European Journal of Medicinal Chemistry 44 (2009) 2611–2620

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Short communication

Studies on chemical structure modification and biology of a natural product, Gambogic acid (I): Synthesis and biological evaluation of oxidized analogues of gambogic acid

Jinxin Wang [a], Li Zhao [b], Yang Hu [b], Qinglong Guo [b], Lei Zhang [a], Xiaojian Wang [a], Nianguang Li [a], Qidong You [a,b,*]

[a] Department of Medicinal Chemistry, China Pharmaceutical University, Nanjing 210009, China
[b] Key laboratory of Carcinogenesis and Intervention of Jiangsu Province, China Pharmaceutical University, Nanjing 210009, China

ARTICLE INFO

Article history:
Received 29 May 2008
Received in revised form 3 September 2008
Accepted 8 September 2008
Available online 7 October 2008

Keywords:
Gambogic acid derivatives
Anti-tumor
Apoptosis inducer
Structure–activity relationship

ABSTRACT

Gambogic acid (GA), a natural product, exhibits high potency in inhibiting cancer cell growth through the effective induction of apoptosis. In order to investigate the structure–activity relationships of GA derivatives, 11 oxidized derivatives of GA were synthesized. Some of them showed strong inhibitory effects on HT-29, Bel-7402, BGC-823, A549, and SKOV 3 cell lines. Moreover, in this paper the cellular growth inhibitor 39-hydroxy-6-methoxy-gambogic acid methyl ester (**10**) was identified as a HepG2 cell apoptosis inhibitor through Annexin-V/PI double staining assay and the expression of the related apoptotic proteins (Bax and Bcl-2). Compound **10** may serve as a potential lead compound for the development of new anticancer drugs. Further SAR studies of GA derivatives indicated that modification of carbon–carbon double bond at C-32/33 or C-37/38 and of the methyl groups at C-39/C-35 can improve antitumor activity.

1. Introduction

Apoptosis, the controlled death of cells, is an important metabolic process in the regulation of cell growth. If apoptosis is blocked in the normal cell, disordered metabolism can cause the development of tumors. The induction of apoptosis in tumor cells has become a chief parameter in the characterization of anti-tumor drugs [1]. Currently, many anticancer drugs used clinically are known to kill tumor cells through the induction of apoptosis. Therefore, the discovery and development of compounds that induce apoptosis are highly desirable goals in the development of therapeutically effective antineoplastic agents and may lead to new anticancer agents.

Between 1980 and 2000, more than 70% of anticancer drugs approved by the US Food and Drug Administration were based on compounds derived from natural sources [2,3]. Gambogic acid (GA, CAS No. 2752-65-0) is the principal active component of gamboge, the resin from various *Garcinia* species, including *Garcinia morella* and *Garcinia hanburyi*. Many modern pharmaceutical studies have been focused on its extensive and potent anti-tumor activities [4–6]. Our previous studies demonstrated that GA can inhibit the growth of a wide variety of tumor cells, including human gastric carcinoma SGC-7901 cells, MGC-803 cells, human lung carcinoma SPC-A1 cells, and human hepatoma SMMC-7721 cells. The potent anticancer activity (both in vitro and in vivo) of GA is mainly attributed to its activation of the impaired apoptotic pathways in cancerous cells via down-regulation of telomerase. Studies have also indicated that GA suppresses the growth of human tumors as a potent apoptosis inducer in these cancer cells [11–14]. Because the exact cytotoxic mechanism was not fully understood, further research has been conducted. GA induced apoptosis through a different mechanism from the taxanes and vinca alkaloids, which induced apoptosis through interaction with tubulin and G2/M cell-cycle arrest [7]. Recent studies on the mechanism of GA action showed that it binds to the transferrin receptor, inducing a unique signal leading to rapid apoptosis of tumor cells [8]. It also serves as a novel ligand for the transferrin receptor and potentiates tumor necrosis factor (TNF)-induced apoptosis through modulation of the nuclear factor-κB signaling pathway [9]. We have recently reported that GA exerted its antiproliferative effect by inhibiting the catalytic activity of Topo IIα, inhibiting telomerase activity by down-regulating the expression of the hTERT gene (a target of c-MYC activity), inducing G2/M phase cell-cycle arrest via disturbance of CDK7-mediated phosphorylation of CDC2/p34 [16]. Furthermore, angiogenesis was inhibited through suppression of vascular endothelial growth factor-induced tyrosine phosphorylation of

* Corresponding author. Department of Medicinal Chemistry, China Pharmaceutical University, Nanjing 210009, China. Fax: +86 025 83271351.
E-mail address: youqidong@gmail.com (Q. You).

0223-5234/$ – see front matter © 2008 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2008.09.034

KDR/Flk-1[10,13,15,17]. Moreover, the selective anticancer activity of GA may also be related to its higher distribution and longer retention time in tumor cells as compared to normal cells. Indeed, our studies suggested that these simultaneous effects of GA may contribute to a highly effective anticancer drug candidate for hepatocellular carcinoma [18].

The elucidation of the new antitumor mechanism of GA and identification of its binding site are good starting points for additional research. At present, the key goal is to create more structural classes of new derivatives of GA for the study of its structure–activity relationships (SARs) and for further exploration of the binding site.

GA contains the xanthone core and a unique 4-oxatricyclo [4.3.1.$0^{3,7}$] decan-2-one ring system [19,20]. Previous reports [7,21–23] examining the SARs showed that the C═C of the α,β-unsaturated ketone in GA is critical for its antitumor activity, while the 6-hydroxy, 8-ketone and 30-carboxy groups could tolerate a variety of modifications. Thus, the syntheses of simple derivatives of GA were undertaken. Based on the particular complexity of its skeleton and the strong biological significance of GA, chemical modifications were focused on exploring the basic pharmacophore of GA for antitumor activity and inducing apoptosis, with the goal of discovering novel derivatives as potential anticancer agents. However, in considering the modification of GA, one must take into account its formidable functional groups, which are sensitive to alkali or acid.

As reported [24], GA is rapidly metabolized in rat liver microsomes and M1 is crucial for the elimination of GA. Cytochrome P-4501A2 is the major rat CYP involved in the metabolism of GA. The important metabolic products, the hydration metabolite and epoxide metabolite of GA, may be more active compounds than the parent drug itself. These results suggested us that oxidative metabolism products of GA may lead to new compounds with greater antineoplastic activity than GA. Therefore, we designed and synthesized 11 oxidized analogues of GA which have been designed and synthesized through modification of the carbon–carbon double bonds at C-32/33 and C-37/38 or by converting the single methyl groups at C-40 and C-34 into the epoxy or hydroxyl group, respectively.

Herein, we describe the synthesis of the gambogic acid analogues **1**–**11** (Fig. 1) and have evaluated them for induction of apoptosis in several cancer cell lines. We also performed SAR studies of gambogic acid as an inducer of apoptosis. We also include preliminary investigations of a promising derivative, compound **10**, as a potent apoptosis inducer in HepG2 cells by detection of growth inhibition, observation of morphological changes, and the expression of the related apoptotic proteins (Bax, Bcl-2 and Actin).

2. Results and discussion

2.1. Chemistry

GA was isolated in overall yield of approximately 5% from available gamboge resin. It was purified by converting the crude gamboge resin extract into the pyridine salt, followed by recrystallization [25]. To investigate the basic SAR of GA with respect to the induction of apoptosis, we planned to regioselectively modify the carbon–carbon double bond at C-32/33, C-37/38, and the single methyl at C-39/C-35 into homologous functional groups, respectively, through different oxidative and reaction conditions. The syntheses of derivatives **1**–**11** are depicted in Schemes 1–5, starting from GA.

Reaction of GA with $(Me)_2CO$ in the presence of K_2CO_3 and MeI produced the methyl ester of GA in 95% yield [23] followed by epoxide formation with CH_2Cl_2 in the presence of *m*-CPBA for a short reaction time, giving the corresponding compound **1** in 36% yield (Scheme 1). The presence of the alkene proton at δ 5.07 (C_{32}-H) in compound **1** suggested that the carbon–carbon double bond at C-32/33 remained intact, because the carbon–carbon double bond at C-32/33 was electronically deficient. Similarly, reaction of GA-methyl ester and GA with *m*-CPBA for a longer reaction time produced the GA derivatives **2** and **3** containing two epoxy groups at C-32/33 and C-37/38. As discussed below, in GA derivatives **1**–**3**, the stereochemistry of C-37 and C-32 was confirmed from the respective spectral data.

Our attempts to use $KMnO_4$/EtOH to prepare compound **4a** (Scheme 2), which contained di-hydroxyl groups at C-37/38, were completely ineffective [26]. However, as demonstrated by spectral

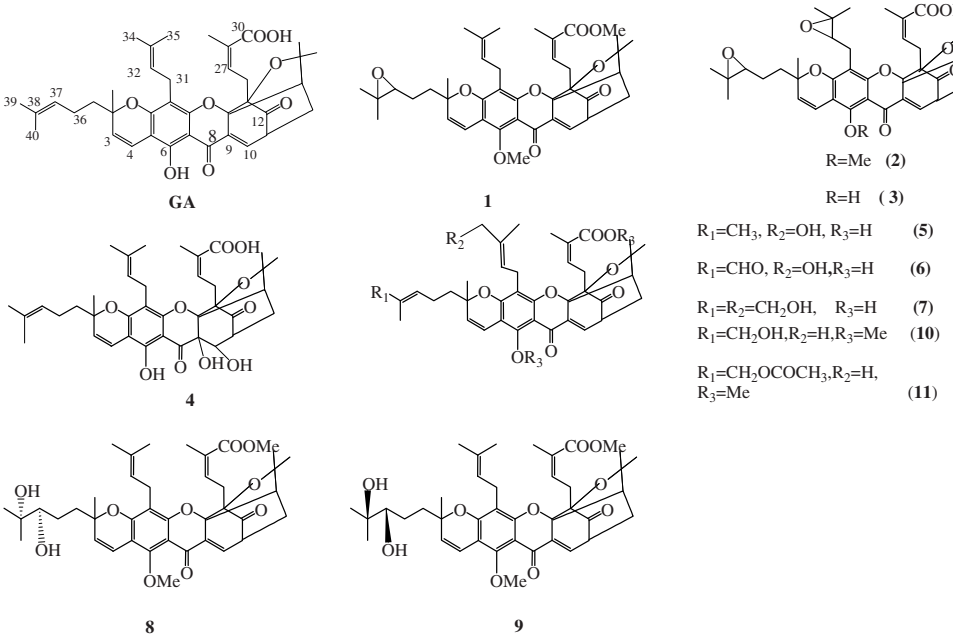
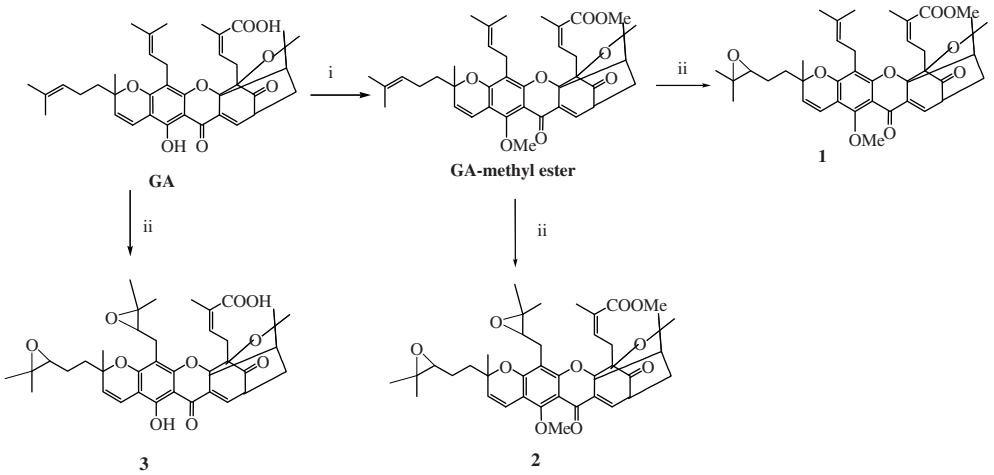


Fig. 1. Structures of gambogic acid (GA) and its oxidative analogues **1**–**11**.



Scheme 1. Reagents and conditions: (i) K_2CO_3, MeI, $(Me)_2CO$, R.T. (ii) *m*-CPBA, CH_2Cl_2, R.T.

data, compound **4** with di-hydroxyl groups at C-9/10 was achieved. Presumably, $KMnO_4$, acting as a nucleophile, attacked the carbon–carbon double bond in the α,β-unsaturated ketone. This oxidation reaction resulted in compound **4**, but not compound **4a**. The observed selectivity can be rationalized by the carbon–carbon double bond at C-9/10 being electronically more deficient than that at C-38/40.

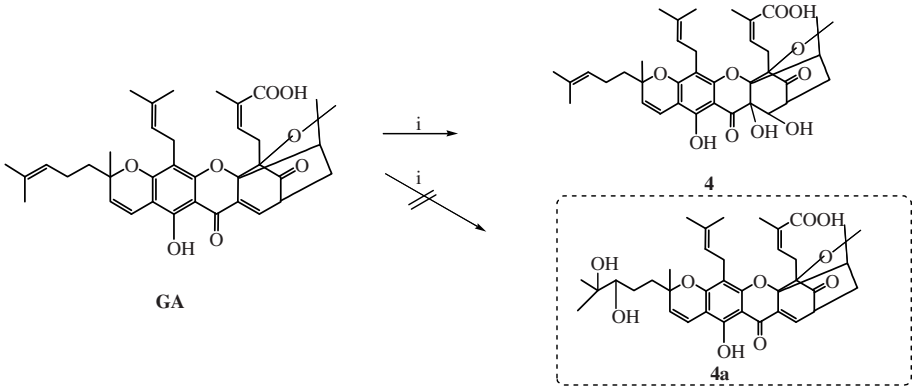
Conventional regioselective oxidations of C–H bonds require neighboring activating groups. Very common in synthesis is the oxidation of allylic methylene or methyl groups to yield allylic alcohols or α,β-unsaturated carbonyl compounds. The successful reagents are selenium dioxide. In following substantial experiments, under very similar reaction conditions, different usage amounts of selenium dioxide resulted in homologous products **5**–**7**, respectively.

The single methyl groups at C-34 and C-39 of GA were, respectively, modified as depicted in Scheme 3. The synthesis of **5** was carried out starting from GA, prepared from SeO_2 and *t*-BuOOH by the oxidation of the methyl at C-34 of GA in 31% yield. Why was the hydroxyl group located at C-34? The observed selectivity can be rationalized by a hydrogen bond formed between the C-30 COOH group and the oxygen of the oxidative reagent SeO_2. The cross-peaks observed in the HMBC spectrum of compound **5** between δ 3.31 (C_H-31a), δ 3.13 (C_H-31b) with 123.7 (C-32), δ 3.91 (C_H-34) with 123.7 (C-32), as well as the correlated signals at δ 5.05 (C_H-37) with 17.51 (C-40)/25.5 (C-39), confirmed the position of the hydroxyl group at C-34 or C-35. The position of the hydroxyl group at C-34 was determined by the ROESY correlations between the signals at δ 3.31 (C_H-31a), δ 3.13 (C_H-31b) and 1.48 (C_H-35). No cross-peak was observed in the ROESY spectrum between δ 3.31 (C_H-31a), δ 3.13 (C_H-31b) and δ 3.91 (C_H-34).

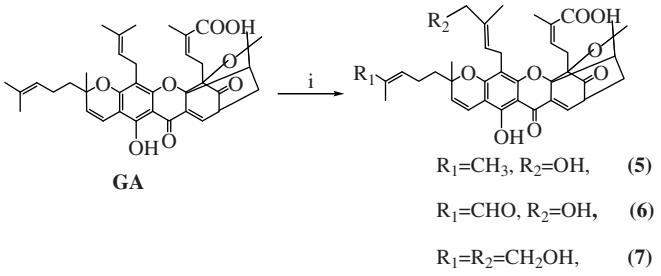
Reaction of GA with SeO_2 and *t*-BuOOH in CH_2Cl_2 produced GA oxidative derivative **6** with an aldehyde group at C-39 and a hydroxyl methyl group at C-35 in 11% yield [27–29]. Another oxidative derivative of GA, compound **7** with hydroxy methyl groups at C-39 and at C-34 was afforded in 35% yield.

The Sharpless asymmetric dihydroxylation (AD) of olefins is an indispensable tool for contemporary organic synthesis. The ADs of GA-methyl ester (in accordance with the literature precedence [29]) provided compounds **8** (75% ee) and **9** (85% ee), of which the di-hydroxyl groups were formed at C-37/38 in the presence of a mixture of *t*-BuOH, H_2O, AD-mix β and methanesulfonyl amide (Scheme 4). Also the ee values were determined by ^1H NMR analysis of the corresponding Moster's ester [36,37]. Why were the di-hydroxyl groups added at C-37/38? A possible reason for the site selectivity was related to the large steric hindrance from the phthalazine class of ligands, $(DHQD)_2$-PHAL, and $(DHQ)_2$-PHAL, and the electronic abundance at C-37/38.

In Scheme 5, oxidation of the methyl at C-39 of GA-methyl ester with CH_2Cl_2 in the presence of SeO_2 and *t*-BuOOH resulted in compound **10** in 61% yield, which was reacted with DMAP and Ac_2O in CH_2Cl_2 to generate acetate **11**. In this synthesis, changed usage amount of selenium dioxide and reaction times resulted in targeted product **10**. Observed side product was much trace. The cross-peaks observed in the HMBC spectrum of compound **10** between



Scheme 2. Reagents and conditions: (i) $KMnO_4$, EtOH, R.T.



Scheme 3. Reagents and conditions: (i) SeO_2, *t*-BuOOH, CH_2Cl_2, R.T.

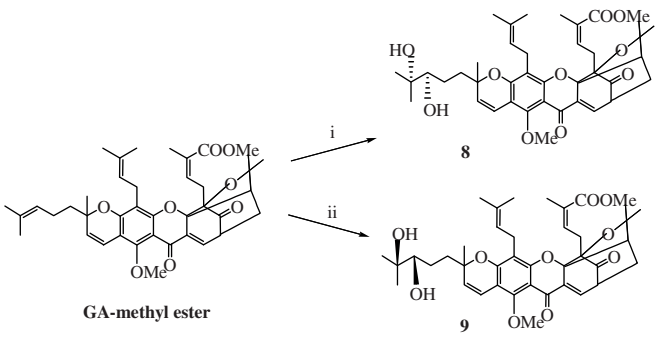
δ 5.32 (C_H-37) and 68.8 (C-39), δ 3.95 (C_H-39) with 20.8 (C-40), δ 5.32 (C_H-37) with 20.8 (C-40) as well as the correlated signals at δ 3.37 (C_H-31a), δ 3.26 (C_H-31b) with 121.9 (C-32) and δ 2.04 (C_H-36) with 125.3 (C-37), confirmed the position of the hydroxyl group at C-39 or C-40. A cross-peak was observed in the ROESY spectrum between δ 2.04 (C_H-36) and 1.68 (C_H-40), confirming that the position of the hydroxyl group was at C-39.

In summary, we have regioselectively synthesized 11 novel oxidative derivatives of GA and identified their structures by spectral techniques.

2.2. Structure–activity relationship

Earlier SAR studies [7] had identified the 9, 10 carbon–carbon double bond of the α,β-unsaturated ketone as critical for cytotoxicity and apoptosis-inducing activity, while the 30-carboxy and 6-hydroxy were not important. We concentrated our synthetic efforts on modifications of the carbon–carbon double bond at C-32/33, C-37/38 and of the methyl groups at C-39/C-35, and the observed structure and activity relationships are summarized in Table 1. The cytotoxic activities of the oxidized derivatives **1**–**11** were determined in vitro by an MTT assay which estimates cell viability by the remaining activity of mitochondrial enzymes [31]. In the A549, Bel7402, HT-29, BGC-823, SKOV3 cell lines, the IC_{50} values for the parent compound, gambogic acid, ranged between 3.0 and 6.0 μM (Table 1). The novel compounds exhibited considerable variation in their cytotoxic activities against the same cell lines.

As a result of cytotoxicity screening, compounds **2**, **7**, and **10** were identified as having a greater effect in the human lung carcinoma cell line (A549) than GA. Compounds **1** and **3** are ~4.0-fold more potent than GA. In contrast, compounds **5**, **9**, and **11** were comparable to GA. In the human hepatoma cell line (Bel-7402), compounds **1**, **2**, and **10** were two- to threefold more cytotoxic than the parent compound. In human gastric carcinoma cell line (BGC823), compounds **3** and **9** had similar cytotoxicity to GA, whereas compounds **1**, **10**, and **11** were ~three- to sixfold more potent than the reference compound. Compound **7** gave the most interesting result in the BGC-823 cell line, with IC_{50} = 0.23 μM, almost 20-fold more potent than GA. In ovarian cancer cell line (SKOV3), five compounds (**2**, **3**, **5**, **6** and **10**) were ~1.6- to 4.0-fold more potent than GA, while compound **1** exhibited the lowest IC_{50} value of 0.64 μM, ~4.8-fold more potent than GA. In a human colorectal cancer cell line (HT-29), seven compounds (**1**–**3**, **5**, **6**, **9**, and **11**) are ~2.1- to 3.0-fold more potent than the parent structure. Compound **10** displayed the strongest cytotoxicity (IC_{50} = 0.89 μM), an almost 6.0-fold increase in potency.



Scheme 4. Reagents and conditions: (i) AD-mix-α, $CH_3SO_2NH_2$, *t*-BuOH-H_2O, R.T.; (ii) AD-mix-β, $CH_3SO_2NH_2$, *t*-BuOH-H_2O, R.T.

Our newly synthesized compounds **1**–**11** exerted significant in vitro inhibitory effects on some cell growth. Compounds **1** and **2** were the most effective analogues in all cell lines, and compound **3** was more potent than GA in all cell lines except Bel7402. It is noteworthy that compounds **1**–**3** possessed one or two epoxy groups at positions C-32/33 or C-37/38. Further SAR studies of the analogues **5**–**7**, **10** and **11** were pursued, wherein the methyl at C-39 or C-35 was modified into hydroxyl or aldehyde groups by oxidation, respectively. The major findings are as follows: (i) compound **10** exhibited greater potency than GA in all cell lines; (ii) compared to GA, compounds **5** and **6** showed better selectivity toward SKOV3 and HT-29 cell lines; (iii) compound **7** had much better selectivity toward the BGC823 and Bel7402 cell lines; and (iv) compound **11**, in which the C-40 hydroxyl group was protected by an acetyl group, showed evident selectivity toward the BGC823 and HT-29 cell lines.

Moreover, compound **4** showed less cytotoxic activities against all cell lines than GA, confirming the earlier report that the 9, 10 carbon–carbon double bond of the α,β-unsaturated ketone is important for biological activity [9]. Remarkably, the two hydroxyl groups at C-37/38 in compounds **8** and **9** had opposite stereochemistry and they displayed evident differences in cytotoxic activities against all cell lines. Evidently the stereochemistry of GA is important for biological activity. Additionally, analogues containing an epoxy group at C-37/38 showed better cytotoxic activities than those containing two hydroxyl groups at the same position.

2.3. Characterization of compound *10* as a potent apoptosis inducer

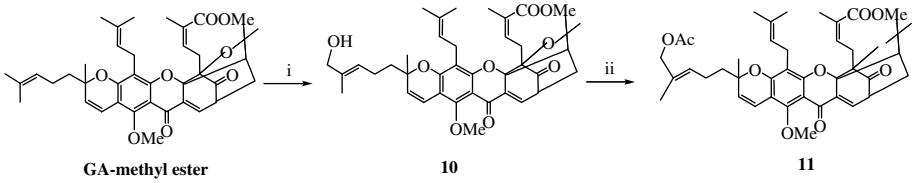
An anticancer leading compound was screened partly by its proapoptotic ability on tumor cells. Currently, human hepatocellular carcinoma (HCC) is the most common liver cancer in the world [32] and discovery of HCC therapeutic agents with greater effect but less toxicity has become one of the main assignments for most pharmaceutical researchers. Inspired by the improved cytotoxicity of compound **10** against the A549, Bel7402, HT-29, BGC-823, and SKOV3 cell lines, especially the Bel7402 cell line, we sought to characterize compound **10** as a potent apoptosis inducer in the human hepatocellular carcinoma cell line HepG2. We evaluated this analogue with an optimized chemical structure by measuring growth inhibition, morphological changes, and the expression of related apoptotic proteins (Bax, Bcl-2).

2.3.1. Cell viability inhibition

Compound **10** inhibited HepG2 cells viability in a concentration-dependent manner as measured by 24 h MTT assay, and its IC_{50} was calculated as 2.35 μM. Over the range of 0–8.0 μM, the inhibitory effect became obvious. (Fig. 2).

2.3.2. Cell morphological assessment

Fig. 3 shows that morphological changes of cells by optical microscopy after treatment with three concentrations (1.0, 2.0, and 3.0 μM) of compound **10** for 20 h. After incubation with compound **10**, HepG2 cells were severely distorted and grew slowly. Some cells became round in shape. The untreated cells displayed normal and healthy shapes, as demonstrated by the clear skeletons.



Scheme 5. Reagents and conditions: (i) SeO_2, *t*-BuOOH, CH2Cl2, R.T.; (ii) DMAP, Ac_2O, CH_2Cl_2, R.T.

Furthermore, the result demonstrated that the inhibitory effects on HepG2 cells were strongly related to dosage of **10**.

2.3.3. AOEB staining

Fluorescence microscopic analysis showed clear morphological changes in the nucleolus, typical of early apoptosis in HepG2 cells following treatment with compound **10** (1.0, 2.0, and 3.0 μM) for a period of 18 h (Fig. 4). Untreated HepG2 cells were stained with uniform green fluorescence which showed the chromatin equably distributed in the nucleolus. After compound **10** treatment, the cells displayed congregated chromatin and nucleolus pyknosis, which emitting bright fluorescence, the early phenomena of apoptosis.

2.3.4. Expressions of Bcl-2, Bax protein

As shown in Fig. 5, after incubation with compound **10** (1.0, 2.0 and 6.0 μM) for 20 h, the expression of Bcl-2 protein decreased and that of Bax was unchanged as compared with control. The current data proved that compound **10** could selectively induce apoptosis of human hepatoma HepG2 cells and the lower expression of the apoptotic Bcl-2 proteins. In previous studies [33–35], it was shown that the ratio of Bcl-2/Bax might be one of the critical factors of a cell's threshold for undergoing apoptosis, and Bax could bind with Bcl-2 and inhibit its function in suppression of apoptosis. So, the observed decrease of Bc1-2/Bax illustrated a possible mechanism of the induction of apoptosis. In our experiments, the ratio of Bc1-2/Bax was 0.469 (control), 0.476 (1.0 μM), 0.478 (2.0 μM) and 0.251 (6.0 μM). It was shown obviously that compound **10** could decrease the ratio of Bc1-2/Bax in HepG2 cells. The apoptosis induction of compound **10** was confirmed in HepG2 cells. Furthermore, how compound **10** works, and the other mechanisms which may contribute to the changes of cell cycle, should be the subject of further research. Further biological in vivo evaluation is underway. Compound **10** may be a promising and significant leading compound for the treatment of cancer diseases in the future.

2.3.5. Annexin-V/PI double-staining assay

The percentage of apoptotic cells (Annexin-V+/PI−) in the control group was 4.3%. After treatment with 1 μM, 2 μM and 6 μM compound **10** for 20 h, the percentages of apoptotic cells were from 4.3% (Control) to 12.9% (6.0 μM) (Fig. 6).

Table 1
The cytotoxicity data of GA and its derivatives [IC_{50} (μM)].

Compound	Cell lines				
	A549	BGC823	SKOV3	HT-29	Bel7402
1	1.48 ± 0.30	0.75 ± 0.22	0.64 ± 0.13	2.19 ± 0.77	1.40 ± 0.29
2	3.61 ± 1.09	3.21 ± 0.7	1.80 ± 0.22	1.86 ± 0.34	1.66 ± 0.4
3	1.56 ± 0.26	4.38 ± 1.0	0.76 ± 0.16	2.08 ± 0.41	3.73 ± 0.41
4	74.2 ± 7.4	29.9 ± 5.1	6.00 ± 1.2	34.7 ± 6.2	33.1 ± 5.8
5	5.87 ± 1.41	4.89 ± 0.98	1.61 ± 0.31	2.66 ± 0.42	4.01 ± 0.86
6	20.5 ± 3.8	6.75 ± 1.41	1.84 ± 0.34	2.08 ± 0.60	15.0 ± 2.9
7	4.24 ± 1.2.	0.23 ± 0.04	3.43 ± 0.80	21.6 ± 4.2	2.32 ± 0.6
8	>100	>100	>100	>100	>100
9	5.86 ± 1.4	3.46 ± 1.0	3.23 ± 1.08	3.75 ± 0.72	3.65 ± 0.65
10	4.38 ± 0.82	0.84 ± 0.18	1.28 ± 0.32	0.89 ± 0.13	0.96 ± 0.28
11	5.25 ± 1.1	1.42 ± 0.43	15.8 ± 3.2	2.41 ± 0.46	3.60 ± 0.61
Gambogic acid	5.81 ± 1.2	4.51 ± 1.3	3.06 ± 1.15	5.61 ± 1.2	3.31 ± 0.92

3. Conclusion

Although these experiments were preliminary, the result of SAR investigation suggests that promising agents with the antitumor activities may be obtained by the conversion of the carbon–carbon double bond at C-32/33, C-37/38 and the methyl at C-39/C-35 into the epoxy and hydroxyl groups, respectively. Compound **10** exhibited much more potent cytotoxic activities against A549, Bel7402, HT-29, BGC-823, SKOV3 cancer cells lines than the reference compound gambogic acid, and the induction of apoptosis on HepG2 cells may be related with the expressions of Bcl-2 and Bax. One of the analogues, compound **10**, may be a highly effective anticancer drug candidate as a potent apoptosis inducer. Moreover, the newly synthesized compounds **1**–**11** can provide abundant materials for biological studies and identification of the binding site of gambogic acid in further research. In brief, our results suggest that gambogic acid and its oxidized derivatives may become attractive molecules for the development of anticancer agents.

4. Experimental protocols

4.1. Chemistry

4.1.1. General methods

Melting points were measured with a Melt-Temp II instruments. IR spectra were recorded on a Nicolet Impact 410 spectrometer. ^1H NMR and ^{13}C NMR spectra were recorded on Bruker AV-300 or AV-500 MHz instruments in $CDCl_3$ using tetramethylsilane (TMS) as the internal standard. EI-MS was recorded Shimadzu GC–MS 2050 apparatus; ESIMS was recorded on Agilent 1100 LC/MSD (70 eV) spectrometers. HREIMS was recorded on a Waters Q-Tof micro.

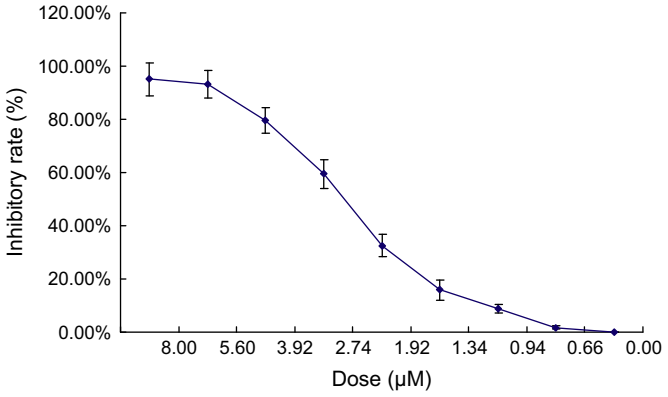


Fig. 2. Inhibitory effects of **10** on primary cultured HepG2 cells. Data are shown as mean ± SD; $n = 3.0$, 0.66, 0.94, 1.34, 1.92, 2.74, 3.92, 5.60 and 8.00 μM of Compound **10** were added, respectively. After 24 h of incubation, the culture medium was removed and MTT was added. Four hours later, the supernatant was discarded and 100 μl of DMSO was added to each well. The mixture was shaken and measured at 570 nm using a Universal Microplate Reader.

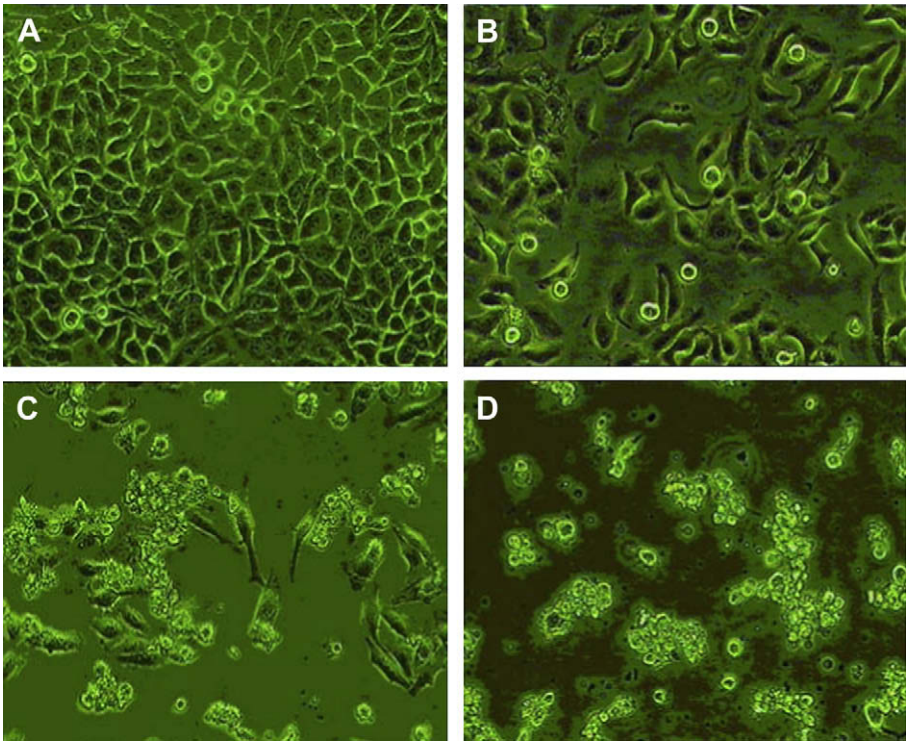


Fig. 3. Morphologic changes of cells observed under inverted microscope: HepG2 cells. A: Control group; B: 1.0 μM compound 10; C: 2.0 μM compound **10**; D: 3.0 μM compound **10**.

Chemical shifts are reported as δ values (parts per million) relative to solvent peak. Coupling constants are reported in hertz. The multiplicity is defined by s (singlet), d (doublet), t (triplet), or m (multiplet).

Column and thin-layer chromatography (CC and TLC, resp.) were performed on silica gel (200–300 mesh) and silica gel GF254, respectively, supplied by Qingdao Marine Chemical Factory. *GA-methyl ester* was prepared by a literature method [23].

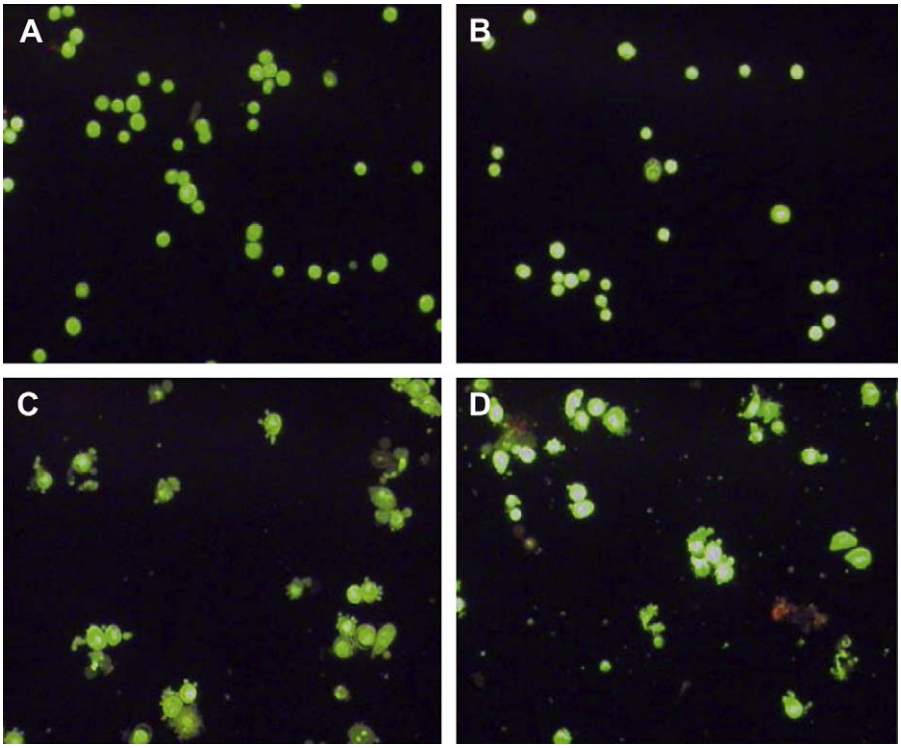


Fig. 4. Nucleolus morphologic changes observed by fluorescence microscope (400×) in HepG2 cells. A: Control group; B: 1.0 μM compound **10**; C: 2.0 μM compound **10**; D: 3.0 μM compound **10**.The early apoptotic cells are observed: the cells were stained green with AO, their membrane retained integrity, and the nuclei exhibited bright condensed chromatin (B). Dead cells were stained equally red with EB. Typical apoptotic blebbing was obvious and late apoptotic cells were observed. Their nuclei exhibited condensed chromatin, and they were stained red with EB; dead cells were stained equally red with EB.

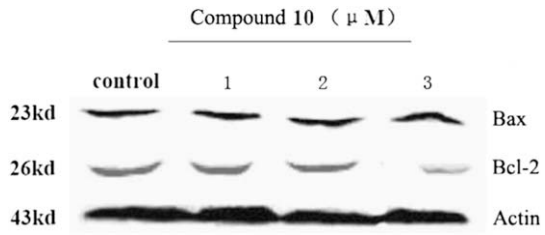


Fig. 5. Effects of **10** on the expression of Bcl-2, Bax and Actin proteins. HepG2 cells were treated with 1.0 μM, 2.0 μM, and 6.0 μM compound **10** for 20 h as described in Section 4. The ratio of Bc1-2/Bax was 0.469 (control), 0.476 (1.0 μM), 0.478 (2.0 μM) and 0.251 (6.0 μM). Proteins were precipitated, solubilized and separated by 10% SDS-PAGE. Proteins were transferred to Immobilon-Pmembranes and quantitated by immunoprecipitation and development with antibodies as described in Section 4.

*4.1.2. (37,38)-Epoxy-6-methoxyl-gambogic acid methyl ester **1** and (32,33), (37,38)-di-epoxyl-6-methoxyl-gambogic acid methyl ester **2***

To a stirred solution of *GA-methyl ester* (328 mg, 0.5 mmol) in CH_2Cl_2 (20 ml) at room temperature, was added *m*-CPBA (104 mg, 0.6 mmol). The resulting mixture was stirred for 6 h at the room temperature. The mixture was extracted with EtOAc and then the organic layer was washed with aqueous saturated Na_2CO_3, H_2O, and brine, respectively, then dried over Na_2SO_4. After removal of the solvent, the residue was subjected to silica gel column chromatography (petroleum ether:EtOAc = 4:1) to give rise to **1** (120 mg, 36%) as a buff-colored oil, and **2** (80 mg, 24%). Compound **1**: Mp: 72–75 °C. IR (film): 2969, 1731, 1708, 1621, 1586, 1460, 1379, 878 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 7.45 (1H, d, J = 6.84 Hz, H-10), 6.67 (1H, d, J = 10.2 Hz, H-4), 5.96 (1H, m, H-27), 5.54 (1H, d, J = 10.2 Hz, H-3), 5.07 (1H, m, H-32), 3.84 (3H, s, 6-OCH_3), 3.45 (3H, s, COO CH_3), 3.43 (1H, m, H-11), 3.30 (1H, d, 31a), 3.15 (1H, m, H-31b), 2.98 (2H, d, J = 6.99 Hz, H-26), 2.68 (1H, t, H-37), 2.51 (1H, d, J = 7.4 Hz, H-22), 2.31 (1H, m, H-21a), 1.85 (2H, m, H-36), 1.76 (3H, s, H-25), 1.69 (3H, s, H-29),1.66 (3H, s, H-34), 1.63 (3H, s, H-35), 1.60 (3H, s, H-39), 1.58 (3H, s, H-40), 1.44 (1H, m, H-21b), 1.47 (3H, m, H-24), 1.28 (3H, s, H-19); ESI-MS: 673 $[M+H]^+$, 695 $[M+Na]^+$.

Compound **2**: Mp: 70–73 °C. IR (film): 2928, 1713, 1708, 1621, 1587, 1461, 1381, 1144, 880 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz): δ 7.46 (1H, d, J = 6.9 Hz, H-10), 6.69 (1H, d, J = 10.1 Hz, H-4), 6.02 (1H, m, H-27), 5.58 (1H, d, J = 10.1 Hz, H-3), 3.83 (3H, s, 6-OCH_3), 3.45 (3H, s, $COOCH_3$), 3.43 (1H, m, H-11), 2.98 (2H, m, H-26), 3.15 (2H, m, H-31), 2.70 (1H, m, H-32), 2.89 (1H, m, H-37), 2.53 (1H, m, H-22), 2.31 (1H, m, H-21a), 1.40 (1H, m, H-21b), 1.98 (2H, m, H-36), 1.77 (3H, s, H-25), 1.72 (2H, m, H-20), 1.67 (3H, s, H-29), 1.48 (3H, s, H-34), 1.43 (3H, s, H-40), 1.47 (6H, br s, H-39, H-24), 1.94 (3H, s, H-35), 1.28 (3H, s, H-19). ESI-MS: 689 $[M+H]^+$, 711 $[M+Na]^+$. HRMS (M + Na) *m/z* 711.3130 (Calcd for $C_{40}H_{48}O_{10}$ Na 711.3145).

*4.1.3. (32,33), (37,38)-Di-epoxyl-gambogic acid **3***

To a stirred solution of GA (110 mg, 0.18 mmol) in CH_2Cl_2 (20 ml) at room temperature, was added *m*-CPBA (36 mg, 0.21 mmol). The resulting mixture was stirred for 12 h at the room temperature. The mixture was extracted with EtOAc and then the organic layer was washed with aqueous saturated Na_2CO_3, H_2O, and brine, respectively, then dried over Na_2SO_4. After removal of the solvent, the residue was subjected to silica gel column chromatography (petrolum:EtOAc = 4:1) to give rise to **3** (40 mg, 36%) as a buff-colored oil. IR (film): 3452, 2969, 2927, 1731, 1713, 1708, 1681, 1586, 1460, 1379 cm^{-1}.^1H NMR ($CDCl_3$, 500 MHz): δ 7.45 (1H, d, J = 6.84 Hz, H-10), 6.67 (1H, d, J = 10.2 Hz, H-4), 5.96 (1H, m, H-27), 5.54 (1H, d, J = 10.2 Hz, H-3), 3.84 (3H, s, 6-OCH_3), 3.45 (3H, s, $COOCH_3$), 3.43 (1H, m, H-11), 3.30 (1H, d, H-31a), 3.15 (1H, m, H-31b), 2.98 (2H, d, J = 6.99 Hz, H-26), 2.70 (2H, m, H-37, H-32), 2.51 (1H, d, J = 7.4 Hz, H-22), 2.31 (1H, m, H-21a), 1.85 (2H, m, H-36), 1.76 (3H, s, H-25), 1.69 (3H, s, H-29), 1.66 (3H, s, H-34), 1.63 (3H, s, H-35), 1.60 (3H, s, H-39), 1.58 (3H, s, H-40), 1.44 (1H, m, H-21b), 1.47 (3H, m, H-24), 1.28 (3H, s, H-19). ESI-MS: 661 $[M+H]^+$, 683 $[M+Na]^+$, 659 $[M-H]^-$. HRMS (M + Na) *m/z* 683.2821 (Calcd for $C_{38}H_{44}O_{10}Na$ 667.2883).

*4.1.4. (9,10)-Dihydroxy-gambogic acid **4***

To a stirred solution of GA (314 mg, 0.5 mmol) in 95% ethanol (15 ml) at room temperature, was added dropwise $KMnO_4$ in

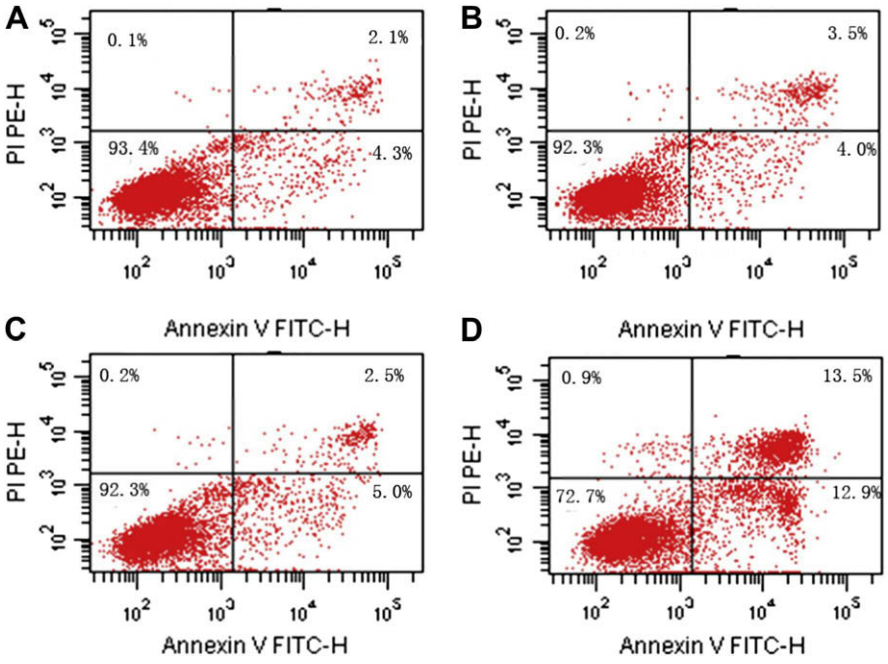


Fig. 6. Fluorescence-activated cell sorter analysis for Annexin-V and PI staining. (A) Control, (B) 1.0 μM, (C) 2.0 μM, (D) 6.0 μM compound **10**, UR (up right): necrotic cells and late apoptotic cells labeled with PI and Annexin V–FITC. LL (lower left): fully viable cells. LR (lower right): early apoptotic cells labeled with Annexin V–FITC but not with PI. After exposure to compound **10**, the cells of LR had increased from 4.3% (Control) to 12.9% (6.0 μM).

water (1.5 ml, 80 mg, 0.5 mmol). The brown suspension was stirred for 6 h, and then the whole mixture was filtered through a silica gel pad, the solvent was removed in vacuo and the residue was taken up in EtOAc, then the organic layer was washed with aqueous saturated Na_2CO_3, H_2O and brine, respectively, then dried over Na_2SO_4 and concentrated. The brown residue was purified by flash chromatography using (petroleum ether:EtOAc = 1:2), and yielded a brown powder **4** (56 mg, 38%). M.p. 238–240 °C. IR (KBr): 3424, 2971, 2927,1742, 1694, 1629,1594, 1446, 1375, 1190, 1086, 1140 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz): δ 11.40 (1H, s, 6-OH), 6.74 (1H, m, H-3), 6.20 (1H, m, H-27), 5.29 (1H, m, H-4), 5.09 (1H, m, H-32), 5.20 (1H, m, H-37), 4.55 (2H, br s, 9-OH, 10-OH), 3.59 (1H, m, 10-H), 3.35 (1H, m, H-11), 3.30 (1H, m, H-31a), 3.21 (1H, m, H-31b), 2.98 (2H, dd, J = 9.7 Hz, 7.9 Hz, H-26), 2.51 (1H, m, H-22), 2.36 (1H, dd, J = 13.0 Hz, 4.6 Hz, H-21a), 2.10 (2H, m, H-36), 1.76 (3H, s, H-25), 1.72 (2H, m, H-20), 1.68 (9H, br s, H-34, H-35, H-39), 1.60 (3H, s, H-40), 1.29 (3H, s, H-19), 1.40 (3H, s, H-24), 1.32 (1H, m, H-21b). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 17.9 (C-40), 18.0 (C-34), 20.2 (C-29), 22.0 (C-31), 27.5 (C-21), 25.7 (C-36), 25.8 (C-35), 27.7 (C-19), 29.8 (C-24), 30.2 (C-25), 33.6 (C-26), 39.0 (C-20), 47.5 (C-11), 48.5 (C-22), 46.5 (C-10), 70.0 (C-9), 82.4 (C-2), 85.2 (C-13), 86.3 (C-23), 92.0 (C-14), 107.43 (C-17), 100.1 (C-7), 105.1 (C-5), 109.0 (C-4), 122.3 (C-32), 126.8 (C-28), 123.5 (C-37), 126.94 (C-3), 131.0 (C-33), 135.82 (C-38), 138.9 (C-27), 155.5 (C-6), 160.6 (C-16), 161.6 (C-18), 171.5 (C-30), 192.5 (C-8), 208.8 (C-12). ESI-MS: 663 $[M + H]^+$, 685 $[M + Na]^+$.

4.1.5. 34-Hydroxy-gambogic acid **5**

GA (628 mg, 1 mmol)) was dissolved in CH_2Cl_2 (2 ml) and added dropwise to a clear solution of SeO_2 (6.0 mg, 0.05 mmol) and *t*-BuOOH (75%, 270 mg, 3 mmol in CH_2Cl_2 (10 ml) at room temperature. After being stirred at room temperature for 5 h, the reaction mixture was diluted with Et_2O and sequentially with aqueous 10% KOH, H_2O and brine, then dried and concentrated. The resulting yellow oil was purified by flash column chromatography on silica gel to yield a yellow oil **5** (130 mg, 21%) M.p. 103–105 °C. IR (KBr): 3457, 3420, 2970, 2923, 1736, 1689, 1623, 1592, 1454, 1436, 1400, 1381, 1330,1176,1138,1047 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz): δ 7.54 (1H, d, J = 6.5 Hz, H-10), 6.59 (1H, d, J = 10 Hz, H-4), 5.75 (1H, m, H-27), 5.43 (1H, d, J = 10.0 Hz, H-3), 5.38 (1H, m, H-37), 5.05 (1H, m, H-32), 3.96 (2H, d, J = 5.5 Hz, H-34), 3.49 (1H, m, H-11), 3.31 (1H, m, H-31a), 3.13 (1H, dd, J = 8.6 Hz, 8.6 Hz, H-31b), 2.93 (2H, dd, J = 4.4 Hz, 4.3 Hz, H-26), 2.50 (1H, d, J = 9.3 Hz, H-22), 2.31 (1H, dd, J = 4.5 Hz, 4.4 Hz, H-21a), 2.03 (2H, m, H-36), 1.78 (3H, m, H-25), 1.72 (3H, s, H-29), 1.70 (3H, m, H-20), 1.48 (3H, s, H-35),1.66 (3H, s, H-39), 1.55 (3H, s, H-40), 1.41 (1H, m, H-24), 1.37 (1H, m, H-21b). 1.25 (3H, s, H-19). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 17.51 (C-40), 20.8 (C-29), 20.9 (C-21), 21.2 (C-31), 22.6 (C-36), 25.3 (C-35), 25.5 (C-39), 27.1 (C-19), 28.8 (C-24), 29.7 (C-26), 30.0 (C-25), 41.7 (C-20), 46.9 (C-11), 49.0 (C-22), 68.3 (C-34), 81.2 (C-2), 83.6 (C-13), 83.8 (C-23), 90.7 (C-14), 100.4 (C-7), 102,8 (C-5), 106.9 (C-17), 115.8 (C-4), 123.7 (C-32), 123.8 (C-37), 124.6 (C-3), 128.5 (C-28), 131.8 (C-38), 133.3 (C-9), 134.2 (C-33), 135.1 (C-10), 136.5 (C-27), 157.4 (C-6), 157.6 (C-16), 161.2 (C-18), 170.3 (C-30), 178.8 (C-8), 203.0 (C-12). ESI-MS: 667 $[M + Na]^+$: 683 $[M + K]^+$. HRMS (M + Na) m/z 667.2888 (Calcd for $C_{38}H_{44}O_9Na$ 667.2883).

4.1.6. 34-Hydroxy-39-formyl-gambogic acid **6** and (34,39)-dihydroxy-gambogic acid **7**

GA (628 mg, 1 mmol) was dissolved in CH_2Cl_2 (2 ml) and added dropwise to a clear solution of SeO_2 (12 mg, 0.1 mmol and *t*-BuOOH (75%, 270 mg, 3 mmol in CH_2Cl_2 (10 ml) at room temperature. After being stirred at room temperature for 5 h, the reaction mixture was diluted with Et_2O and sequentially with aqueous 10% KOH, H_2O and brine, then dried and concentrated. The resulting yellow oil was purified by flash column chromatography on silica gel to yield the yellow powder **6** (40 mg, 6%) and **7** (100 mg, 15%). Compound **6**: M.p. 105–107 °C. IR (KBr): 3470, 3418, 2972, 2924, 2854, 1736, 1687, 1634, 1592, 1454, 1436, 1400, 1380, 1331, 1240, 1176, 1138, 1046, 1018 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz): δ 12.80 (1H, s, 6-OH), 9.32 (1H, s, CHO), 7.57 (1H, d, J = 6.9 Hz, H-10), 7.32 (1H, m, H-32), 6.68 (1H, d, J = 10.1 Hz, H-4), 6.50 (1H, d, J = 10.1 Hz, H-3), 6.3 (1H, m, H-27), 5.4 (1H, m, H-3), 3.95 (2H, br d, J = 12.7 Hz, H-34), 3.50 (1H, m, H-11), 3.30 (2H, m, H-31), 2.98 (2H, m, H-26), 2.07 (1H, m, H-36a), 2.72 (1H, d, J = 9.3 Hz, H-22), 2.37 (1H, m, H-21a), 2.15 (1H, m, H-36b), 1.45 (3H, s, H-24), 1.28 (3H, s, H-19), 1.55 (3H, s, H-40), 1.68 (3H, s, H-35), 1.72 (2H, m, H-20), 2.31 (1H, m,H-21a), 1.40 (1H, m, H-21b), 1.70 (3H, s, H-29). ESI-MS: 657 $[M-H]^+$. HRMS (M + Na) m/z 681.2643 (Calcd for $C_{38}H_{42}O_{10}Na$ 681.2676). Compound **7**: M.p. 85–87 °C. IR (KBr): 3474, 3416, 2963, 2923, 1734, 1714, 1635, 1592, 1435, 1401, 1330, 1176, 1260, 1095,1022, 802 cm^{-1}.^1H NMR ($CDCl_3$, 300 MHz): δ 137.61 (1H, d, J = 6.9 Hz, H-10), 6.65 (1H, d, J = 9.9 Hz, H-4), 5.9 (1H, m, H-27), 5.58 (1H, d, J = 10.0 Hz, H-3), 5.39 (2H, m, H-32, 37), 3.9 (4H, br d, J = 9.6 Hz, H-34, H-40), 3.52 (1H, m, H-11), 3.33 (2H, m, H-31a), 3.10 (1H, dd, J = 14.7 Hz, 7.6 Hz, H-31b), 2.93 (2H, d, J = 7.23 Hz, H-26), 2.51 (1H, d, J = 9.0 Hz, H-22), 2.31 (1H, dd, J = 4.5 Hz, 4.8 Hz, H-21a), 2.13 (2H, m, H-36), 1.46 (1H, m, H-24), 1.45 (1H, m, H-21b), 1.80 (3H, m, H-25),1.78 (3H, s, H-29), 1.72 (3H, m, H-20), 1.68 (3H, s, H-35), 1.69 (3H, s, H-39), 1.56 (3H, s, H-19). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 21.2 (C-29), 22.0 (C-31), 23.1 (C-36), 25.3 (C-21), 25.7 (C-35), 27.8 (C-19), 29.2 (C-24), 29.7 (C-26), 30.0 (C-25), 42.3 (C-20), 48.0 (C-11), 50.0 (C-22), 68.2 (C-34), 68.3 (C-40), 82.1 (C-2), 84.4 (C-13), 84.5 (C-23), 92.3 (C-14), 101.3 (C-7), 103.4 (C-5), 108.1 (C-17), 116.7 (C-4), 123.4 (C-32), 123.4 (C-37), 124.6 (C-3), 129.0 (C-28), 134.1 (C-9), 136.0 (C-38), 136.8 (C-33), 135.1 (C-10), 136.5 (C-27), 158.5 (C-6), 158.8 (C-16), 162.0 (C-18), 168.4 (C-30), 180. 5 (C-8), 203.9 (C-12). ESI-MS: 661 $[M + H]^+$, 683 $[M + Na]^+$. HRMS (M + Na) m/z 683.2820 (Calcd for $C_{38}H_{44}O_{10}Na$ 683.2832).

4.1.7. (37R,38)-Dihydroxy-6-methoxy-gambogic acid methyl ester **8**

To *GA-methyl ester* (1 g, 1.59 mmol) was added a mixture of *t*-BuOH (5 ml), H_2O (5 ml), AD-mix-α (2.67 g, 1.91 mmol), and methanesulfonyl amide (182 mg, 1.91 mmol). The solution was stirred for 48 h at 0 °C. After the addition of saturated Na_2SO_3 (2.0 g, 1.38 mmol), the mixture was stirred for 40 min and diluted with EtOAc. The organic layer was washed with H_2O and brine, dried over $MgSO_4$ and concentrated to give the crude product purified by silica gel column chromatography, yielded yellow solid **8** (90 mg, 11%). Unreacted *GA-methyl ester* (220 mg). M.p. 108–110 °C. IR (KBr): 3315, 3034, 3012, 2934, 2927, 1713, 1641, 1589, 1435, 1413, 1316, 1143, 1086, 1029 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz): δ 7.46 (1H, d, J = 6.9 Hz, H-10), 6.65 (1H, d, J = 10.1 Hz, H-4), 6.06 (1H, t, H-27), 5.56 (1H, d, J = 10.2 Hz, H-3), 5.07 (1H, br t, H-32), 4.74 (2H, br s, 37-OH, 38-OH), 3.82 (3H, s, 6-OCH_3), 3.76 (1H, d, J = 10.9 Hz, 37-H), 3.43 (1H, m, H-11), 3.41 (3H, s, $COOCH_3$), 3.34 (2H, m, H-31a), 3.10 (1H, dd, J = 14.7 Hz, 7.6 Hz, H-31b), 2.92 (2H, d, J = 7.23 Hz, H-26), 2.51 (1H, d, J = 9.0 Hz, H-22), 2.31 (1H, dd, J = 13.5 Hz, 4.8 Hz, H-21a), 1.46 (1H, m, H-24), 1.39 (1H, m, H-21b), 1.76 (5H, m, H-20, H-25), 1.65 (3H, s, H-35), 1.69 (6H, s, H-29, H-34), 1.67 (3H, s, H-39), 2.03 (2H, m, H-36), 1.28 (3H, s, H-19). ESI-MS: 691 $[M + H]^+$, 713 $[M + Na]^+$, 729 $[M + K]^+$. HRMS (M + H) m/z 691.3483 (Calcd for $C_{40}H_{51}O_{10}$ 691.3482).

4.1.8. (37S,38)-Dihydroxy-6-methoxy-gambogic acid methyl ester **9**

To *GA-methyl ester* (250 mg, 0.38 mmol) was added a mixture of *t*-BuOH (3.5 ml), H_2O (3.5 ml), AD-mix-β (590 mg, 0.42 mmol), and methanesulfonyl amide (36 mg, 0.38 mmol). The solution was stirred for 48 h at 0 °C. After the addition of saturated Na_2SO_3 (72 mg, 0.57 mmol), the mixture was stirred for 40 min and diluted with EtOAc. The organic layer was washed with H_2O and brine, dried over $MgSO_4$ and concentrated to give the crude product purified by silica gel column chromatography, yielded yellow solid **9** (62 mg, 48%). Unreacted *GA-methyl ester* (120 mg). M.p.

110–113 °C. IR (KBr): 3315, 3034, 3012, 2934, 2927, 1713, 1641, 1589, 1435, 1413, 1316, 1143, 1086, 1029 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz): δ 7.45 (1H, d, $J = 6.9$ Hz, H-10), 6.65 (1H, d, $J = 10.1$ Hz, H-4), 6.06 (1H, t, H-27), 5.56 (1H,d, $J = 10.2$ Hz, H-3), 5.07 (1H, br t, H-32), 4.76 (2H, br s, 37-OH, 38-OH), 3.82 (3H, s, 6-OCH_3), 3.75 (1H, d, $J = 10.9$ Hz, 37-H), 3.43 (1H, m, H-11), 3.41 (3H, s, $COOCH_3$), 3.33 (1H, m, H-31a), 3.10 (1H, dd, $J = 14.7$ Hz, 7.6 Hz, H-31b), 2.93 (2H, d, $J = 7.23$ Hz, H-26), 2.51 (1H, d, $J = 9.0$ Hz, H-22), 2.31 (1H, dd, $J = 13.5$ Hz, 4.8 Hz, H-21a), 1.46 (1H, m, H-24), 1.39 (1H, m, H-21b), 1.76 (5H, m, H-20, H-25), 1.65 (3H, s, H-35), 1.69 (6H, s, H-29, H-34), 1.67 (3H, s, H-39), 2.03 (2H, m, H-36), 1.28 (3H, s, H-19). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 18.14 (C-34), 20.9 (C-29), 22.0 (C-31), 23.6 (C-36), 25.6 (C-21), 25.4 (C-35), 26.8 (C-19), 28.8 (C-24), 29.2 (C-25), 29.8 (C-26), 29.5 (C-40), 38.9 (C-39), 43.4 (C-20), 46.5 (C-11), 46.7 (C-37), 49.0 (C-22), 51.1 (30-$COOCH_3$), 62.1 (6-OCH_3), 72.9 (C-38), 80.4 (C-2), 83.6 (C-13), 83.7 (C-23), 91.1 (C-14), 107.4 (C-17), 109.9 (C-7), 113.0 (C-5), 116.8 (C-4), 121.7 (C-32), 127.2 (C-3), 127.6 (C-28), 131.8 (C-33), 133.9 (C-9), 135.7 (C-10), 136.4 (C-27), 155.2 (C-6), 158.5 (C-16), 159.6 (C-18), 167.6 (C-30), 174.6 (C-8), 203.8 (C-12), 68.76 (C-40). ESI-MS: 691$[M + H]^+$, 713$[M + Na]^+$, 729$[M + K]^+$. HRMS (M + H) m/z 691.3483 (Calcd for $C_{40}H_{51}O_{10}$ 691.3482).

4.1.9. 39-Hydroxy-6-methoxy-gambogic acid methyl ester **10**

GA-methyl ester (450 mg, 0.69 mmol) was dissolved in CH_2Cl_2 (2 ml) and added dropwise to a clear solution of SeO_2 (7.6 mg, 0.068 mmol) and *t*-BuOOH (75%, 185 mg, 2.06 mmol in CH_2Cl_2 (10 ml) at room temperature. After being stirred at room temperature for 19 h, the reaction mixture was diluted with Et_2O and sequentially with aqueous 10% NaOH, H_2O and brine, then dried and concentrated. The resulting yellow oil was purified by flash column chromatography on silica gel to yield a yellow sheet solid **10** (280 mg, 61%) .IR (KBr): 3467, 3416, 2966, 2925, 1734, 1709, 1655, 1607, 1586, 1463, 1427, 1384, 1225, 1143, 1046 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz): δ 7.53 (1H, d, $J = 6.70$ Hz, H-10), 6.68 (1H, d, $J = 2.3$ Hz, H-4), 5.95 (1H, m, H-27), 5.53 (1H, d, $J = 6.1$ Hz, H-3), 5.32 (1H, m, H-37), 5.16 (1H, m, H-32), 4.11 (2H, s, H-40), 3.81 (3H, s, 6-OCH_3), 3.43 (3H, s, $COOCH_3$), 3.41 (1H, m, H-11), 3.37 (1H, m, H-31a), 3.26 (1H, m, H-31b), 2.96 (2H, m, H-26), 2.51 (1H, d, $J = 9.31$ Hz, H-22), 2.30 (1H, m, H-21a), 2.04 (2H, m, H-36), 1.75 (3H, s, H-25), 1.69 (3H, s, H-34), 1.65 (3H, s, H-35), 1.68 (3H, s, H-40), 3.95 (2H, s, H-39), 1.28 (3H, s, H-19), 1.38 (1H, m, H-21b), 1.44 (3H, s, H-24). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 18.1 (C-34), 20.7 (C-29), 20.8 (C-40), 22.1 (C-31), 22.3 (C-36), 25.5 (C-21), 25.7 (C-35), 27.9 (C-19), 28.9 (C-24), 29.4 (C-25), 29.9 (C-26), 41.7 (C-20), 46.8 (C-11), 49.0 (C-22), 50.9 (30-$COOCH_3$), 62.1 (6-OCH_3), 68.8 (C-39), 80.5 (C-2), 83.6 (C-13), 83.8 (C-23), 90.9 (C-14), 107.4 (C-17), 100.7 (C-7), 112.6 (C-5), 116.9 (C-4), 121.9 (C-32), 125.3 (C-37), 126.9 (C-3), 12, 158.6 (C-16), 159.7 (C-18), 174.6 (C-8), 203.9 (C-12), 167.3 (C-30). ESI-MS: $(M + H)^+$ m/z 673.3369 (Calcd for $C_{40}H_{49}O_9$ 673.3377).

4.1.10. 40-Acetoxy-6-methoxy-gambogic acid methyl ester **11**

Compound **10** (85 mg, 0.13 mmol) was dissolved in CH_2Cl_2 (5 ml), to the resulting solution was added pyridine (0.25 ml), freshly distilled Ac_2O (0.29 ml) and DMAP (2 mg, 0.016 mmol) at room temperature with vigorous stirring for 4 h. The reaction mixture was diluted with Et_2O and sequentially with 10% HCl aqueous solution, H_2O, and brine, then dried and concentrated. The resulting crude was purified by flash column chromatography on silica gel to yield a yellow oil characterized as **11** (30 mg, 33%). ^1H NMR ($CDCl_3$, 300 MHz): δ 7.53 (1H, d, $J = 6.70$ Hz, H-10), 6.68 (1H, d, $J = 2.3$ Hz, H-4), 5.95 (1H, m, H-27), 5.53 (1H, d, $J = 6.1$ Hz, H-3), 5.12 (1H, m, H-37), 5.09 (1H, m, H-32), 4.45 (2H, s, H-39), 4.11 (2H, s, H-40), 3.81 (3H, s, 6-OCH_3), 3.43 (3H, s, $COOCH_3$), 3.41 (1H, m, H-11), 3.37 (1H, m, H-31a), 3.26 (1H, m, H-31b), 2.96 (2H, m, H-26), 2.51 (1H, d, $J = 9.31$ Hz, H-22), 2.30 (1H, m, H-21a), 2.04 (2H, m, H-36), 1.90 (3H, s, $-CH_3CO-$), 1.75 (3H, s, H-25),1.69 (3H, s, H-34), 1.65 (3H, s, H-35), 1.69 (3H, s, H-40), 1.28 (3H, s, H-19), 1.38 (1H, m, H-21b), 1.44 (3H, s, H-24). ESI-MS: 715 $[M + H]^+$ 737 $[M + Na]^+$, 753 $[M + K]^+$.

4.2. Biological materials and methods

4.2.1. Biology

Compounds **1**–**11** and gambogic acid (supplied by the School of Pharmacy, China Pharmaceutical University) were dissolved in DMSO (Sigma) (0.1 ml) to make a final concentration of 10^{-2} M and was stored at −20 °C. The solutions of compounds **1**–**11** and GA were freshly diluted with RPMI-1640 (GIBCO, USA) to the appropriate final concentrations at time of use.

Tumor cells: HT-29, Bel-7402, BGC-823, A549 and SKOV3, HepG2 cell lines were purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science. Cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated calf serum (Sijiqing, Hangzhou, China), 100 U/ml penicillin G, and 100 U/ml streptomycin, pH 7.4, in a water jacketed CO_2 incubator (Thermo Forma, USA) with a humidified atmosphere of 5% CO_2 at 37 °C.

Reagents: MTT (Fluka, USA) was dissolved in 0.01 M PBS. Primary antibody of actin was obtained from CalBiochem. Primary antibodies of Bcl-2 and Bax were obtained from Santa Cruz Biotechnology Inc., USA. Secondary antibodies were all obtained from Rockland Inc., USA. CycleTest™ Plus DNA reagent lit was obtained from Becton-Dickinson, USA. Annexin V-EGFP Apoptosis Detection kit was obtained from KeyGen, USA.

4.2.2. Methods

4.2.2.1. MTT assay of growth inhibition [30]. Cells (5×10^3 per well in a 96-well plate) were treated with **1**–**11** (0.01, 0.1, 1.0, 10, 100 and 1000 μM) and GA (0.01, 0.1, 1.0, 10, 100 and 1000 μM) for 48 h, respectively. Then, 0.5% MTT was added into each well and incubated for 4 h at 37 °C. The supernatant was discarded and 0.1 ml DMSO was added to dissolve the precipitate. The mixture was shaken on a micro-vibrator for 5 min and the absorbance was measured at 570 nm by an Automated Microplate Reader ELx800 (Bio Tek). Inhibition ratio (IR%) was calculated by the following method:

$$1 - \frac{\text{Average absorbance of treated group}}{\text{Average absorbance of control group}} \times 100$$

The IC_{50} was defined as the concentration that caused 50% inhibition of cell proliferation, and was calculated by SAS statistical software. The data are presented as the mean ± standard deviation of three observations.

4.2.3. Cell morphological assessment

HepG2 cell line was cultured in RPMI-1640 until mid-log phase. Compound **10** (1, 2, or 3 μM) was then added to the culture media and the cells were further incubated for 20 h. At the end of incubation, the morphology of the cells was monitored under an inverted light microscope. All floating and attached cells were harvested with 0.02% (w/v) EDTA and 0.25% (w/v) trypsinase. The cell suspension was fixed with ice-cold 4% paraformaldehyde for 20 min and washed with ice-cold PBS. The cell suspension was permeabilized with 0.3% Triton X-100 and washed with ice-cold PBS, stained with fluorochrome dye DAPI (Santa Cruz, USA), and observed under a fluorescence microscope (OlympusIX51, Japan) with a peak excitation wavelength of 340 nm.

4.2.4. AOEB staining

Aliquots of compound **10** (1.0–3.0 μM) were added to HepG2 cells and incubated for an additional 18 h. Paraformaldehyde (4%, 500 μl) was then added and fixed for 20 min followed by addition of 0.1% Triton-100 (300 μl), permeabilizing for 20 min, and addition of

300 μl AOEB in PBS for staining. Five minutes later, the cells were observed under a fluorescence microscope (Olympus, Japan) with peak excitation wavelength of 490 nm.

4.2.5. Expression of Bcl-2, Bax, and Actin proteins

After incubation with compound **10** (1.0–6.0 μM), proteins of cells were isolated by lysis buffer (100 mM Tris-Cl, pH6.8, containing 4% (m/v) SDS, 20% (v/v) glycerol, 200 mM β-mercaptoethanol, PMSF 1 mM, Aprotinin 1 μg/ml, Sigma) and measured by the Bradford assay with a Bio Photometer (Bio Photometer 6131 GB/HK, Eppendorf) at 595 nm. Proteins (96 μg applied per well) were separated by SDS-PAGE on 10% gels and electrotransferred to an Immobilon-Pmembrane (Millipore, USA). Immune complexes were formed by incubation of the proteins with the primary antibodies mouse anti-Bax, rabbit anti-Bcl-2, rabbit and mouse anti-Actin overnight at 4 °C. Blots were washed and incubated for 1 h with IRDye™800 conjugated anti-mouse and anti-rabbit secondary antibodies. All antibodies were obtained from Santa Cruz Biotechnology, CA, USA. Immunoreactive protein bands were detected with an Odyssey Scanning System (LI-COR Inc., USA).

4.2.6. Annexin-V/PI double-staining assay

HepG2 cells labeled with Annexin-V/PI were early apoptotic cells. Compound **10** (1.0, 2.0 and 6.0 μM) was directly added into the flasks and incubated for 20 h. Cells were harvested and 1×10^6 cells were washed and resuspended with PBS. Apoptotic cells were identified by double supravital staining with recombinant FITC (fluorescein isothiocyanate)-conjugated Annexin-V and PI, using the Annexin V–FITC Apoptosis Detection kit (BioVision, USA) according to the manufacturer's instructions. Flow cytometric analysis was performed immediately after supravital staining. Data acquisition and analysis were performed in a Becton–Dickinson FACSCalibur flow cytometer using CellQuest software.

4.2.7. Statistical evaluation

All results shown represent the mean ± SD from triplicate experiments performed in a parallel manner unless otherwise indicated. Statistical analyses were performed using an unpaired, two-tailed Student's *t*-test. All comparisons are made relative to untreated controls and significance of difference is indicated as $^{*}P < 0.05$ and $^{**}P < 0.01$.

Acknowledgments

The authors thank the 863 High-Tech Project of China (2002AA2Z3112) (2004AA2Z3A10), the Key Projects of Science and Technology Research of Ministry of Education of PRC (2006-106094) and the Projects of Natural Science Foundation of Jiangsu Province (BK2006149) for financial support.

References

[1] J.F.R. Kerr, Cancer 73 (1994) 2013–2026.
[2] B.B. Aggarwal, H. Ichikawa, P. Garodia, Expert Opin. Ther. Targets 10 (2006) 87–118.
[3] D.J. Newman, G.M. Cragg, K.M. Snader, J. Nat. Prod. 66 (2003) 1022–1037.
[4] L. Juian, L.Z. Lin, J.M. Pezzuto, G.A. Cordell, *Garcinia hanburyi*, Magn. Reson. Chem. 31 (1993) 340–347.
[5] H. Auterhoff, H. Frauendorf, W. Liesenklas, C. Schwandt, Arch. Pharm. 295 (1962) 833–846.
[6] W. Liesenklas, H. Auterhoff, Arch. Pharm. Ber. Dtsch. Pharm.Ges. 299 (1966) 797–798.
[7] H.Z. Zhang, S. Kasibhatla, Y. Wang, J. Herich, J. Guastella, B. Tseng, J. Drewe, S.X. Cai, Biomed. Chem. 12 (2004) 309–317.
[8] S. Kasibhatla, K.A. Jessen, S. Maliartchouk, J.Y. Wang, N.M. English, J. Drewe, L. Qiu, S.P. Archer, A.E. Ponce, N. Sirisoma, S.C. Jiang, H.Z. Zhang, K.R. Gehlsen, S.X. Cai, D.R. Green, B. Tseng, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 12095–12100.
[9] M.K. Pandey, B. Sung, K.S. Ahn, A.B. Kunnumakkara, M.M. Chaturvedi, B.B. Aggarwal, Blood 110 (2007) 3517–3525.
[10] Q.L. Guo, S.S. Lin, Q.D. You, Life Sci. 78 (2006) 1238–1245.
[11] L. Zhao, Q.L. Guo, Q.D. You, Z.Q. Wu, H.Y. Gu, Biol. Pharm. Bull. 27 (2004) 998–1003.
[12] W. Liu, Q.L. Guo, Q.D. You, L. Zhao, H.Y. Gu, S.T. Yuan, World J. Gastroenterol. 11 (2005) 3655–3659.
[13] Z.Q. Wu, Q.L. Guo, Q.D. You, L. Zhao, H.Y. Gu, Biol. Pharm. Bull. 27 (2004) 1769–1774.
[14] Q.L. Guo, Q.D. You, Z.Q. Wu, S.T. Yuan, L. Zhao, Acta Pharmacol. Sin. 25 (2004) 769.
[15] J. Yu, Q.L. Guo, Q.D. You, Cancer Chemother. Pharmacol. 58 (2006) 434–443.
[16] J. Yu, Q.L. Guo, Q.D. You, Carcinogenesis 28 (2007) 632–638.
[17] N. Lu, Y. Yang, Q.D. You, Y. Ling, Y. Gao, H.Y. Gu, L. Zhao, X.T. Wang, Q.L. Guo, Cancer Lett. 258 (2007) 80–89.
[18] Y. Yang, L. Yang, Q.D. You, F.F. Nie, H.Y. Gu, L. Zhao, X.T. Wang, Q.L. Guo, Cancer Lett. 256 (2007) 259–266.
[19] W.D. Ollis, M.V.J. Ramsay, I.O. Sutherland, S. Mongkolsuk, Tetrahedron 21 (1965) 1453–1470.
[20] J. Asano, K. Chiba, M. Tada, T. Yoshii, Phytochemistry 41 (1996) 815–820.
[21] S.W. Jang, M. Okada, I. Sayeed, G. Xiao, D. Stein, P. Jin, K. Ye, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 16329–16334.
[22] T. Ben, S.N. Sudath, WO 2,004,002,428.
[23] S.X. Cai, H.Z. Zhang, US 2,003,078,292.
[24] Y.T. Liu, K. Hao, X.Q. Liu, G.J. Wang, Acta Pharmacol. Sin. 27 (2006) 1253–1258.
[25] Q.L. Guo, L. Zhao, Q.D. You, Chin. J. Nat. Med. 2 (2004) 106–110.
[26] D. Brewster, M. Myers, P. Ormerod, J. Chem. Soc., Perkin Trans. 1 (1973) 2796–2801.
[27] U.T. Bhalerao, H. Rapoport, J. Am. Chem. Soc. 93 (1971) 4835–4840.
[28] J.E. McMurry, R.G. Dushin, J. Am. Chem. Soc. 112 (1990) 6942–6947.
[29] N.K.N. Yee, R.M. Coates, J. Org. Chem. 57 (1992) 4598–4603.
[30] Y.D. Wu, Y. Wang, K.N. Houk, J. Org. Chem. 57 (1992) 1362–1368.
[31] M.F. Florian, A.J. Claude, A. Markus, Appl. Environ. Microbiol. 65 (1999) 3727–3729.
[32] D.M. Parkin, F. Bray, J. Ferlay, Int. J. Cancer 94 (2001) 153–156.
[33] F. Pettersson, A.G. Dalgleish, R.P. Bissonnette, Br. J. Cancer 87 (2002) 555–561.
[34] X.M. Yin, Nature 369 (1994) 321–323.
[35] B.Antonsson, Exp. Cell. 256 (2000) 50–57.
[36] A. Hassner, V. Alexanian, Tetrahedron Lett. 46 (1978) 4475–4478.
[37] H. Hagiwara, H.J. Uda, Org. Chem. 53 (1988) 2308–2313.