Journal of Molecular Catalysis B: Enzymatic 62 (2010) 242–247

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Cytotoxic biotransformed products from andrographolide by *Rhizopus stolonifer* ATCC 12939

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ARTICLE INFO

Article history:
Received 23 June 2009
Received in revised form 3 November 2009
Accepted 3 November 2009
Available online 10 November 2009

Keywords:
Andrographolide
Microbial transformation
Rhizopus stolonifer
Oxidative products
Antiproliferative activities

ABSTRACT

Biotransformation of andrographolide (**1**) by *Rhizopus stolonifer* ATCC 12939 was investigated. Ten bioconversion products were isolated and identified. Their structures were elucidated by high resolution mass spectroscopy (HR-MS), extensive NMR techniques, including ^{1}H NMR, ^{13}C NMR, DEPT, ^{1}H–^{1}H correlation spectroscopy (COSY), two dimensional nuclear Overhauser effect correlation spectroscopy (NOESY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond coherence (HMBC). Their structures were identified to be 12(*R*),13(*R*)-12-hydroxyandrographolide (**2**), 12(*S*),13(*S*)-12-hydroxyandrographolide (**3**), isoandrographolide (**4**), 3-dehydro-isoandrographolide (**5**), 14-deoxy-11,12-didehydroandrographolide (**6**), 3-*oxo*-14-deoxy-11,12-didehydroandrographolide (**7**), 3-dehydroandrographolide (**8**), 14-deoxyandrographolide (**9**), 3-dehydro-14-deoxyandrographolide (**10**) and 3-dehydro-14-deoxyandrographolide-19-oic acid (**11**). Among them, compounds **5** and **11** are novel compounds. The biosynthetic pathways of andrographolide by *R. stolonifer* were proposed. Most of the products showed potential antiproliferative activities against human breast cancer (MCF-7), human colon cancer (HCT-116) and human leukemia (HL-60) cell lines, and their structure–activity relationships (SAR) were discussed in detail.

1. Introduction

Andrographolide 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. It 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), which exhibited 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 exerted immunomodulatory effects by interfering with NFAT activation and ERK1 and ERK5 phosphorylation in T-cells [6]. It also had 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 importance of microbial biotechnology in the production of steroid drugs and hormones was realized for the first time in 1952 when Murray and Peterson patented the process of 11α-hydroxylation of progesterone by a *Rhizopus* species. Since then, microbial transformations have provided adequate tools for the large scale productions of natural or modified steroid analogues. Microbial transformation of natural products are currently favored when compared to their natural counterparts due to some therapeutic advantages, such as an increased potency, longer half-lives in the blood stream, simpler delivery methods and reduced side effects. The preferential use of whole cells over enzymes as biocatalysts for the production of these pharmaceutical derivatives mostly results from the costs of the latter enzyme isolation, purification and stabilization. Furthermore, the use of microbial models to mimic mammalian metabolism is well known [14,15].

In the continuing research, the metabolites of andrographolide in rats [16–18] and human being [19–21] had been carried out in our research group, and series of sulfate and dehydrate metabolites of andrographolide were isolated and identified. Meanwhile, the microbial conversions of andrographolide were screened and

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doi:10.1016/j.molcatb.2009.11.002

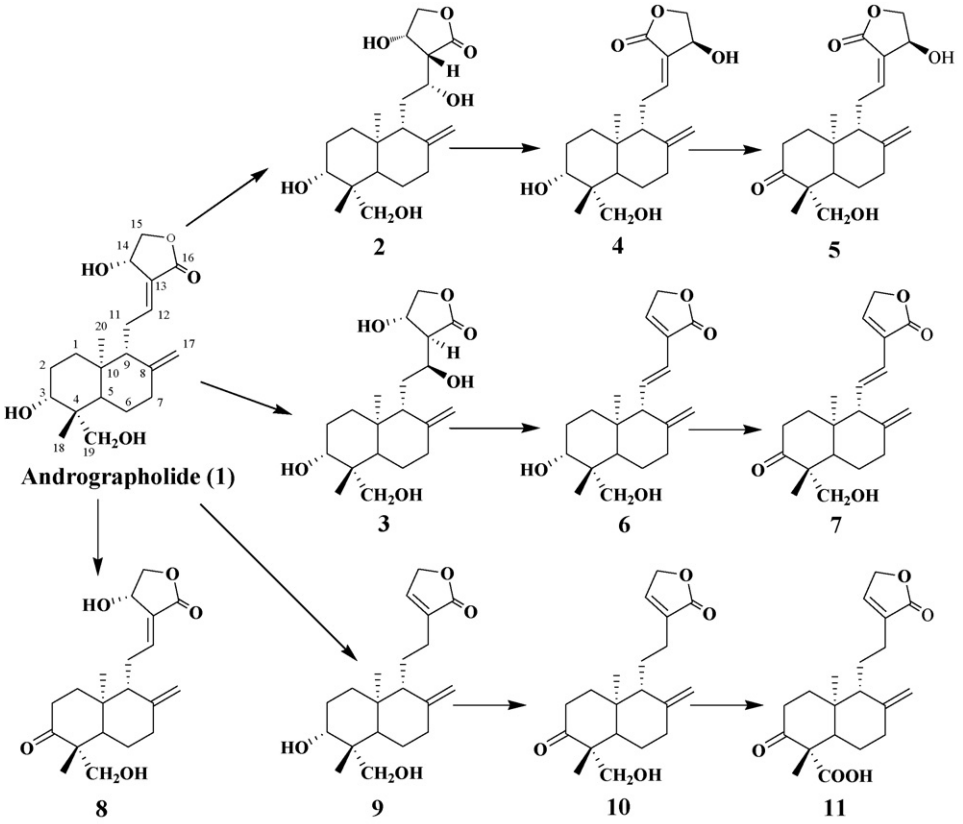

Fig. 1. Biotransformation pathways of andrographolide (**1**) by *R. stolonifer*.

several fungi were selected for further studies. The present work was an attempt to get the microbial transformation products of andrographolide by *Rhizopus stolonifer* ATCC 12939 and provide some structure–bioactivity relationships against human tumor cell proliferation of andrographolide.

2. Results

Thirty-two fungi were screened for the biotransformation of andrographolide and *R. stolonifer* ATCC 12939 was chosen for further study. After 96 h incubation, ten biotransformation products (Fig. 1) were isolated and identified from the broth of *R. stolonifer* in potato medium. Some of them have considerable *in vitro* antitumor activities compared to the substrate–andrographolide (**1**).

2.1. Identification of biotransformation products

Ten metabolites of andrographolide were identified by chemical and spectroscopic ways. Among them, two were novel compounds and were elucidated to be 3-dehydro-isoandrographolide (**5**) and 3-dehydro-14-deoxyandrographolide-19-oic acid (**11**). Others were known compounds, which were identified as 12(*R*),13(*R*)-12-hydroxyandrographolide (**2**) [22], 12(*S*),13(*S*)-12-hydroxyandrographolide (**3**) [22], isoandrographolide (**4**) [23], 14-deoxy-11,12-didehydroandrographolide (**6**) [18], 3-*oxo*-14-deoxy-11,12-didehydroandrographolide (**7**) [22,24], 3-dehydroandrographolide (**8**) [25], 14-deoxyandrographolide (**9**) [18], and 3-dehydro-14-deoxyandrographolide (**10**) [22], respectively.

Compound **5** was obtained as white amorphous powder and was positive for the Legal and Kedde reactions, suggesting the presence of an α,β-unsaturated lactone. The high resolution ESI-MS gave the ion peak $[M-H]^-$ at *m/z* 347.1835 (calcd 347.1858), according to its molecular formula of $C_{20}H_{28}O_5$.

In the ^{1}H NMR of compound **5**, there was a pair of olefinic protons at δ 4.85 (H-17a, br. s) and 4.55 (H-17b, br. s), which were exocyclic methylene protons of *Andrographis* diterpenoids. The lowest field proton signal at δ 6.55 (H-12, t, J = 7.1 Hz) was designated to the β-olefinic proton of α,β-unsaturated lactone. The proton signal linked to oxygenated carbon of H-3 at δ 3.38 in andrographolide was disappeared. In the lowest field of its ^{13}C NMR, there was a new carbonyl carbon signal at δ 213.1. The carbon of C-12 (151.0) was shifted to lower field about 2 ppm compared to andrographolide (**1**), which was appeared at δ 149.3. In the HMBC spectrum, the carbon signal at δ 213.1 correlated with the signals at δ 2.80 (H-2a, dt, J = 15.1, 3.8 Hz), 2.40 (H-2b, m), 0.91 (20-CH_3, s) and 1.60 (H-5), suggested C-3 was a carbonyl carbon. The carbonyl carbon of lactone at δ 171.5 correlated with the signals at δ 6.55 (H-12) and 4.76 (H-14, dd, J = 6.2, 3.0 Hz), indicated the carbon–carbon double bond of the α,β-unsaturated lactone located at C-12(13). In the NOESY spectrum, the signal of δ 6.55 (H-12) had NOE correlations with 4.76 (H-14). Compared the spectroscopic data with those of compound **4** and the literature [23], the carbon–carbon double bond at C-12(13) was determined as *Z*-form, instead of *E*-form in andrographolide. When compared the signals of compound **5** with those of andrographolide reported in the literature [23], the proton signals of H-11 to H-15 and the carbon signals of C-11 to C-15 were almost identical. Therefore, the C-14 was determined as *S*-configuration, which the configuration of C-14 in the literature was established from single-crystal X-ray analysis. Based on above analysis, compound **5** was elucidated to be 3-dehydro-isoandrographolide, which was a novel compound. The full assignments of proton and carbon signals were given in Table 1.

Table 1
^{1}H and ^{13}C NMR data of compounds **5** and **11**.

Position	Compound **5**		Compound **11**	
	δ_C	δ_H (H, multiple, *J* in Hz)	δ_C	δ_H (H, multiple, *J* in Hz)
1	39.5	1.79 (H, m) 1.38 (H, m)	39.2	1.81 (H, br. d, 12.1) 1.22 (H, br. t, 12.1)
2	36.3	2.80 (H, dt, 15.1, 3.8) 2.40 (H, m)	36.2	2.76 (H, m) 2.35 (H, m)
3	213.1	–	212.0	–
4	55.0	–	60.1	–
5	57.1	1.60 (H, o)	56.5	1.26 (H, br. d, 9.6)
6	24.8	1.76 (H, m) 1.68 (H, m)	25.0	2.06 (H, m) 1.89 (H, br. d, 11.3)
7	38.8	2.38 (H, m) 2.03 (H, br. d, 12.1)	38.6	2.40 (H, br. d, 11.3) 2.13 (H, m)
8	148.9	–	148.7	–
9	58.0	2.00 (H, m)	57.6	1.75 (H, br. d, 8.2)
10	40.0	–	40.0	–
11	24.5	2.85 (2H, t, 7.1)	23.6	1.71 (H, br. t, 9.8) 1.53 (H, o)
12	151.0	6.55 (H, t, 7.1)	25.7	2.60 (H, m) 2.21 (H, o)
13	129.2	–	131.2	–
14	69.8	4.76 (H, dd, 6.2, 3.0)	147.3	7.23 (H, br. s)
15	75.1	4.36 (H, dd, 10.1, 6.2) 4.06 (H, dd, 10.1, 3.0)	72.5	4.70 (2H, s)
16	171.5	–	175.2	–
17	108.4	4.85 (H, br. s) 4.55 (H, br. s)	108.5	4.86 (H, br. s) 4.67 (H, br. s)
18	22.4	1.30 (3H, s)	23.3	1.53 (3H, s)
19	64.8	4.20 (H, d, 11.1) 4.56 (H, d, 11.1)	180.2	
20	15.5	0.91 (3H, s)	13.5	1.03 (3H, s)

Notes: (1) All spectra were recorded on Bruker AV-400, 400 MHz for ^{1}H and 100 MHz for ^{13}C in CD_3OD. (2) The carbon signals were assigned unambiguously on ^{1}H NMR, ^{13}C NMR, COSY, TOCSY, NOESY, HMQC, and HMBC spectra. (3) "m" indicates multiples, "o" indicates overlapped.

Compound **11** was obtained as colorless needle crystals (in methanol) and also an α,β-unsaturated lactone judged from its positive for the Legal and Kedde reactions. The molecular formula $C_{20}H_{26}O_5$ was drawn from its HR-ESI-MS, which gave the ion peak $[M-H]^-$ at *m*/*z* 345.1733. In the ^{1}H NMR, an olefinic proton of α,β-unsaturated lactone appeared at δ 7.23 (H-14, br. s). The spectroscopic data of compound **11** were very similar to those of compound **10**. There was also a pair of olefinic protons at δ 4.86 (H-17a, br. s) and 4.67 (H-17b, br. s) of the exocyclic methylene protons for *Andrographis* diterpenoids. There were 7 sp^2 hybrid carbon signals in the ^{13}C NMR, which included three carbonyl carbons at δ 212.0 (C-3), 180.2 (C-19) and 175.2 (C-16). In the HMBC spectrum, the carbon signal at δ 212.0 correlated with the signals at δ 2.76 (H-2a, m), 2.35 (H-2b, m), 1.53 (18-CH_3, s) and 1.26 (H-5), suggested C-3 was a carbonyl carbon. The carboxylic carbon at δ 180.2 (C-19) had long range correlation with δ 1.53 (18-CH_3, s), suggesting the hydroxymethyl group at C-19 of andrographolide was oxygenated to a carboxylic acid. In the NOESY spectrum, the β-orientation signal at 1.26 (H-5, br.d., *J* = 9.6 Hz) has correlation with the signal at 1.53 (18-CH_3, s), suggested the 18-CH_3 was β-orientation and the carboxylic group linked at C-4 was α-orientation. The signal of lactone carbonyl carbon at δ 175.5 correlated with δ 7.23 (H-14), 2.60 (H-12a) and 2.21 (H-12b), suggested the α,β-unsaturated bond of lactone located at C-13(14). Based on above evidences, compound **11** was elucidated to be 3-dehydro-14-deoxyandrographolide-19-oic acid, which was a novel compound as far as known. The full assignments of proton and carbon were given in Table 1.

2.2. Antiproliferative activities against human tumor cells in vitro

The antiproliferative activities against human tumor cell lines of the bioconversion products were evaluated. Most bioconversion products showed considerable cytotoxic activities against human breast cancer (MCF-7), human colon cancer (HCT-116) and leukemia (HL-60) cell lines, and the results were shown in Table 2.

3. Discussion

Most biotransformation products of andrographolide by *R. stolonifer* were oxygenated or dehydrated products. Nucleophilic reaction could be occurred easily at the β-carbon since the double bond at C-12(13) of andrographolide was an α,β-unsaturated lactone, and compounds **2**–**3** were a pair of hydrated products of andrographolide at C-12(13) through a nucleophilic reaction. After hydration of the double bond at C-12(13), the configuration of the double bond could be converted to *Z*-form through dehydration (compounds **4** and **5**). The hydroxyl group at C-14 could undergo dehydration easily and formed conjugated double bonds (Compounds **6**, **7**, **9**–**11**). Compound **6** was a double dehydration product of andrographolide, which was the main microbial conversion product of andrographolide by *R. stolonifer*. On other side, the lactone ring is very stable, which the result is similar to those of andrographolide metabolites *in vivo* by either rats or human being [16–22]. From above analysis, the proposed biosynthetic pathways of microbial conversion products of andrographolide were

Table 2
IC_{50} values for the inhibition of human hepatoma and leukemia cells of andrographolide (**1**) and its metabolites 2–11 (mean ± SD, $n = 8$).

Compounds	IC_{50} (μmol/L)		
	MCF-7	HCT-116	HL-60
1	9.5 ± 1.3	6.0 ± 0.8	7.5 ± 1.2
2	45.2 ± 5.1	53.1 ± 6.2	17.6 ± 2.1
3	56.3 ± 7.2	45.6 ± 5.7	28.3 ± 1.9
4	2.1 ± 0.4	1.8 ± 0.2	23.1 ± 1.9
5	53.1 ± 6.1	66.3 ± 5.8	41.5 ± 6.0
6	15.1 ± 1.8	6.8 ± 1.0	10.5 ± 1.6
7	95.2 ± 10.1	188.0 ± 21.0	163.1 ± 15.2
8	71.5 ± 8.0	56.2 ± 6.3	105.6 ± 12.0
9	10.1 ± 1.6	8.6 ± 1.1	15.3 ± 2.1
10	172.1 ± 15.0	88.3 ± 10.2	167.6 ± 18.1
11	190.7 ± 20.0	151.7 ± 16.8	131.0 ± 16.7

shown in Fig. 1. Compounds **6** and **9** were also the andrographolide metabolite in rats, which suggested microbial conversion could be regarded as reference for *in vivo* metabolite study [18].

Most metabolites showed obvious *in vitro* antitumor activities. Compound **4**, isoandrographolide, showed the most potential antiproliferative activities against MCF-7 and HCT-116 cell lines, with IC_{50} values were 2.1 ± 0.4 and 1.8 ± 0.2 μmol/L, respectively. The antiproliferative activities against the selected cell lines were decreased significantly after the β-hydroxyl group has been oxidized to the keto group (compounds **5**, **7**, **8**, **10** and **11**). Hydration at 12(13)-en of andrographolide (compounds **2** and **3**) could slightly decrease the antiproliferative activities against MCF-7, HCT-116 and HL-60 cell lines. Epimerization of 12(13)-en of andrographolide (compound **4**) could increase the target bioactivity of MCF-7 and HCT-60, while had an adverse result against HL-60 cell. Dehydration of the hydroxyl at C-14 almost had no effect to the target bioactivities. These SAR results provided useful clues in the process of andrographolide development.

4. Experimental

4.1. General experimental procedures

IR spectra were determined on a Bruker IFS 55 spectrometer (Bruker Inc., Fällanden, Switzerland) in KBr pellets. UV spectra were measured on a Cary 5000 UV–Vis spectrophotometer (Varian Inc., Palo Alto, CA). ESI-MS spectra were recorded on a Bruker Esquire 2000 mass spectrometer. High resolution ESI-MS spectra were recorded on a Bruker mass spectrometer (Bruker Inc., Fällanden, Switzerland). NMR spectra were measured on a Bruker AV-400 spectrometer (Bruker Inc., Fällanden, Switzerland). Analytical and semi-preparative HPLC were carried on Waters 600 instruments equipped with RI and PDA detectors (Waters Corp., Milford, MA). Silica gels for column chromatography were products of Qingdao Marine Chemical Factory (Qingdao, China). Normal-phase and reverse-phase preparative thin-layer chromatography were products of Merck (Darmstadt, Germany).

Andrographolide (**1**) was isolated from *Andrographis paniculata* Nees, and its purity was above 98% determined by HPLC method.

4.2. Microorganisms and culture

All microbials, including *R. stolonifer* ATCC 12939 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 for half an hour. Then the solution was filtered and the filtrate was added with water and 20 g glucose to 1 L.

4.3. Culture and biotransformation procedures

Screening scale biotransformations of andrographolide were carried out in 250 mL Erlenmeyer flasks, which contained 60 mL of potato medium. Microorganisms were transferred into the flasks from the slants. The flasks were placed on rotary shakers, operating at 180 rpm at 28 °C. The substrate 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 these flasks were maintained under the same conditions for additional 4 days. Culture controls consisted of fermentation blanks in which microorganisms were grown without substrate but adding the same amount 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 and the residues were dissolved in methanol. The solutions were spotted on silica gel 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 analyses revealed that *R. stolonifer* ATCC 12939 could biotransform the substrate.

Preparative scale biotransformation of andrographolide by *R. stolonifer* was carried out in 1000 mL Erlenmeyer flasks containing 250 mL of potato medium, operating at 180 rpm at 28 °C. A total of 1500 mg of substrate was transformed. Other procedures were the same as screening scale biotransformation.

4.4. Extraction and isolation

Brown residue (15.3 g) was obtained from the fermented broth and cells of *R. stolonifer*. The residues were resolved in 2000 mL distilled water and extracted with the same volume of 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 (3.3 g) was subjected to an open silica gel column (30 mm × 350 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 **6** (210.3 mg, 14.0% yield) and **9** (31.3 mg, 2.1% yield) were obtained through recrystallization using acetone. The residue of the chloroform/methanol 50:1 elution was applied to an ODS column (20 mm × 250 mm) and eluted with methanol/water, and the methanol/water (7:3) fraction was subjected to a semi-preparative RP-HPLC (Zorbax Rx-C18, 5 μm, 9.4 mm × 250 mm, Agilent) and eluted with methanol/water (80:20, v/v) to obtain compounds **10** (17.0 mg, 1.1% yield) and **7** (11.2 mg, 0.7% yield). The chloroform/methanol 20:1 elution was subjected to a Sephadex LH-20 column (20 mm × 550 mm) and eluted with chloroform/methanol 4:6, and the second fraction was purified by the semi-preparative RP-HPLC and eluted with methanol/water (65:35, v/v), and got compounds **5** (18.1 mg, 1.2% yield), **4** (128.4 mg, 8.5% yield), and **2** (36.0 mg, 2.4% yield), respectively. The fourth fraction of the Sephadex LH-20 column was subjected to the semi-preparative RP-HPLC and eluted with methanol/water (70:30, v/v), and got compounds **3** (28.0 mg, 1.9% yield) and **8** (25.7 mg, 1.7% yield). The chloroform/methanol 10:1 elution was applied to an ODS column (20 mm × 250 mm) and eluted with methanol/water, and the methanol/water 60/40 fraction got compound **11** (8.5 mg, 0.6% yield) after recrystallization using methanol.

12(R), 13(R)-12-hydroxyandrographolide (**2**): white powder, $C_{20}H_{32}O_6$. Legal and Kedde Reactions: positive. IR υ_{max} (KBr) cm^{-1}: 3443, 2932, 1755, 1532, 1061, 891, 786. High resolution ESI-MS (negative) m/z: 367.2137 $[M-H]^-$ (calcd for $C_{20}H_{31}O_6$, 367.2121). ^1H NMR (400 MHz, CD_3OD): δ 4.97 (1H, br. t, $J = 3.3$ Hz, H-14), 4.94 (1H, t, $J = 8.1$ Hz, H-15a), 4.90 (1H, br. s, H-17a), 4.81 (1H, br. s,

H-17b), 4.62 (1H, dd, J = 8.1, 2.6 Hz, H-15b), 4.51 (1H, d, J = 9.2 Hz, H-12), 4.30 (1H, d, J = 11.2 Hz, H-19a), 3.72 (1H, m, H-3), 3.60 (1H, d, J = 11.2 Hz, H-19b), 1.33 (3H, s, 18-CH_3), 0.69 (3H, s, 20-CH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 177.3 (C-16), 148.5 (C-8), 107.2 (C-17), 80.3 (C-3), 76.0 (C-15), 73.0 (C-14), 70.5 (C-12), 64.0 (C-19), 55.9 (C-5), 54.2 (C-9), 54.1 (C-13), 43.1 (C-4), 39.6 (C-10), 38.6 (C-7), 38.2 (C-1), 31.2 (C-11), 29.0 (C-2), 24.9 (C-6), 23.6 (C-18), 15.2 (C-20).

12(S), 13(S)-12-hydroxyandrographolide (**3**): white powder, $C_{20}H_{32}O_6$. Legal and Kedde Reactions: positive. IR υ_{max} (KBr) cm^{-1}: 3451, 2920, 1750, 1520, 1053, 931, 803. High resolution ESI-MS (negative) m/z: 367.2130 [M−H]$^-$ (calcd for $C_{20}H_{31}O_6$, 367.2121). ^{1}H NMR (400 MHz, CD_3OD): δ 5.10 (1H, br. s, H-17a), 5.03 (1H, t, J = 2.1 Hz, H-14), 4.92 (1H, br. s, H-17b), 4.66 (1H, d, J = 7.1 Hz, H-12), 4.54 (1H, br. d, J = 9.5 Hz, H-15a), 4.51 (1H, br. d, J = 9.5 Hz, H-15b), 4.42 (1H, d, J = 11.0 Hz, H-19a), 3.62 (1H, m, H-3), 3.51 (1H, d, J = 11.0 Hz, H-19b), 1.38 (3H, s, 18-CH_3), 0.72 (3H, s, 20-CH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 178.3 (C-16), 148.5 (C-8), 107.5 (C-17), 80.4 (C-3), 75.4 (C-15), 69.6 (C-14), 66.0 (C-12), 65.0 (C-19), 56.0 (C-5), 52.8 (C-9), 51.5 (C-13), 43.2 (C-4), 38.8 (C-7), 38.7 (C-10), 38.2 (C-1), 31.0 (C-11), 29.0 (C-2), 24.9 (C-6), 23.6 (C-18), 15.4 (C-20).

Isoandrographolide (**4**): colorless amorphous powder, $C_{20}H_{30}O_5$. Legal and Kedde Reactions: positive. IR υ_{max} (KBr) cm^{-1}: 3441, 2932, 1742, 1613, 1043, 950, 821. ESI-MS (negative) m/z: 367 [M−H]$^-$. ^{1}H NMR (400 MHz, CD_3OD): δ 7.03 (1H, s, H-12), 5.03 (1H, br. s, H-17a), 4.86 (1H, br. s, H-17b), 4.72 (2H, s, H-15), 4.60 (1H, t, J = 3.6 Hz, H-14), 4.22 (1H, d, J = 11.2 Hz, H-19a), 3.41 (1H, m, H-3), 3.23 (1H, d, J = 11.2 Hz, H-19b), 1.20 (3H, s, 18-CH_3), 0.71 (3H, s, 20-CH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 171.8 (C-16), 151.2 (C-12), 149.1 (C-8), 129.4 (C-13), 108.6 (C-17), 81.0 (C-3), 75.0 (C-15), 69.9 (C-14), 65.0 (C-19), 57.9 (C-9), 56.5 (C-5), 43.7 (C-4), 40.1 (C-10), 39.1 (C-7), 38.1 (C-1), 29.0 (C-2), 25.3 (C-6), 24.8 (C-11), 23.4 (C-18), 15.6 (C-20).

3-Dehydro-isoandrographolide (**5**): white amorphous powder, $C_{20}H_{28}O_5$. Legal and Kedde Reactions: positive. IR υ_{max} (KBr) cm^{-1}: 3432, 2928, 1751, 1720, 1650, 1021, 975, 831. High resolution ESI-MS (negative) m/z: 347.1835 [M−H]$^-$ (calcd for $C_{20}H_{27}O_5$, 347.1858). ^{1}H and ^{13}C NMR see Table 1.

14-Deoxy-11,12-didehydroandrographolide (**6**): colorless plate (in acetone), $C_{20}H_{28}O_4$. Legal and Kedde Reactions: positive. IR υ_{max} (KBr) cm^{-1}: 3442, 2928, 1751, 1656, 1543, 1062, 945, 821. ESI-MS (negative) m/z: 331 [M−H]$^-$. ^{1}H NMR (400 MHz, CD_3OD): δ 7.40 (1H, br. t, J = 6.2 Hz, H-14), 6.80 (1H, dd, J = 15.6, 9.8 Hz, H-11), 6.13 (1H, d, J = 15.6 Hz, H-12), 4.71 (1H, d, J = 2.3 Hz, H-17a), 4.50 (1H, d, J = 2.3 Hz, H-17b), 4.43 (2H, m, H-15), 4.25 (1H, d, J = 11.5 Hz, H-19a), 3.38 (1H, m, H-3), 3.21 (1H, d, J = 11.5 Hz, H-19b), 1.26 (3H, s, 18-CH_3), 0.75 (3H, s, 20-CH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 174.8 (C-16), 150.1 (C-8), 146.6 (C-14), 136.5 (C-11), 129.6 (C-13), 122.5 (C-12), 109.1 (C-17), 81.2 (C-3), 71.6 (C-15), 65.0 (C-19), 62.8 (C-9), 55.8 (C-5), 43.8 (C-4), 39.6 (C-10), 38.5 (C-1), 37.8 (C-7), 28.9 (C-2), 24.4 (C-6), 23.3 (C-18), 15.8 (C-20).

3-Oxo-14-deoxy-11,12-didehydroandrographolide (**7**): colorless powder, $C_{20}H_{26}O_4$. Legal and Kedde Reactions: positive. IR υ_{max} (KBr) cm^{-1}: 3432, 2923, 1747, 1712, 1661, 1530, 1041, 930, 850. ESI-MS (negative) m/z: 329 [M−H]$^-$. ^{1}H NMR (400 MHz, CD_3OD): δ 7.42 (1H, br. t, J = 6.3 Hz, H-14), 6.72 (1H, dd, J = 15.2, 9.3 Hz, H-11), 6.10 (1H, d, J = 15.2 Hz, H-12), 4.78 (1H, d, J = 2.1 Hz, H-17a), 4.61 (1H, d, J = 2.1 Hz, H-17b), 4.52 (1H, d, J = 12.1 Hz, H-19a), 4.50 (2H, m, H-15), 4.11 (1H, d, J = 12.1 Hz, H-19b), 1.28 (3H, s, 18-CH_3), 0.93 (3H, s, 20-CH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 213.3 (C-3), 172.5 (C-16), 148.7 (C-8), 145.8 (C-14), 135.3 (C-11), 128.9 (C-13), 122.4 (C-12), 109.1 (C-17), 71.0 (C-15), 64.7 (C-19), 61.0 (C-9), 56.7 (C-5), 55.2 (C-4), 39.5 (C-1), 39.0 (C-10), 38.0 (C-7), 36.3 (C-2), 24.5 (C-6), 21.9 (C-18), 15.5 (C-20).

3-Dehydroandrographolide (**8**): white powder, $C_{20}H_{28}O_5$. Legal and Kedde Reactions: positive. IR υ_{max} (KBr) cm^{-1}: 3430, 2928, 1731, 1718, 1652, 1510, 1023, 917, 836. High resolution ESI-MS (negative) m/z: 347.1840 [M−H]$^-$ (calcd for $C_{20}H_{27}O_5$, 347.1858). ^{1}H NMR (400 MHz, CD_3OD): δ 6.81 (1H, dd, J = 7.1, 5.4 Hz, H-12), 5.01 (1H, d, J = 5.6 Hz, H-14), 4.81 (1H, br. s, H-17a), 4.66 (1H, br. s, H-17b), 4.61 (1H, d, J = 11.2 Hz, H-19a), 4.40 (H, br. d, J = 11.2 Hz, H-15a), 4.25 (1H, d, J = 11.2 Hz, H-19b), 4.11 (H, br. d, J = 11.2 Hz, H-15b), 1.25 (3H, s, 18-CH_3), 0.92 (3H, s, 20-CH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 212.9 (C-3), 172.1 (C-16), 149.3 (C-12), 149.0 (C-8), 129.4 (C-13), 109.1 (C-17), 75.8 (C-15), 66.3 (C-14), 64.5 (C-19), 57.8 (C-9), 56.5 (C-5), 43.5 (C-4), 40.5 (C-10), 39.2 (C-1), 39.0 (C-7), 36.0 (C-2), 25.3 (C-11), 25.2 (C-6), 23.0 (C-18), 15.3 (C-20).

14-Deoxyandrographolide (**9**): colorless needle crystals (in acetone), $C_{20}H_{30}O_4$. Legal and Kedde Reactions: positive. IR υ_{max} (KBr) cm^{-1}: 3433, 2947, 1744, 1643, 1055, 957, 761. ESI-MS (negative) m/z: 333 [M−H]$^-$. ^{1}H NMR (400 MHz, CD_3OD): δ 7.23 (1H, s, H-14), 4.92 (1H, br. s, H-17a), 4.86 (1H, br. s, H-17b), 4.38 (2H, m, H-15), 4.23 (1H, d, J = 11.2 Hz, H-19a), 3.41 (H, m, H-3), 3.25 (1H, d, J = 11.2 Hz, H-19b), 1.22 (3H, s, 18-CH_3), 0.72 (3H, s, 20-CH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 175.2 (C-16), 148.5 (C-8), 147.2 (C-14), 131.2 (C-13), 108.7 (C-17), 80.6 (C-3), 72.5 (C-15), 64.7 (C-19), 57.6 (C-9), 56.1 (C-5), 43.4 (C-4), 39.8 (C-10), 38.7 (C-7), 38.1 (C-1), 29.0 (C-2), 25.5 (C-12), 25.2 (C-6), 23.5 (C-11), 23.1 (C-18), 15.3 (C-20).

3-Dehydro-14-deoxyandrographolide (**10**): white powder, $C_{20}H_{28}O_4$. Legal and Kedde Reactions: positive. IR υ_{max} (KBr) cm^{-1}: 3424, 2940, 1748, 1720, 1651, 1039, 921, 805. ESI-MS (negative) m/z: 331 [M−H]$^-$. ^{1}H NMR (400 MHz, CD_3OD): δ 7.20 (1H, s, H-14), 4.86 (1H, br. s, H-17a), 4.80 (1H, br. s, H-17b), 4.50 (1H, d, J = 12.1 Hz, H-19a), 4.41 (2H, m, H-15), 4.18 (1H, d, J = 12.1 Hz, H-19b), 1.25 (3H, s, 18-CH_3), 0.92 (3H, s, 20-CH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 213.0 (C-3), 175.3 (C-16), 149.0 (C-8), 147.2 (C-14), 131.3 (C-13), 108.6 (C-17), 72.6 (C-15), 64.7 (C-19), 57.6 (C-9), 56.6 (C-5), 43.6 (C-4), 40.4 (C-10), 39.3 (C-1), 39.1 (C-7), 36.2 (C-2), 25.5 (C-12), 25.2 (C-6), 23.4 (C-11), 23.0 (C-18), 15.5 (C-20).

3-Dehydro-14-deoxyandrographolide-19-oic acid (**11**): colorless needle crystals (in methanol), $C_{20}H_{26}O_5$. Legal and Kedde Reactions: positive. IR υ_{max} (KBr) cm^{-1}: 3425, 2936, 1753, 1725, 1643, 1068, 1021, 842. High resolution ESI-MS (negative) m/z: 345.1733 [M−H]$^-$ (calcd for $C_{20}H_{27}O_5$, 347.1702). ^{1}H and ^{13}C NMR see Table 1.

4.5. 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 [26].

Acknowledgements

This research was partly supported by the Start Fund of Wuhan University and the starting scientific research fund for returned overseas students of Ministry of Education of China.

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