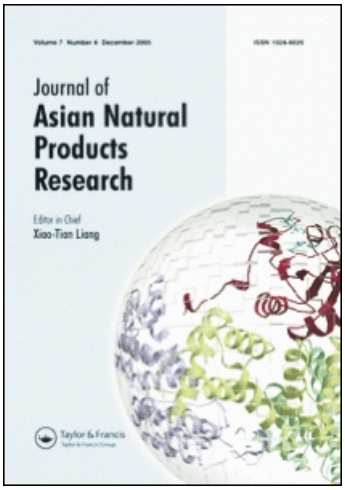

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ORIGINAL ARTICLE

Antitumor activity of two gelsemine metabolites in rat liver microsomes

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Gelsemine is one of the major alkaloids from *Gelsemium elegans* Benth., which has been used as an antitumor remedy in clinic. In this paper, metabolism of gelsemine has been investigated *in vitro* in phenobarbital-treated rat liver microsomes. The metabolites of gelsemine were separated and evaluated using the flash silica gel column, preparative HPLC, using NMR and MS methods. According to the spectral data, two metabolites, M1 and M2, were identified as 4-*N*-demethylgelsemine and 21-oxogelsemine, respectively. By the MTT method *in vitro*, the antitumor activities between gelsemine and its metabolites were compared in the HepG2 and HeLa cell lines. Moreover, the main metabolic pathway was further proposed.

Keywords: gelsemine; 4-*N*-demethylgelsemine; 21-oxogelsemine; antitumor; liver microsomes

1. Introduction

Gelsemium elegans Benth., of which Gou--wen is the Chinese name, has been used in folk medicine as an analgesia, immunoenhancement, antispasmodic, and especially as an antitumor remedy in clinic for many years [1–3]. More attention was paid to this plant and more than 40 alkaloids separated from *G. elegans* Benth. have hitherto been reported [4], but all of these alkaloids do not show strong antitumor activity. Many researchers have been trying to find other antitumor compounds from this plant. This result suggested that two possible reasons were necessarily considered. One was unknown non-alkaloid compounds, exhibiting antitumor bioactivities, and the other was the known alkaloids' metabolites *in vivo*, playing an important role in biological activities. While in our previous work, some non-alkaloid compounds were found and their anti-tumor activities were evaluated [5–11], in this paper, we present the study of alkaloids' metabolism.

Of the total alkaloid compounds from *G. elegans* Benth., gelsemine is the main content. Our research focused on the changes in antitumor bioactivity between gelsemine and its metabolites. As gelsemine showed toxicity leading to respiratory depression, it is unsuitable for the study of metabolism *in vivo*. The rat liver microsomal model has been established as an important method for drug metabolism research *in vitro* for many years. In the present study, we made use of this model to evaluate the metabolism of gelsemine. According to our investigation, two

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metabolites, M1 (4-*N*-demethylgelsemine) and M2 (21-oxogelsemine), were obtained, the chemical structures of which were determined by spectroscopic analysis. Furthermore, the antitumor bioactivities between the parent compound and its corresponding metabolites were compared in both HepG2 and HeLa cell lines.

2. Results and discussion

2.1 HPLC-DAD analysis of gelsemine and its metabolites

After incubation with an NADPH-generating system, the dichloromethane extract was collected and filtered by a 0.45 μm filter for the sample analysis. The HPLC-DAD chromatograms (Figure 1) showed two main metabolites in the simplified liver microsomes in comparison with blank samples and the parent drug. Metabolites **1** (M1) and **2** (M2) have a similar UV profile to that of gelsemine. These evidences showed that the metabolites have a similar parent nucleus [12].

2.2 Spectral analysis of gelsemine and its metabolites

To structurally identify the metabolites in the liver microsomal metabolism system, enough metabolites were collected for NMR detection by preparative HPLC (PHPLC).

Metabolite **1** (M1) was obtained as a white amorphous powder (MeOH), mp 224–226°C, UV (MeOH) data (see Figure 1). M1 showed an HPLC retention time of 12.5 min, and ESI-MS values at *m/z* 309 (100) $[M + H]^+$, 360 (10), 274 (10), 242 (3) indicated the loss of a $-CH_3$ group from gelsemine. ^{1}H NMR spectral data (Table 1) showed that the signal at δ 2.25 (4-*N*-CH_3 group) disappeared, and the

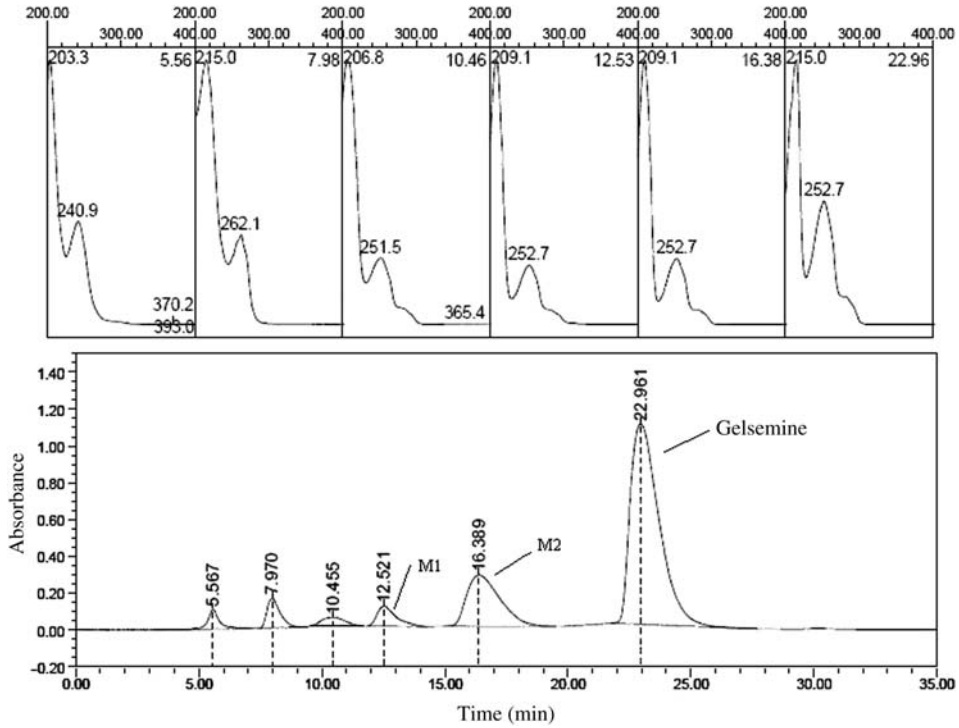

Figure 1. HPLC chromatogram and UV spectrum of liver microsome metabolites extracted by dichloromethane. M1, M2: metabolites; gelsemine: parent drug.

Table 1. ^{1}H NMR spectral data[a] of gelsemine, M1, and M2.

Position	Gelsemine	M1	M2
3	3.81 s	3.83 s	3.87 s
5	3.46 br s	3.83 br s	3.87 br s
6	1.98 br s	1.76 br s	2.07 br s
9	7.40 d ($J = 7.6$)	7.48 d ($J = 7.5$)	7.39 d ($J = 7.5$)
10	6.99 t ($J = 7.2$)	7.03 t ($J = 7.5$)	7.06 t ($J = 7.5$)
11	7.17 t ($J = 7.2$)	7.22 t ($J = 7.5$)	7.27 t ($J = 7.5$)
12	6.74 d ($J = 7.6$)	6.81 d ($J = 7.5$)	6.86 d ($J = 7.5$)
14a	2.83 dd ($J = 14.2$, 2.8)	2.86 dd ($J = 14.4$, 2.8)	2.97 dd ($J = 14.7$, 2.7)
14b	2.01 ddd ($J = 14.2$, 6.1, 3.2)	2.01 ddd ($J = 14.6$, 5.6, 2.8)	2.15 ddd ($J = 14.7$, 5.6, 2.7)
15	2.30 m	2.43 m	2.50 m
16	2.43 br d ($J = 8.0$)	2.31 br d ($J = 8.3$)	2.22 m
17a	3.92 dd ($J = 11.0$, 2.0)	3.97 dd ($J = 11.0$, 2.0)	4.01 dd ($J = 11.4$, 2.0)
17e	4.11 dd ($J = 11.0$, 2.0)	4.06 dd ($J = 11.0$, 2.0)	4.15 dd ($J = 11.4$, 2.0)
18a	4.95 dd ($J = 17.8$, 1.1)	4.97 d ($J = 17.8$)	5.21 d ($J = 17.8$)
18e	5.09 dd ($J = 11.0$, 1.1)	5.13 d ($J = 11.0$)	5.50 d ($J = 11.1$)
19	6.26 dd ($J = 17.8$, 11.0)	6.29 dd ($J = 17.8$, 11.0)	6.06 dd ($J = 17.8$, 11.0)
21a	2.78 d ($J = 10.4$)	3.01 d ($J = 11.1$)	
21b	2.32 d ($J = 10.4$)	2.69 d ($J = 11.1$)	
N-CH_3	2.25 s		2.79 s
1-NH	8.71 s	7.70 br s	7.59 br s
4-NH		2.31 br s	

Note: [a] Measured in $CDCl_3$ at 300 MHz (J in Hz).

signal at δ 2.31 indicated the existence of the NH group. Due to the effect of 4-*N*-demethylation, the chemical shifts of H-5 and H-21 shifted downfield, while H-6 and H-16 shifted upfield. ^{13}C NMR spectral data (Table 2) showed 19 carbon signals, one less than gelsemine. The absence of the signal at δ 40.4 (N-CH_3 group) was consistent with the above conclusion. These evidences proved 4-*N*-demethylation. Compared with the reference [13], the structure of M1 was elucidated as 4-*N*-demethylgelsemine.

Metabolite **2** (M2) was obtained as a white amorphous powder (MeOH), mp 222–224°C, UV (MeOH) data (see Figure 1). M2 showed an HPLC retention time of 16.4 min, and ESI-MS values at m/z 337 (50) $[M + H]^+$, 695 (100) $[2M + Na]^+$, 359 (80) $[M + Na]^+$, 275 (20), 229 (5) indicated 14 units more than gelsemine. ^{1}H NMR spectral data (Table 1) showed that the signals at δ 2.78 (H-21a) and 2.32 (H-21b) disappeared

Table 2. ^{13}C NMR spectral data[a] of gelsemine, M1, and M2.

Position	Gelsemine	M1	M2
2	179.2	178.6	177.4
3	69.2	69.5	69.0
5	71.8	54.6	66.2
6	50.6	52.7	53.8
7	54.0	54.0	53.2
8	131.7	131.9	130.4
9	128.0	128.3	127.9
10	121.4	121.9	122.1
11	127.7	127.9	128.5
12	108.0	109.0	109.5
13	140.5	140.2	140.2
14	22.6	22.8	23.1
15	35.5	35.8	31.7
16	37.9	43.8	42.6
17	61.3	61.2	60.7
18	111.8	112.5	117.4
19	138.5	138.5	133.2
20	53.8	57.5	60.5
21	65.9	65.7	176.9
CH_3	40.4		27.9

Note: [a] Measured in $CDCl_3$ at 75 MHz.

compared with the parent drug, together with the presence of the carbonyl at δ 177.4 and the absence of the carbon at δ 66.2, suggesting that C-21 was oxidized. By comparison of physical and spectral data with those in the literature, M2 was identified as 21-oxogelsemine [14].

2.3 Bioactivity evaluation of the metabolites in vitro

The antitumor bioactivities of the two metabolites, M1 and M2, were compared with their parent drug gelsemine using the MTT method. M1 and M2 were shown to induce HepG2 and HeLa cell death in a time- and concentration-dependent manner (Figure 2). M1 and M2 from 40 to 160 μmol/l exhibited potent inhibitory effects on HepG2 cells and HeLa cell growth. However, gelsemine in the same concentration range exerted no effects on both cells (data not shown). After incubating with 160 μmol/l M1 for 24 h, the inhibitory ratio of M1 reached 28.3% in HepG2 cells and 24.0% in HeLa cells, respectively. As for M2, 160 μmol/l treatment for 24 h caused 67.0 and 48.1% cell growth inhibition in HepG2 and HeLa cells, respectively. The IC_{50} values at 24 h were 340.3 μmol/l in M1-treated HepG2 cells, 338.9 μmol/l in M1-treated HeLa cells, 107.1 μmol/l in M2-treated HepG2 cells, and 169.8 μmol/l in M2-treated HeLa cells, respectively. Therefore, M2 showed more potent cytotoxicity than M1, whereas their parent drug, gelsemine, exerted no antitumor effect until the concentration of 600 μmol/l, and the IC_{50} value was not calculated.

To characterize the M1- and M2-induced HepG2 and HeLa cell growth inhibition, we observed the morphologic changes in the cells. When tumor cells were

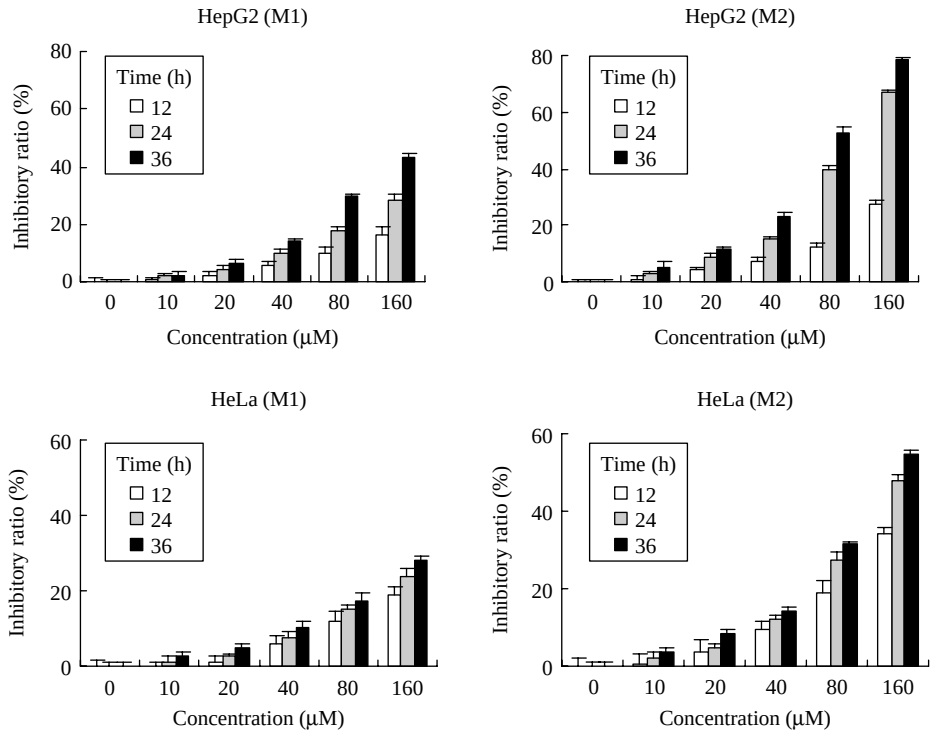

Figure 2. Cytotoxic effects of M1 and M2 on HepG2 and HeLa cells. HepG2 and HeLa cells were treated with various doses of M1 and M2 for 12, 24, and 36 h. $n = 3$, mean ± SD.

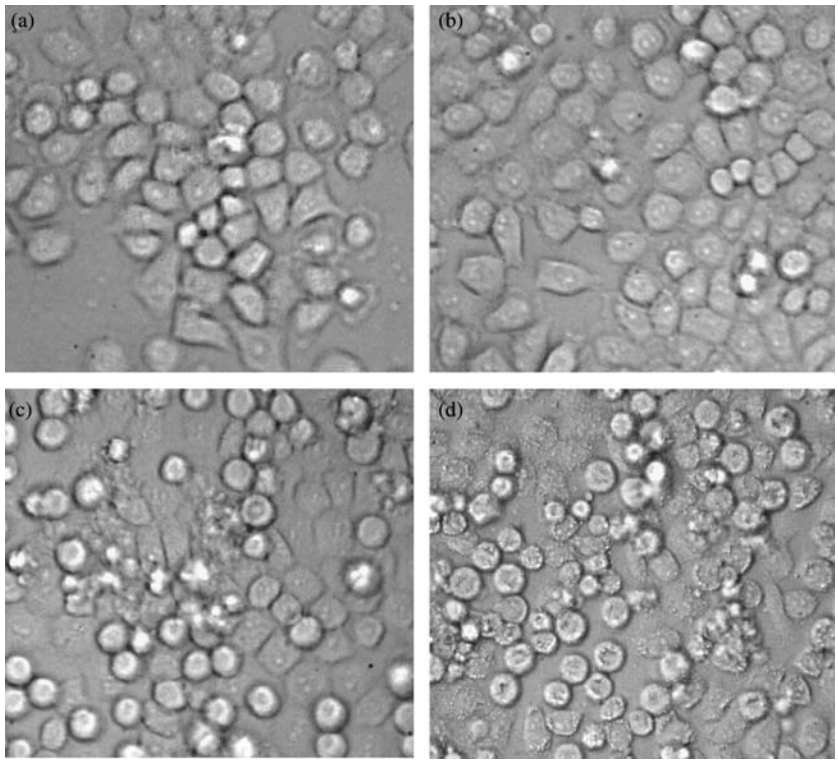

Figure 3. Morphologic changes in gelsemine-, M1-, and M2-treated HepG2 cells: (a) medium control; (b–d) the cells were incubated with gelsemine, M1, and M2 for 24 h at 160 μmol/l, respectively.

treated with M1 or M2 at 160 μmol/l for 24 h, morphologic changes were observed compared with the control group and the gelsemine group under phase contrast microscopy (Figures 3 and 4). By 24 h, the morphologic changes including membrane blebbing and nuclear condensation were observed in the M1- and M2-treated HepG2 and HeLa cells. Moreover, the cells treated with M2 were shown to exhibit more morphologic changes than M1-incubated cells. The cells in the control group and gelsemine-treated group did not show any typical cytotoxic features.

2.4 Metabolism pathway of gelsemine

Liver microsomes contained an enzyme system, cytochrome P450 (CYP450), which controlled the main metabolism of drugs [15]. In our research, phenobarbital-induced rat microsomes, incubated with an NADPH-generating system *in vitro,* were used for the metabolism of gelsemine at a high rate. In conclusion, the present study gave clear evidences of two main metabolic pathways, *N*-demethylation and oxidation (Figure 5), involving the CYP450 enzyme. In the *N*-demethylation metabolic pathway, gelsemine lost a methyl group and was metabolized to M1 (4-*N*-demethylgelsemine) by demethylase, and in the oxidation metabolic pathway, gelsemine was metabolized to M2 (21-oxogelsemine) by liver microsomal oxidase [16]. According to this result, it was proposed that gelsemine was metabolized by *N*-demethylation and oxidation *in vivo,*

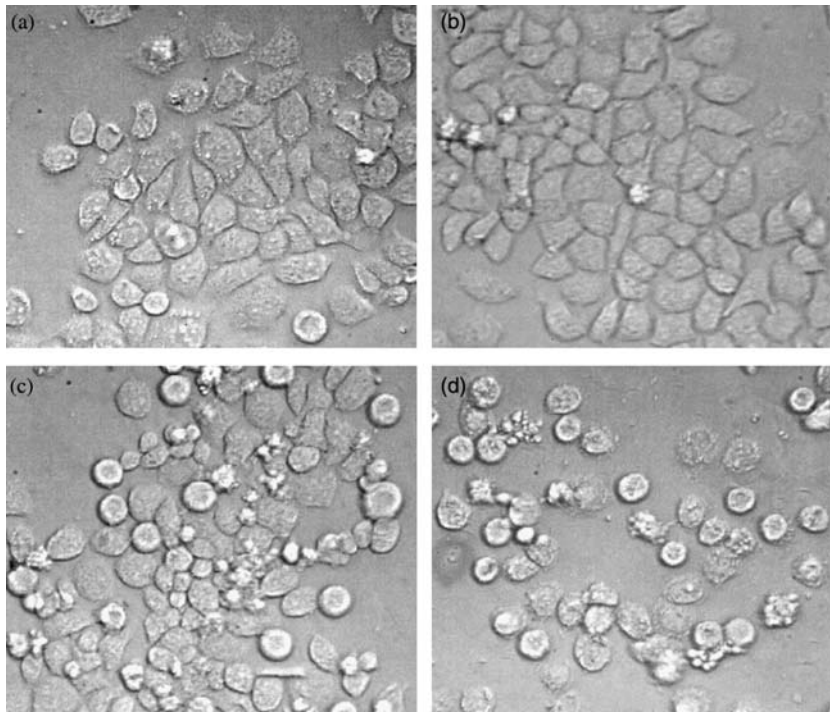

Figure 4. Morphologic changes in gelsemine-, M1-, and M2-treated HeLa cells: (a) medium control; (b–d) the cells were incubated with gelsemine, M1, and M2 for 24 h at 160 μmol/l, respectively.

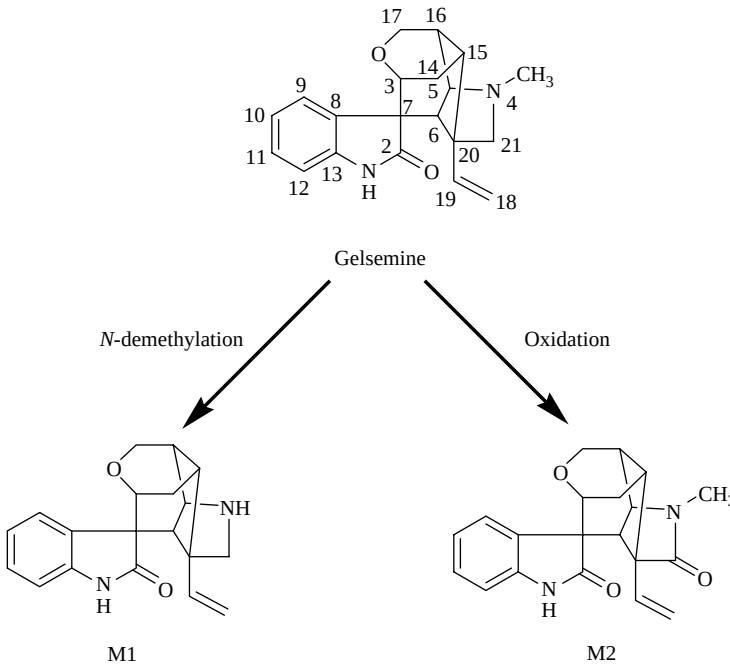

Figure 5. Main metabolism pathway of gelsemine in rat liver microsomes.

which mainly generated phase I metabolites. Furthermore, we will continue to focus on the CYP450 subtype enzyme, which generates the *N*-demethylation and oxidation accurately.

3. Experimental

3.1 General experimental procedures

Melting points were determined on a PHMK05 micromelting point apparatus and are uncorrected. HPLC analysis was carried out on a Waters 2996 PDA detector and a 600 pump using a Kromasil C18 column (4.6 × 200 mm, 5 μm) at room temperature. The flow rate was 0.4 ml/min. Detection was carried out at 254 nm with peak scanning from 200 to 400 nm. HPLC was performed with a JASCO PU-2087 Plus pump and a UV-2075 Plus detector using a HiQ Sil C18 column (10 × 250 mm, 5 μm). The flow rate was 1.0 ml/min. ESI-MS spectra were recorded on an Agilent 1100 spectrometer. NMR spectra were recorded on a Bruker-ARX-300 spectrometer.

3.2 Plant material

Gelsemine was separated from *G. elegans* Benth. and identified by NMR (Tables 1 and 2) and MS spectral data were compared with reference [17]. The purity was 99.0% detected by HPLC. NADP Na_2, NADH Na_2, glucose-6-phosphate Na_2, and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co., Shanghai, China. $MgCl_2$ and KCl were purchased from Sinopharm Chemical Reagent Co. Ltd, Shanghai, China. All other reagents were of HPLC grade. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical, St Louis, MO, USA.

3.3 Animals

Male Sprague–Dawley rats (230–250 g) were obtained from Lab Animal Center of Institute of Shenyang Pharmaceutical University. The animals were housed at 20–23°C with free access to tap water and a normal standard diet. Rats were given phenobarbital at a dose of 60 mg/kg per day for 4 days. After the last dose, rats were fasted but had free access to water for 24 h and used for preparation of liver microsomes.

3.4 Preparation of liver microsomes

Male SD rats were killed under ether anesthesia. After perfusion with ice-cold (4°C) 0.1 M PBS (pH 7.4) to remove residual blood, the livers were immediately excised and homogenized in a Fluko homogenizer with 2 g liver tissue in 10 ml ice-cold PBS. The homogenates were centrifuged at 9000*g* for 20 min, and the resulting supernatants were centrifuged at 10,000*g* for 60 min at 4°C. The supernatant part was used as the simplified liver microsomes [18,19].

3.5 Incubation conditions

The flesh simplified liver microsomes (about 40 ml) were vibrated at a constant temperature water bath of 37°C for 5 min in a conical flask. Gelsemine (60 mg) was dissolved in 100 μl methanol and added dropwise into liver microsomal solution with vibration for 10 min. Then, reactions were initiated by the addition of a pre-warmed NADPH-generating system [20] (0.8 mM NADP Na_2, 8 mM glucose-6-phosphate Na_2, 1 unit/ml glucose-6-phosphate dehydrogenase, and 6 mM $MgCl_2$), and incubation was continued for up to 3 h. After completion of the reaction, 40 ml dichloromethane was used to terminate the reaction and to extract the metabolites.

3.6 Extraction and isolation

The crude extracts were first separated by a silica gel column with dichloromethane–methanol gradient elution (100:0 → 1:1) to

obtain four fractions. Fraction 1 (CH_2Cl_2:MeOH, 99:1) was separated by PHPLC to give metabolite 1 (M1, 10.0 mg). Fraction 3 (CH_2Cl_2:MeOH, 95:5) was separated by PHPLC to afford metabolite 2 (M2, 8.0 mg).

3.7 Cell culture

HepG2, human hepatocellular carcinoma cell line (ATCC, #HB-8065, Manassas, VA, USA), and HeLa, human cervical carcinoma cell line (ATCC, #CCL-2, Manassas, VA, USA), were obtained from American Type Culture Collection. Cells were cultured in RPMI-1640 medium supplemented with 10% heat inactivated (56°C, 30 min) fetal calf serum (Beijing Yuanheng Shengma Research Institution of Biotechnology, Beijing, China), 2 mM L-glutamine (Gibco, Grand Island, NY, USA), 100 kU/l penicillin, and 100 mg/l streptomycin (Gibco) at 37°C in a 5% CO_2 humidified atmosphere. Cells in the exponential phase of growth were used in the experiments.

3.8 Cell growth assay

HepG2 and HeLa cells were dispensed in 96-well flat-bottomed microtiter plates (NUNC, Roskilde, Denmark) at a density of 1×10^4 cells/well. After 12 h incubation, the cells were treated with gelsemine, M1, and M2 at 80 μmol/l for 24 h. Four hours before the end of incubation, 20 μl MTT solution (5.0 mg/l) was added to each well. The resulting crystals were dissolved in DMSO. Results were measured by the MTT assay as described previously [21] with a plate reader (Bio-Rad, Hercules, CA, USA).

The percentage of cell growth inhibition was calculated as follows:

Inhibitory ratio (%) = $(A_{490,\text{control}} - A_{490,\text{sample}})/(A_{490,\text{control}} - A_{490,\text{blank}}) \times 100$.

3.9 Observation of morphologic changes

HepG2 and HeLa cells in RPMI-1640 containing 10% FBS were seeded into 25 ml culture bottles and incubated overnight. The cells were incubated with gelsemine, M1, or M2 at 160 μmol/l for 24 h, and the cellular morphology was observed using phase contrast microscopy (Leica, Wetzlar, Germany).

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