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Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

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Online publication date: 13 September 2010

To cite this Article Zhao, Hu-Yi , Wu, Fu-Kai , Qiu, Ying-Kun , Wu, Zhen , Jiang, Yong-Tao and Chen, Ji-Yong(2010) 'Studies on cytotoxic constituents from the skin of the toad *Bufo bufo gargarizans*', Journal of Asian Natural Products Research, 12: 9, 793 — 800

To link to this Article: DOI: 10.1080/10286020.2010.505187
URL: http://dx.doi.org/10.1080/10286020.2010.505187

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Journal of Asian Natural Products Research
Vol. 12, No. 9, September 2010, 793–800

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ORIGINAL ARTICLE

Studies on cytotoxic constituents from the skin of the toad *Bufo bufo gargarizans*

Hu-Yi Zhao[a], Fu-Kai Wu[b], Ying-Kun Qiu[a]*, Zhen Wu[a], Yong-Tao Jiang[c] and Ji-Yong Chen[c]

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(*Received 2 February 2010; final version received 28 June 2010*)

To study the chemical composition of the skin of *Bufo bufo gargarizans*, many kinds of chromatography methods were used in the isolation procedures, while the structures of isolated compounds were determined on the basis of their NMR and MS spectral analysis. As a result, two new compounds were isolated from its ethanolic extract and characterized as cinobufotalin 3-nonanedioylarginine ester (**8**) and bufotalin 3-pimeloylarginine ester (**14**). Furthermore, 13 known compounds were obtained. Isolated bufadienolides showed significant inhibition effect against SMMC-7721 cell lines *in vitro*.

Keywords: *Bufo bufo gargarizans*; toad skin; cinobufotalin 3-nonanedioylarginine ester; bufotalin 3-pimeloylarginine ester; cytotoxicity

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1. Introduction

Toads, particularly members of the genus *Bufo*, are identified as a convenient and useful source of granular gland secretions, which commonly contain biogenic amines, bufadienolides, alkaloids, steroids, peptides, and proteins. Including the genus *Bufo*, the different amphibian skin possessed a wide range of biologically active substances with therapeutic potential. In the traditional Chinese medicine, amphibian skin had been used for the alleviation of human sufferings. Chan'Pi, the skin of a Chinese toad (*Bufo bufo gargarizans*), whose extract preparation known as 'Hua Chan Su', was used in the treatment of various diseases, including cancer, arrhythmia, and heart diseases [1]. Many of these effects are attributed to bufadienolides [2], one of the active compounds in this drug. Recently, new biological activities of bufadienolides, such as an effect of bufalin on inducing apoptosis in human leukemia U937 cells by modulating the MAPK pathways, an inhibitory effect of several bufadienolides on tumor cell growth, and an inhibitory effect on the IL-6 activities by 20,21-epoxy resibufogenin, have been reported [3]. In order to find new and bioactive bufadienolides from Chan'Pi, we investigated the skin of *B. bufo gargarizans*. From its ethanolic extract, we obtained two new bufadienolides, named cinobufotalin 3-nonanedioylarginine ester (**8**) and bufotalin 3-pimeloylarginine ester (**14**), and 13 known compounds (Table 1). Moreover, the isolated bufadienolides showed significant inhibition effects against SMMC-7721 cell lines *in vitro*.

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ISSN 1028-6020 print/ISSN 1477-2213 online
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DOI: 10.1080/10286020.2010.505187
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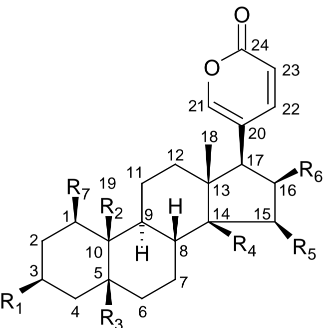
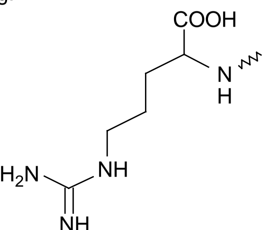
Table 1. Structures of compounds **1–15**.



Arg:



	R_1	R_2	R_3	R_4		R_5	R_6	R_7
1	OH	CH_3	H	OH		H	OAc	H
2	OH	CHO	OH	OH		H	H	H
3	OH	CHO	OH		—O—		OH	H
4	OH	CH_3	OH	OH		H	H	H
5	OH	CH_3	H		—O—		H	H
6	OH	CHO	OH	OH		H	H	H
7	OH	CH_3	OH	OH		H	H	OH
8	OCO $(CH_2)_7$COArg	CH_3	OH		—O—		OAc	H
9	OCO $(CH_2)_2$COArg	CH_3	H		—O—		OAc	H
10	OCO $(CH_2)_2$COArg	CH_3	H	OH		H	OAc	H
11	OCO $(CH_2)_6$COArg	CHO	OH	OH		H	H	H
12	OCO $(CH_2)_2$COArg	CH_3	H	OH		H	H	H
13	OCO $(CH_2)_6$COArg	CH_3	OH	OH		H	H	H
14	OCO $(CH_2)_5$COArg	CH_3	H	OH		H	OAc	H
15	OCO $(CH_2)_6$COArg	CH_3	H	OH		H	H	H

2. Results and discussion

Compound **8** gave a quasi-molecular ion peak at *m/z* 784.9353 $[M + H]^+$ by HR-ESI-MS, which is consistent with the molecular formula of $C_{41}H_{60}N_4O_{11}$. The UV spectrum of **8** showed absorption maximum due to an α-pyrone group at 295 nm. The IR absorption bands at 3399, 2942, 2935, 1724, 1715, 1638, and $1535\,cm^{-1}$ revealed the existence of the hydroxyl, carboxyl, methyl, amide, and α-pyrone groups. The ^1H NMR spectral data at δ_H 8.16, 7.54, 6.44, and ^{13}C NMR spectral data at δ 161.5, 152.6, 148.9, 116.7, and 113.9 in the low field of **8** also indicated a *mono*-substituted α-pyrone residue. All the data suggested that compound **8** was a bufadienolide. Five oxygen-bearing carbon signals (δ 75.3, 72.6, 70.9, 60.0, and 50.4) appeared in the ^{13}C NMR spectrum of **8**. The methyl signals could also be observed at δ 20.2, 17.2, and 17.1 in the ^{13}C NMR spectrum. On the basis of its ^1H and ^{13}C NMR spectral data, the aglycone of **8** was identified as cinobufotalin, whose ^1H and ^{13}C NMR spectral data were assigned by comparing with those in the literature and further confirmed by the experiments of DEPT and 2D NMR as shown in Figure 1, except for the acylation shifts around the C-3 of **8**. The arginine structure of **8** was established by comparison of their NMR chemical shifts and proton–proton correlations with those of **9**. Remaining signals (δ 173.1, 34.7, 25.1, 29.4, 29.2, 26.0, 25.2, 36.6, and 172.8) in the ^{13}C NMR spectrum belong to the azelaic acid fragment. With the help of ^1H–^1H COSY, HSQC, and DEPT experiments, the carbon and proton signals could be attributed one by one. After acid hydrolysis, three fragments, cinobufotalin, arginine, and azelaic acid could be obtained. With the aid of ^1H–^1H COSY and ^{13}C–^1H COSY spectra, the structure of **8** could be drawn as the combination of an aszelaic acid residue, a

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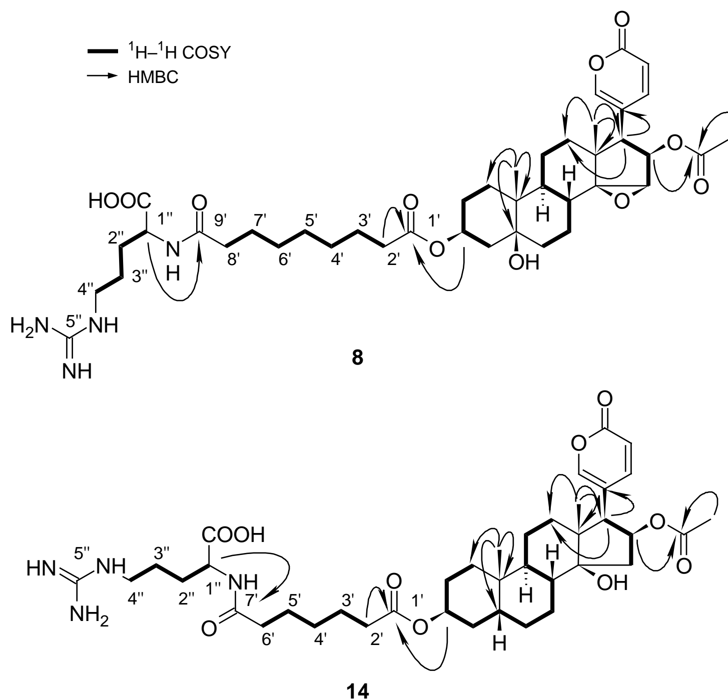

Figure 1. Key ^1H–^1H COSY, HMBC, NOE correlations of compounds **8** and **14**.

cinobufotalin unit, and an arginine moiety. In the HMBC spectrum, long-range correlations could be found between H-1′ (δ 4.83) and C-9′ (δ 172.8), as well as H-3 (δ 5.33) and C-1′ (δ 173.1). Thus, compound **8** was determined to be cinobufotalin 3-nonanedioylarginine ester (**8**), as shown in Figure 1.

For compound **14**, ESI-MS gave its quasi-molecular ion peak at *m/z* 743 $[M + H]^+$. The extract molecular weight [743.8986, $(M + H)^+$, calcd 743.8984] and the molecular formula ($C_{39}H_{58}N_4O_{10}$) were given by high-resolution ESI-MS measurement. The UV spectrum of **14** showed absorption maximum at 295 nm. The IR absorption bands at 3426, 2930, 2854, 1739, 1721, 1631, and 1535 cm^{-1} revealed the existence of the hydroxyl, carboxyl, methyl, amide, and α-pyrone groups. The ^1H and ^{13}C NMR spectral data in the low field of **14** (Table 2) also indicated a *mono*-substituted α-pyrone residue. All the data suggested that compound **14** was a bufadienolide, too. Three carbon signals bearing oxygen (δ 70.5, 57.5, and 41.0) and two signals of the methyl group (δ 24.0 and 17.1) appeared in the ^{13}C NMR spectrum. Furthermore, the structure of arginine was supported by comparison of the corresponding ^{13}C NMR spectral data of **9**. There was a set of signals (δ 172.9, 34.7, 25.3, 29.2, 26.0, 36.6, and 172.3) belonging to a pimelic acid

Table 2. ^1H NMR spectral data of compounds **8** and **14**.

No.	**8**	**14**
1	2.14 (1H, m), 1.72 (1H, m)	1.57 (2H, m)
2	1.75 (1H, m), 1.68 (1H, m)	1.69 (2H, m)
3	5.33 (1H, br s)	5.31 (1H, br s)
4	2.33 (1H, m), 1.85 (1H, m)	2.01 (1H, m), 1.50 (1H, m)
5		1.79 (1H, m)
6	1.94 (1H, m), 1.52 (1H, m)	1.88 (1H, m), 1.29 (1H, m)
7	1.48 (1H, m), 1.36 (1H, m)	2.22 (1H, m), 1.36 (1H, m)
8	1.69 (1H, m)	1.85 (1H, m)
9	2.13 (1H, m)	1.68 (1H, m)
11	1.77 (1H, m), 1.03 (1H, m)	1.36 (1H, m), 1.20 (1H, m)
12	1.72 (1H, m), 1.50 (1H, m)	1.53 (1H, m), 1.36 (1H, m)
15	3.92 (1H, br s)	2.72 (1H, dd, $J = 9.1$, 15.2 Hz), 2.13 (1H, m)
16	5.66 (1H, d, $J = 9.2$ Hz)	5.76 (1H, t-like)
17	3.05 (1H, d, $J = 9.2$ Hz)	3.08 (1H, d, $J = 8.8$ Hz)
18	0.85 (3H, s)	0.99 (3H, s)
19	1.17 (3H, s)	0.96 (3H, s)
21	7.54 (1H, br s)	7.62 (1H, br s)
22	8.16 (1H, br d, $J = 9.9$ Hz)	8.66 (1H, br d, $J = 9.7$ Hz)
23	6.44 (1H, d, $J = 9.9$ Hz)	6.39 (1H, d, $J = 9.7$ Hz)
2′	2.36 (2H, m)	2.38 (2H, m)
3′	1.73 (2H, m)	1.76 (2H, m)
4′	1.31 (2H, m)	1.42 (2H, m)
5′	1.38 (2H, m)	1.78 (2H, m)
6′	1.44 (2H, m)	2.39 (2H, m)
7′	1.70 (2H, m)	
8′	2.41 (2H, m)	
1″	4.83 (1H, br s)	4.93 (1H, d, $J = 5.8$ Hz)
2″	2.14 (1H, m), 1.95 (1H, m)	2.22 (1H, m), 2.01 (1H, m)
3″	1.67 (2H, m)	1.69 (2H, m)
4″	3.51 (1H, br s), 3.58 (1H, br s)	3.62 (1H, br s), 3.41 (1H, br s)
COCH_3	1.84 (3H, s)	1.79 (3H, s)

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fragment. Acid hydrolysis of **14** yielded bufotalin, arginine, and pimelic acid which could be identified by comparing their NMR spectral data and Rf (retardation factor) values with authentic samples. With the aid of $^{1}H-^{1}H$ COSY and $^{13}C-^{1}H$ COSY spectra (Figure 1), the structure of **14** could be drawn as the combination of a pimelic acid residue, a bufotalin unit (**1**), and an arginine. In the HMBC experiment, correlations were observed between H-3 (δ 5.31) and C-1′ (δ 172.9), H-1′ (δ 4.93) and C-7′ (δ 172.3), so the above moieties could be linked with each other to form a whole structure of **14**. Thus, compound **14** was elucidated as bufotalin 3-pimeloylarginine ester as shown in Figure 1.

Other known compounds were identified by their melting points and ^{1}H, ^{13}C NMR spectral data with those reported in the literature. They were characterized as bufotalin (**1**) [4], hellebrigenin (**2**) [5], telocinobufagin (**3**) [6], 16-desacetyl-19-oxocinobufotalin (**4**) [7], resibufogenin (**5**) [4], daigredorigenin (**6**) [8], arenobufagin (**7**) [9], cinobufagin 3-succinoylarginine ester (**9**) [10], 3-argininylsuccinoyl bufotalin (**10**) [10], hellebritoxin (**11**) [11], bufalin 3-succinoylarginine ester (**12**) [10], telocinobufatoxin (**13**) [12], and bufalitoxin (**15**) [13].

3. Experimental

3.1 General experimental procedures

Melting points were determined on an XT-4 micromelting point apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer digital polarimeter. UV spectra were recorded on a Shimadzu UV-260 spectrometer. IR spectra were determined on a Perkin-Elmer 683 infrared spectrometer in KBr pellets. NMR spectra were taken with TMS as the internal standard on a Bruker Avance 400 FT-NMR spectrometer. HR-ESI-MS were measured on a Bruker FT-MS Apex III spectrometer and ESI-MS on a Finnigan Lcq advantage spectrometer. Column chromatography was performed on silica gel (Marine Chemical Factory, Qingdao, China), Sephadex LH-20 (Amersham Pharmacia Biotech AB, Uppsala, Sweden), and Cosmosil 75 C_{18}-OPN (Nakalai Tesque Co. Ltd, Tokyo, Japan). TLC was conducted on silica GF254 (Marine Chemical Factory) and RP-18 F254 (Merck, Darmstadt, Germany) plates. Detection was done by spraying 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4, followed by heating. HPLC was performed using an ODS column (Phenomenex LUNA C_{18} 20×250 mm; Los Angeles, CA, USA).

3.2 Animal material

The skin of *B. bufo gargarizans* was purchased in Shandong Province, China, in April 2005, and identified by Dr Yanyan Zhao, School of Pharmacy, Yantai University. A voucher specimen (20050201-CA) has been deposited at Medical College, Xiamen University.

3.3 Extraction and isolation

The dried skin of *B. bufo gargarizans* (20.0 kg) was finely minced and extracted with 95% EtOH under reflux. Evaporation of the solvent under reduced pressure gave the EtOH extract (660 g). The EtOH extract was partitioned with acetic ether and H_2O. Removal of the solvent under reduced pressure from the acetic ether and H_2O soluble fractions yielded 450 and 200 g of residues, respectively. The acetic ether soluble fraction (120 g) was subjected to a normal-phase silica gel column [1.2 kg, $CHCl_3$–MeOH (100:0 → 0:100, v/v)] to give 24 fractions. Each fraction was subjected to further separation using repeated ODS (eluted successively with H_2O, 30% MeOH, 50% MeOH, 70% MeOH, and MeOH), silica gel [eluted successively with $CHCl_3$–acetone (20:1 → 15:1 → 10:1 → 5:1 → acetone, v/v)], Sephadex LH-20 (eluted with MeOH) column chromatographies, and

preparative HPLC (Phenomenex LUNA C_{18} 20 × 250 mm, $CH_3CN–H_2O$ 45:55) to give 15 compounds, identified as: bufotalin (**1**, 156 mg), hellebrigenin (**2**, 36 mg), telocinobufagin (**3**, 367 mg), 16-desacetyl-19-oxocinobufotalin (**4**, 12 mg), resibufogenin (**5**, 986 mg), daigredorigenin (**6**, 15 mg), and arenobufagin (**7**, 381 mg). Macroporous resin column (HPD-100) was applied in the separation of the water soluble extract. Three fractions were afforded after eluting gradually with water and alcohol, and they were H_2O eluent, 30% EtOH eluent, and 95% EtOH eluent. The extract of the 95% EtOH eluent was further separated using repeated ODS (eluted successively with H_2O, 30% MeOH, 50% MeOH, 70% MeOH, and MeOH), and preparative HPLC (Phenomenex LUNA C_{18} 20 × 250 mm, $CH_3CN–H_2O$ 35:65) to give eight bufatoxins, characterized as cinobufotalin 3-nonanedioylarginine ester (**8**, 33 mg), cinobufagin 3-succinoylarginine ester (**9**, 30 mg), 3-argininylsuccinoyl bufotalin (**10**, 145 mg), hellebritoxin (**11**, 238 mg), bufalin 3-succinoylarginine ester (**12**, 73 mg), telocinobufatoxin (**13**, 274 mg), bufotalin 3-pimeloylarginine ester (**14**, 15 mg), and bufalitoxin (**15**, 16 mg).

Table 3. ^{13}C NMR spectral data of compounds **1**, **8**, **9**, and **14**.

No.	**1**	**8**	**9**	**14**
1	30.4	25.9	30.5	30.8
2	28.7	26.2	26.3	25.3
3	66.0	70.9	70.6	70.5
4	34.2	35.7	31.3	31.0
5	36.6	72.6	37.2	37.5
6	27.3	35.2	26.0	27.0
7	42.4	21.4	20.9	21.7
8	35.8	42.4	39.3	42.3
9	35.8	32.5	33.3	35.8
10	35.7	40.9	35.5	35.4
11	41.0	23.7	21.1	21.5
12	41.0	39.8	39.7	40.3
13	83.6	45.2	45.3	50.0
14	83.6	72.5	72.5	83.5
15	74.6	60.0	59.9	41.0
16	74.6	75.3	75.4	74.6
17	17.1	50.4	50.5	57.5
18	17.1	17.2	17.3	17.1
19	24.1	17.1	23.7	24.0
20	118.0	116.7	116.7	118.0
21	151.8	152.6	152.6	152.0
22	150.5	148.9	148.9	150.5
23	112.8	113.9	113.9	112.9
24	161.9	161.5	161.5	161.9
1′		173.1	172.6	172.9
2′		34.7	30.7	34.7
3′		25.1	30.6	25.3
4′		29.4	170.0	29.2
5′		29.2		26.0
6′		26.0		36.6
7′		25.2		172.3
8′		36.6		
9′		172.8		
1″		55.0	54.9	54.6
2″		30.6	30.5	30.8
3″		25.0	25.1	26.4
4″		41.5	41.3	41.3
5″		158.9	159.0	159.1
COCH_3	170.0	170.0	171.1	170.0
CO*CH_3*	20.7	20.2	20.2	20.7
—COOH		178.2	177.8	177.8

3.3.1 Cinobufotalin 3-nonanedioylarginine ester (8)

White powder; mp 177–178°C; $[\alpha]_D^{24}$ + 60.0 (c = 1.0, MeOH); UV λ_{max}^{MeOH} nm (log ε) (MeOH): 295.0 (3.42); IR ν_{max} (KBr): 3399, 2942, 2935, 1724, 1715, 1638, 1535 cm^{-1}; ^1H NMR and ^{13}C NMR spectral data, see Tables 2 and 3; positive ion ESI-MS: m/z 785 $[M + H]^+$, HR-ESI-MS: m/z 785.4328 $[M + H]^+$ (calcd for $C_{41}H_{61}N_4O_{11}$, 785.4331).

3.3.2 Bufotalin 3-pimeloylarginine ester (14)

White powder; mp 198–199°C; $[\alpha]_D^{24}$ + 40.0 (c = 1.0, MeOH); UV λ_{max}^{MeOH} nm (log ε) (MeOH): 295.2 (3.29); IR ν_{max} (KBr): 3426, 2930, 2854, 1739, 1721, 1631, 1535 cm^{-1}; ^1H NMR and ^{13}C NMR spectral data, see Tables 2 and 3; positive ion ESI-MS: m/z 743 $[M + H]^+$, HR-ESI-MS: m/z 743.4229 $[M + H]^+$ (calcd for $C_{39}H_{59}N_4O_{10}$, 743.4226).

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*3.3.3 Acid hydrolysis of **8***

Compound **8** (2.0 mg) in 10% HCl–dioxane (1:1, 1 ml) was heated at 80°C for 4 h in a water bath. The reaction mixtures were neutralized with Ag_2CO_3, filtered, and then extracted with $CHCl_3$ (1 ml × 3). After concentration, cinobufotalin was found in the $CHCl_3$ layer and the H_2O layer was examined by TLC with *n*-BuOH–AcOH–H_2O (4:1:5, upper layer) as the development solvent and compared with arginine and azelaic acid.

*3.3.4 Acid hydrolysis of **14***

Compound **14** (2.1 mg) in 10% HCl–dioxane (1:1, 1 ml) was heated at 80°C for 4 h in a water bath. The reaction mixtures were neutralized with Ag_2CO_3, filtered, and then extracted with $CHCl_3$ (1 ml × 3). After concentration, the $CHCl_3$ layer afforded the bufotalin and H_2O layer was examined by TLC with *n*-BuOH–AcOH–H_2O (4:1:5, upper layer) as the development solvent, from which arginine and pimelic acid were observed.

3.4 MTT assay

Tumor cells (1×10^4 cells/ml) were seeded in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and kanamin (0.1 mg/ml) at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. Test compounds, as well as the positive control, taxol, were added to this culture and incubated at 37°C for a further 120 h without medium change. Cell viability was then evaluated by the MTT-reducing test and compared with that of the control culture where the cells were treated without adding test compounds.

3.5 Statistics

Values are shown as mean ± SE. Statistical analysis was done by Student's *t*-test and one-way ANOVA, and $p < 0.05$ was considered significant.

Table 4. Cytotoxicities of isolated bufadienolides (IC_{50}, μg/ml).

Compound	Taxol	**1**	**3**	**5**	**7**	**8**	**9**	**10**	**11**	**12**	**14**	**15**
SMMC-7721	0.02	0.4	0.35	0.69	0.03	0.62	2.5	1.29	0.45	0.74	5.37	0.77

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3.6 *Biological activity*

Most of the compounds showed significant cytotoxicities against the SMMC-7721 cancer cell lines. The results are shown in Table 4.

Acknowledgements

The project was supported by the Natural Science Foundation of Fujian Province of China (No. 2010J05087). The authors gratefully acknowledge Li Shen, Centre of Analysis and Test, School of Pharmacy, Yantai University for her kind help in the determination of the samples.

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