (3.89), 310 (2.95), 335 (sh), 353 (sh). ^{1}H- and ^{13}C-NMR: *Table 1*. ESI-MS (pos.): 306 ($[M+Na]^+$). HR-ESI-MS (pos.): 306.0740 ($[M+Na]^+$, $C_{16}H_{13}NNaO_4^+$; calc. 306.0742).

Asiaticumine B (= *(1*S*,2*R*,13b*S*,13c*R*)-2,3,4,5,13b,13c-Hexahydro[*J*][1,3]dioxolo-1*H*,8*H*-[1,2]oxazino[4,3,2-*de*]phenanthridine-1,2-diol*; **2**). Light yellow powder, $C_{16}H_{17}NO_5$. $[\alpha]_D^{20} = -69$ (*c* = 0.27, MeOH). UV (MeOH): 217 (3.70), 236 (2.85), 291 (3.01). ^{1}H- and ^{13}C-NMR: *Table 1*. ESI-MS (neg.): 338 ($[M + Cl]^-$). HR-ESI-MS (neg.): 338.0782 ($[M+Cl]^-$, $C_{16}H_{17}ClNO_5^-$; calc. 338.0795).

Crinumaquine (= *5,6-Dihydro-9,12-dimethoxybenzo[*g*][1,3]-benzodioxolo[5,6-*a*]quinolizinium*; **3**). Yellow powder. UV (MeOH): 230 (4.30), 267 (4.29), 347 (4.25). ^{1}H- and ^{13}C-NMR: *Table 1*. ESI-MS: 336 (M^+).

Assay for Cytotoxicity. A MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium hydrobromide) colorimetric assay was performed in 96-well plates. Cell cultures were diluted with fresh medium consisting of RPMI1640 with 15% newborn bovine serum (NBS), 100 IU/ml penicillin, and 100 IU/ml phytomycin to 5×10^4 cells/ml and plated in 96-well microplates at 100 μl/well. After 24 h

incubation at 37° in a 5% CO_2 atmosphere, the tested compounds at six different concentrations (10^{-2}–10^2 μg/ml) were added to the microplates in 10-μl amounts. The four tumor cell lines, A549, LOVO, 6T-CEM, and HL-60, were exposed to the drugs for another 72 h. Then, 20 μl of MTT soln. (5 mg/ml) were added to each well, and the plate was incubated for 4 h at 37° with 5% CO_2. The OD of each well was measured on a plate reader (*Wellscan MK-2*, *Labsystems*, Finland) at 570 nm. Adriamycin (purchased from *Nanjing Tianzun Zezhong Chemical Co. Ltd.*, P. R. China) was used as positive reference substance with concentrations of 10^{-3}–10^2 μg/ml. The cell lines were all preserved in Shanghai Institute for Pharmaceutical Industry, P. R. China. The experiment was performed three times independently. The data are represented as mean ± S.D.

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