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Novel bioconversion products of andrographolide by *Aspergillus ochraceus* and their cytotoxic activities against human tumor cell lines

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ABSTRACT

Andrographolide (**1**), a major labdane diterpenoidal constituent of a famous traditional Chinese of *Andrographis paniculata*, exhibits a wide spectrum of biological activities including antibacterial, anti-inflammatory, and antitumor properties. Bioconversion of andrographolide (**1**) by *Aspergillus ochraceus* (ATCC 1008) was investigated. Five bioconversion products were isolated and identified. Their structures were identified to be 8β-hydroxy-8(17)-dihydroandrographolide (**2**), 8β-hydroxy-8(17)-dihydro-14-deoxy-11,12-didehydroandrographolide (**3**), 8β-hydroxy-8(17)-dihydro-14-deoxy-11,12-didehydroandrographolide 19-oic acid (**4**), 14-deoxy-11,12-didehydroandrographolide (**5**), and 14-deoxy-11,12-didehydroandrographolide 19-oic acid (**6**). Metabolites **2**–**4** were novel compounds. The proposed biosynthetic pathways of andrographolide by *A. ochraceus* were drawn. Most bioconversion products showed potential cytotoxic activities against human breast cancer (MCF-7), human colon cancer (HCT-116) and leukemia (HL-60) cell lines.

1. Introduction

Andrographolide, chemically designated as 2(3H)-furanone, 3-[2-[decahydro-6-hydroxy-5-(hydroxymethyl)-5, 8a-dimethyl-2-methylene-1-napthalenyl] ethylidene] dihydro-4-hydroxy (Fig. 1), was the major active constituent of *Andrographis paniculata* Nees, a famous traditional Chinese and Ayurvedic medicine for the treatment of gastric disorders, infectious diseases and common colds. Andrographolide exhibits several anti-inflammatory properties, including inhibition of NF-κB activation through covalent modification of reduced Cysteine 62 of p50 [1], inhibition of intercellular adhesion molecule-1 expression in monocytes activated by tumor necrosis factor-α [2], suppression of cyclooxygenase-2 (COX-2) expression in neutrophils and microglial cells [3], and IFN-γ and IL-2 production [4,5]. Andrographolide exerts immunomodulatory effects by interfering with NFAT activation and ERK1 and ERK5 phosphorylation in T-cells [6]. It also shows hepatoprotective [7], and anti-HIV [8] activities. Andrographolide is also a potential cancer therapeutic agent [9], which could induce cell cycle arrest and mitochondrial-mediated apoptosis in human leukemic HL-60 cells [10] and human hepatoma HepG2 cells via alteration of reactive oxygen species (ROS) [11], induce apoptosis via caspase-8-dependent pathway in human cancer cells [12], enhance 5-fluorouracil-induced apoptosis via caspase-8-dependent mitochondrial pathway involving p53 participation in hepatocellular carcinoma (SMMC-7721) cells [13].

The microbial conversions of andrographolide were screened and several fungi were selected for biotransformation in our previous research [14]. In the present work, bioconversion of andrographolide (**1**) by *Aspergillus ochraceus* was investigated. Five metabolites, including four new compounds, were isolated and identified. The extraction, isolation, identification and bioactivity evaluation of the metabolites were described in detail.

2. Materials and methods

2.1. Apparatus and reagents

IR spectra were determined on a Bruker IFS 55 spectrometer (Bruker Daltonics Inc., Swiss) in KBr pellets. ESI-MS spectra were recorded on a Bruker Esquire 2000 mass spectrometer. High-resolution ESI-MS spectra were recorded on a Bruker second ionization mass spectrometer. NMR spectra were measured on a Bruker AV-400 spectrometer. Analytical and preparative HPLC were carried on Waters 600 instruments equipped with RI and PDA detectors. Silica gels for column chromatography were products of Qingdao Marine Chemical Factory (Qingdao, China). Normal-phase and reverse-phase preparatory thin-layer chromatography were products of Merck (Darmstadt, Germany).

All analytical reagents were purchased from Tianjin Bodi Chemical Company (Tianjin, China). Methanol for HPLC was product of Hanbang Chemical Company (Taian, China). Andrographolide was

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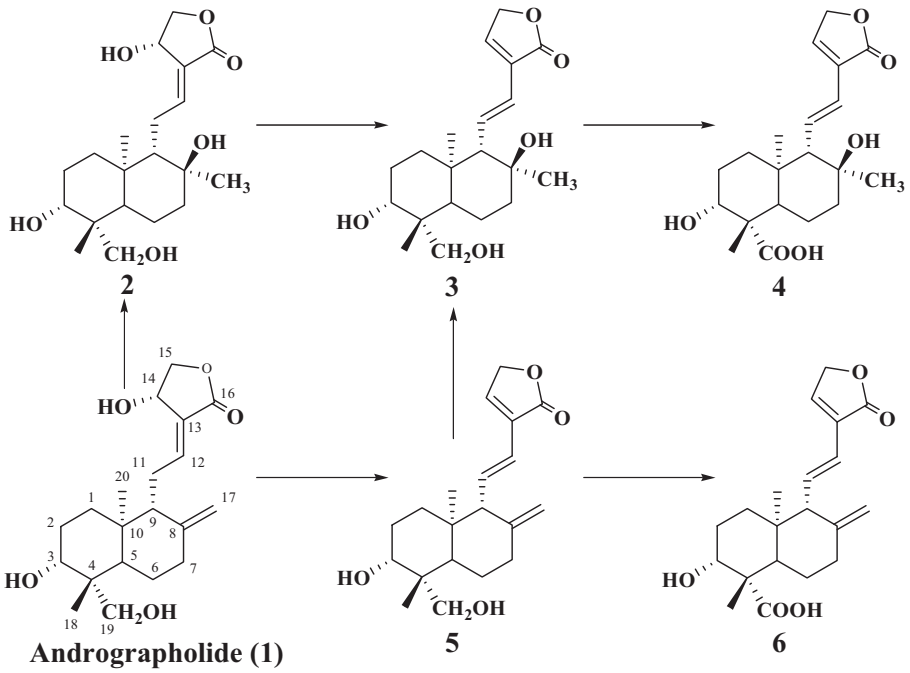

Fig. 1. Proposed biotransformation pathways of andrographolide by *Aspergillus ochraceus*.

isolated and purified from *A. paniculata* Nees in our laboratory, and its purity was above 98.5% determined by HPLC method.

2.2. Microorganism and culture medium

All microbials, including *A. ochraceus* (ATCC 1008) was purchased from the American Type Culture Collection (ATCC, Rockville, MD). All culture and biotransformation experiments were performed in potato medium. Potato medium was prepared by the following procedure: 200 g of mincing husked potato, added 800 mL water, was boiled in water for half an hour. Then the solution was filtered and the filtrate was added with water and 20 g glucose to 1 L.

2.3. Culture and biotransformation procedures

Screening scale bioconversion of andrographolide by *A. ochraceus* was carried out in 250 mL Erlenmeyer flasks containing 80 mL of potato medium. Microorganisms were transferred into the flasks from the slants. The flasks were placed on rotary shakers, which operated at 180 rpm at 28 °C. Andrographolide was dissolved in methanol with a concentration of 10 mg/mL. After 48 h of culture, 0.5 mL of the solution was added into the fermentation flasks and the flasks were maintained under the same conditions for additional 96 h. Culture controls consisted of fermentation blanks in which microorganisms were grown without substrate but adding the same volume of methanol. When the fermentation finished, the broths were filtered and the filtrates were extracted with the same volume of ethyl acetate for three times. The cells were refluxed with methanol. The extracts were evaporated to dryness under reduced pressure. The residues were dissolved in distilled water and extracted with ethyl acetate. The two ethyl acetate extracts were condensed and the residue was dissolved in methanol. The methanol solution was spotted on silica gel TLC plates which were developed by chloroform–methanol system and visualized by spraying with 10% H_2SO_4 solution, followed by heating at 110 °C for 10 min. TLC analysis revealed that *A. ochraceus* could transform the substrate. The fermentation was duplicated and had a good reproducibility.

Preparative scale biotransformation of andrographolide by *A. ochraceus* was carried out in 1000 mL Erlenmeyer flasks containing 250 mL of potato medium. A total of 1200 mg of andrographolide was transformed. The following procedures were the same as screening scale biotransformation.

2.4. Extraction, purification and identification of biotransformation products

Twelve grams of brown residue was obtained from the fermented broth and cells of *A. ochraceus*. The residues were resolved in 1500 distilled water and extracted with the same volume ethyl acetate and water-saturated n-butanol for three times, respectively. From TLC analysis, the n-butanol fraction did not contain any metabolite of andrographolide. The ethyl acetate fraction (2.5 g) was subjected to an open silica gel column (25 mm × 300 mm) and eluted with chloroform/methanol gradiently (chloroform, 50:1, 20:1, 10:1, 5:1, 2:1, and methanol, v/v). From chloroform/methanol 50:1 elution, compound **5** (372.0 mg, 31.0% yield) was gotten through recrystallization using methanol. Compound **6** (25.0 mg, 2.1% yield) was obtained from chloroform/methanol 20:1 elution. The chloroform/methanol 10:1 elution was applied to an ODS MPLC column (20 mm × 250 mm) and eluted with methanol/water. The methanol/water 70:30 fraction was performed on a semi-preparative Rp-HPLC (Zorbax Rx-C18, 5 μm, 9.4 mm × 250 mm, Agilent) and eluted with methanol/water (67:33, v/v), and got compound **4** (13.2 mg, 1.1% yield), **2** (42.0 mg, 3.5% yield), and **3** (28.0 mg, 2.3% yield), respectively.

8β-Hydroxy-8(17)-dihydroandrographolide (**2**): white powder, $C_{20}H_{32}O_6$. Legal and Kedde reactions: positive. IR υ_{max} (KBr) cm^{-1}: 3460, 2927, 1750, 1552, 1083, 900, 790. HR-ESI-MS (negative) *m/z*: 367.2128 $[M-H]^-$ (calcd. for $C_{20}H_{31}O_6$, 367.2121). ^{13}C NMR (100 MHz for carbon, in CD_3OD) data see Table 1, and ^1H NMR (400 MHz for proton, in CD_3OD) data see Table 2.

8β-Hydroxy-8(17)-dihydro-14-deoxy-11,12-didehydroandrographolide (**3**): colorless needle crystals (in methanol), $C_{20}H_{30}O_5$. Legal and Kedde reactions: positive. IR υ_{max} (KBr) cm^{-1}: 3432, 2923, 1780, 1621, 1551, 1100, 903, 775.

Table 1
^{13}C NMR spectral data of andrographolide (**1**) and its metabolites **2**–**6**.

Position	1	2	3	4	5	6
1	38.0	38.3	38.2	38.1	38.5	38.2
2	28.9	29.0	29.0	29.6	28.9	28.9
3	80.5	80.3	80.4	79.5	81.2	79.0
4	43.5	43.1	43.2	49.3	43.8	48.8
5	56.2	56.0	56.3	55.7	55.8	55.8
6	25.2	24.6	24.8	25.1	24.4	24.6
7	38.7	46.6	46.3	46.5	37.8	36.9
8	148.6	74.5	74.8	75.0	150.1	150.0
9	57.5	61.3	60.9	61.3	62.8	62.5
10	39.8	39.1	40.3	40.1	39.6	39.7
11	25.5	27.2	136.2	136.3	136.5	136.8
12	149.3	148.5	122.6	122.7	122.5	122.1
13	129.5	128.2	129.4	129.4	129.6	129.9
14	66.5	67.0	146.9	146.8	146.6	146.6
15	76.0	76.2	71.3	71.4	71.6	71.2
16	172.5	172.3	174.5	174.8	174.8	174.6
17	109.3	25.0	24.8	25.0	109.1	109.3
18	23.1	23.6	23.6	23.4	23.3	23.5
19	64.7	64.5	64.1	180.2	65.0	180.5
20	15.5	15.3	15.4	13.6	15.8	13.3

Notes: All spectra were recorded on a BRUKER AV-400, 400 MHz for ^{1}H and 100 MHz for ^{13}C in CD_3OD.

HR-ESI-MS (negative) *m/z*: 349.2027 $[M-H]^-$ (calcd. for $C_{20}H_{29}O_5$, 349.2015). ^{13}C NMR (100 MHz for carbon, in CD_3OD) data see Table 1, and ^{1}H NMR (400 MHz for proton, in CD_3OD) data see Table 2.

8β-Hydroxy-8(17)-dihydro-14-deoxy-11,12-didehydroandrographolide-19-oic acid (**4**): white amorphous powder, $C_{20}H_{28}O_6$. Legal and Kedde reactions: positive. IR υ_{max} (KBr) cm^{-1}: 3430, 2925, 1786, 1720, 1635, 1550, 1102, 897, 788. HR-ESI-MS (negative) *m/z*: 363.1821 $[M-H]^-$ (calcd. for $C_{20}H_{27}O_6$, 363.1808). ^{13}C NMR (100 MHz for carbon, in CD_3OD) data see Table 1, and ^{1}H NMR (400 MHz for proton, in CD_3OD) data see Table 2.

14-Deoxy-11,12-didehydroandrographolide (**5**): white powder, $C_{20}H_{28}O_4$. Legal and Kedde reactions: positive. IR υ_{max} (KBr) cm^{-1}: 3445, 2922, 1760, 1655, 1561, 1086, 930, 805. ESI-MS (negative) *m/z*: 331 $[M-H]^-$. ^{1}H NMR (400 MHz, CD_3OD) *δ*: 7.43 (1H, br t, *J* = 6.0 Hz, H-14), 6.82 (1H, dd, *J* = 15.8, 10.1 Hz, H-11), 6.15 (1H, d, *J* = 15.8 Hz, H-12), 4.80 (1H, d, *J* = 2.1 Hz, H-17a), 4.56 (1H, d, *J* = 2.1 Hz, H-17b), 4.48 (2H, m, H-15), 4.20 (1H, d, *J* = 11.2 Hz, H-19a), 3.40 (1H, m, H-3), 3.24 (1H, d, *J* = 11.2 Hz, H-19b), 1.22 (3H, s, 18-CH_3), 0.73 (3H, s, 20-CH_3). ^{13}C NMR data see Table 1.

14-Deoxy-11,12-didehydroandrographolide-19-oic acid (**6**): colorless cubic crystals (in methanol), $C_{20}H_{26}O_5$. Legal and Kedde Reactions: positive. IR υ_{max} (KBr) cm^{-1}: 3450, 2925, 1762, 1703, 1665, 1552, 1080, 912. HR-ESI-MS (negative) *m/z*: 345.1715 $[M-H]^-$ (calcd. for $C_{20}H_{25}O_5$, 345.1702). ^{1}H NMR (400 MHz, CD_3OD) *δ:* 7.42 (1H, br t, *J* = 5.8 Hz, H-14), 6.80 (1H, dd, *J* = 15.6, 9.6 Hz, H-11), 6.14 (1H, d, *J* = 15.6 Hz, H-12), 4.86 (1H, d, *J* = 1.8 Hz,

Table 2
^{1}H NMR spectral data of metabolites **2**–**4** at 400 MHz in CD_3OD, *δ* (H, multiple, *J* in Hz).

Position	2	3	4
1	1.80 (H, m) 1.23 (H, m)	1.77 (H, m) 1.21 (H, m)	1.81 (H, m) 1.25 (H, m)
2	1.72 (2H, m)	1.70 (2H, m)	1.75 (2H, m)
3	3.46 (H, m)	3.41 (H, m)	3.43 (H, m)
4	–	–	–
5	1.30 (H, m)	1.35 (H, m)	1.26 (H, m)
6	1.34 (H, m) 1.28 (H, m)	1.30 (2H, m)	1.33 (2H, m)
7	2.50 (H, m) 2.24 (H, m)	2.54 (H, m) 2.26 (H, m)	2.50 (H, m) 2.21 (H, m)
8	–	–	–
9	2.03 (H, m)	2.30 (H, m)	2.27 (H, m)
10	–	–	–
11	2.60 (2H, m)	6.76 (H, dd, 15.6, 8.6)	6.70 (H, dd, 15.2, 8.2)
12	6.56 (H, dd, 7.2, 5.1)	6.08 (H, d, 15.6)	6.00 (H, d, 15.2)
13	–	–	–
14	4.98 (H, t, 6.3)	7.26 (H, br t, 6.0)	7.23 (H, br t, 6.2)
15	4.35 (H, dd, 12.2, 6.3) 4.10 (H, dd, 12.2, 6.3)	4.72 (2H, m)	4.66 (2H, m)
16	–	–	–
17	1.32 (3H, s)	1.31 (3H, s)	1.30 (3H, s)
18	1.25 (3H, s)	1.26 (3H, s)	1.28 (3H, s)
19	4.08 (H, d, 12.0) 3.36 (H, d, 12.0)	4.12 (H, d, 11.8) 3.30 (H, d, 11.8)	–
20	0.90 (3H, s)	0.86 (3H, s)	0.75 (3H, s)

H-17a), 4.62 (1H, d, J = 1.8 Hz, H-17b), 4.51 (2H, m, H-15), 3.36 (1H, m, H-3), 1.30 (3H, s, 18-CH_3), 0.76 (3H, s, 20-CH_3). ^{13}C NMR data see Table 1.

2.5. Cell culture and assay for cytotoxic activity

Human breast cancer (MCF-7), human colon cancer (HCT-116) and leukemia (HL-60) were used for bioactivity evaluation of andrographolide and its metabolites based on established protocols [15].

3. Results

3.1. Identification of biotransformation products

Thirty-five fungi were screened for the bioconversion ability and *A. ochraceus* was chosen for andrographolide biotransformation. Five metabolites (Fig. 1) were isolated from the broth of *A. ochraceus* in potato medium. The assignments of carbon and proton signals of all metabolites were given in Tables 1 and 2.

Compound **2** was obtained as white powder and was positive for the Legal and Kedde reactions, suggesting the presence of an α,β-unsaturated lactone in the molecule. An ion peak $[M-H]^-$ at m/z 367 showed in the negative ESI-MS. The HR-ESI-MS gave the ion peak $[M-H]^-$ at m/z 367.2128 (calcd. 367.2121), accordingly to its molecular formula of $C_{20}H_{32}O_6$. In the ^{1}H NMR of compound **2**, the olefinic protons at δ 4.86 and 4.63 were disappeared comparing to the substrate-andrographolide, and there was an additional methyl group signal at δ 1.32 (3H, s) in the high field, suggested the carbon–carbon double bond at 8(17) was hydrated in the compound **2**. In the ^{13}C NMR, there were 3 sp^2-hybrid carbon signals. The signals of carbon-carbon double bond at 8(17) of andrographolide were disappeared, and there was an additional oxygenated carbon signal at δ 74.5. The above evidences in the ^{1}H and ^{13}C NMR spectra suggested compound **2** was a hydration product at the C-8(17) of andrographolide and there was a hydroxyl group at the C-8 in the molecule [16]. In the HMBC spectrum, the methyl group at 1.32 ppm (17-CH_3) has correlations with δ 74.5 (C-8), 46.6 (C-7) and 61.3 (C-9), which confirmed the carbon–carbon double bond at 8(17) in the andrographolide was hydration and a novel hydroxyl group linked at C-8. In the NOESY spectrum, the signal at δ 1.32 (17-CH_3) has NOE correlation with the signal of δ 0.90 (20-CH_3), suggested the hydroxyl group linked at C-8 located at equatorial orientation. Based on above analysis, compound **2** was elucidated to be 8β-hydroxy-8(17)-dihydroandrographolide, which was a novel compound as far as we knew.

Compound **3** was obtained as colorless needle crystals (in methanol), which was positive for the Legal and Kedde reactions. The HR-ESI-MS gave the ion peak $[M-H]^-$ at m/z 349.2027, accordingly to its molecular formula of $C_{20}H_{30}O_5$. Compared the mass spectrum with compound **2**, compound **3** lost an 18 amu, which suggested it was a dehydrate product of compound **2**. In the ^{13}C NMR, it had 20 carbon signals, including 5 sp^2-hybrid carbons. The carbon NMR data of compound **3** were similar to those of **2**, except the signals of C-9 side chain. Compared to the metabolite of andrographolide in rats, the C-9 side chain was the same to 14-deoxy-11, 12-didehydroandrographolide [17]. In the NOESY spectrum, there were NOE correlations between the signal at δ 1.31 (17-CH_3) and 0.86 (20-CH_3), suggested the hydroxyl group linked at C-8 located at equatorial orientation [16]. Based on above analysis, compound **3** was elucidated to be 8β-hydroxy-8(17)-dihydro-14-deoxy-11,12-didehydroandrodrapholide, which was a novel compound as far as we knew.

Compound **4** was gotten as white amorphous powder and also an α,β-unsaturated lactone judged from its positive for the Legal and Kedde reactions. The molecular formula $C_{20}H_{28}O_6$ was drawn from its HR-ESI-MS, which gave the ion peak $[M-H]^-$ at m/z 363.1821. In the lower field of ^{13}C NMR, there were two carboxyl signals, suggested there was an additional carboxyl group (δ 180.2) in the molecular. Compound **4** and **3** had a very similar ^{13}C NMR spectrum, except the signal at C-19, C-4, C-3 and C-5. The C-19 hydroxymethyl group (δ 64.1) in compound **3** was disappeared, which was substituted by a carboxylic acid. Therefore, compound **4** was identified as 8β-hydroxy-8(17)-dihydro-14-deoxy-11, 12-didehydroandrodrapholide-19-oic acid and was a new compound.

Compound **5** was identified to be 14-deoxy-11, 12-didehydroandrodrapholide through ESI-MS, ^{1}H and ^{13}C NMR, and compared with authentic metabolite of andrographolide which isolated from rats' urine [17].

Compound **6**, colorless cubic crystals in methanol, was positive to the Legal and Kedde reactions. The molecular formula of compound **6** was established as $C_{20}H_{26}O_5$ according to its ion $[M-H]^-$ at m/z 345.1715 in the HR-ESI-MS, suggesting it was an oxygenated product of compound **5**. There was an additional carboxyl group (δ 180.5) in compound **6** compared to **5**. In the HMBC spectrum, the carboxyl group signal at δ 180.5 has long correlations with 18-CH_3 (δ 1.30) and H-5 (δ 1.32), which suggested the hydroxymethyl group at C-19 in compound **5** was replaced by a carboxyl group. Based on above analysis, compound **6** was identified as 14-deoxy-11, 12-didehydroandrodrapholide-19-oic acid.

Table 3
IC_{50} values for the inhibition of human tumor cells of andrographolide (**1**) and its metabolites **2**–**6** (mean ± SD, n = 8).

Compound	IC_{50} (μmol/L)		
	MCF-7	HCT-116	HL-60
1	8.3 ± 1.0	4.1 ± 0.5	7.8 ± 1.4
2	12.1 ± 2.2	10.3 ± 0.8	3.5 ± 0.6
3	15.3 ± 2.1	11.0 ± 1.5	9.5 ± 0.9
4	2.2 ± 0.3	1.5 ± 0.2	7.0 ± 0.9
5	13.8 ± 2.1	6.0 ± 0.8	11.3 ± 2.0
6	10.1 ± 3.1	7.5 ± 1.0	4.9 ± 0.8
Cisplatin (positive control)	5.2 ± 0.7	11.0 ± 1.3	1.6 ± 0.1

3.2. Cytotoxic activity

The cytotoxic activities of the bioconversion products of andrographolide were evaluated by MTT assay. Their cytotoxic activities were given in Table 3.

4. Discussion

Most biotransformation products of andrographolide were hydroxylated or dehydrated products. Compounds **2**–**4** were hydrated products of the carbon-carbon double bond at C-8(17), followed by dehydration or oxidation. 14-Deoxy-11, 12-didehydroandrographolide (compound **5**) was the main microbial conversion product of andrographolide by *A. ochraceus*. From chemical aspect, compound **5** is more stable than andrographolide. Compounds **6** to **9** were the derivatives of compound **5**, through dehydration, oxidation or hydration of the lactone ring. However, the lactone ring was stable and no change after andrographolide was metabolized *in vivo* by either rats or human being [17].

Compound **5** was also the one of andrographolide metabolite in rats [17], which suggested microbial conversion could provide some useful clues for *in vivo* metabolism study. The proposed biosynthetic pathways of the microbial conversion products of andrographolide were shown in Fig. 1.

Most bioconversion products showed considerable cytotoxic activities against human breast cancer (MCF-7), human colon

cancer (HCT-116) and leukemia HL-60 cell lines compared to substrate–andrographolide (Table 3). Among these metabolites, compound **4** showed the most potential cytotoxic activities against the selected cell lines. Hydration at 8(17)-en of andrographolide (compound **2**) had no obvious effect on the cytotoxicity against MCF-7 cells, while could increase the target activity against HL-60. Dehydration of the hydroxyl at C-14 had no obvious effect to the target bioactivity (compounds **3**–**6**). These results provided useful clues in the process of andrographolide development.

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