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Triterpenes and Saponins from Leaves of *Camellia nitidissima*, and Cytotoxic Activities Against Bel-7402 and SMMC-7721 Human Liver Cancer Cells

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Abstract: *Camellia nitidissima* is commonly used for making tea to prevent cancer in China, but its phytochemicals and bioactivity was insufficiently reported. In this work, the total content of saponins from leaves of *C. nitidissima* was investigated, and the high level of total saponins varied obviously with the augment of leafage. There are 12 triterpenes and saponins, i.e., β-daucosterol (**1**), α-spinasterol-β-D-glucoside (**2**), lupeol (**3**), 17,3β-acetoxy-20-lupanol (**4**), 3β,6α,13β-trihydroxyolean-7-one (**5**), oleanolic acid (**6**), oleanolic acid 3-acetate (**7**), ginsenoside Rg_1 (**8**), ginsenoside F_5 (**9**), ginsenoside F_1 (**10**), ginsenoside Rd (**11**), and (3β,6α,12β)-3,6,12-trihydroxydammar-24-en-20-yl-2-O-β-D-glucopyranosyl-(2→1)-O-β-D-glucopyranosyl-(2→1)-O-α-L-rhamnopyranoside (**12**) from *C. nitidissima* leaves were isolated and identified. Specially, five dammarane tetracyclic triterpenoids derived from 20(*S*)-protopanaxatriols (compounds **8**-**12**) obtained from *C. nitidissima* were the first reported, and compound **12** is a new dammarane triterpenoid. Based on scratch assay and cell counting kit-8 (CCK-8) assay, compound **12** and the extracts of *C. nitidissima* leaves showed potential cytotoxic activities against Bel-7402 and SMMC-7721 human liver cancer cells *in vitro*.

Keywords: *Camellia nitidissima*; triterpenes and saponins; cytotoxic activities; liver cancer.

1. Introduction

Yellow *Camellia*, is also known as Golden *Camellia*, and belongs to the *Theaceae* family that comprise approximately 42 species and 5 variants. *Camellia nitidissima* is one of the most well-known species of yellow *Camellia* with golden flowers, and its leaves and flowers have been used to make tea, as well as folk medicines to treat hypertension, sore throat and prevent cancer, and they have been

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recognized as new food sources by the State Food & Drug Administration of China in 2010. Though *C. nitidissima* is rare and endemic to a narrow region of Guangxi province in Southern China and Northern Vietnam, it has been widely cultivated due to high economic value and potential as tea and functional food sources since 2010. However, there is insufficient information about its biological activities and phytochemicals.

During these years, interesting biological activities of the yellow *Camellia* have been reported, including anticancer activity [1-4], antioxidant activity [5,6], and inhibiting the formation of advanced glycation end-products[7,8], which may relate to their chemical components of yellow *Camellia*, such as flavonoids, triterpenoids and polyphenols *etc*. The chemical constituents from *C. nitidissima* have been partially analyzed and identified so far [9-14], but the information about its compositions and biological activities is still inadequate. In this study, we measured the total content of saponins in *C. nitidissima* leaves with different leafage, and obtained twelve triterpenes and saponins from *C. nitidissima*. It is interesting that five dammarane tetracyclic triterpenoids derived from 20(*S*)-protopanaxatriols were isolated from *C. nitidissima* for the first time, and a new dammarane triterpenoid (compound **12)** has not been reported before. In addition, we evaluated the cytotoxic activitie of the extracts of *C. nitidissima* and the new compound **12** against Bel-7402 and SMMC-7721 human liver cancer cells.

2. Materials and Methods

2.1. Plant Material

Leaves of *Camellia nitidissima* with different leafage were picked from a group of trees and pooled together in 2016 from Fangcheng Yellow *Camellia* National Natural Reserve in Guangxi Province, China. This plant was identified by Professor Mouxiang Lai from Guangxi University of Chinese medicine, and a herbarium specimen of *C. nitidissima* (JHC2016010801) was deposited at the Guangxi Institute of Botany, Chinese Academy of Sciences. Human liver tumor cell lines of Bel-7402 and SMMC-7721 were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

2.2. General

^{1}H-NMR and ^{13}C-NMR spectra data were recorded on a Bruker AVANCE III HD 600 MHz spectrometer with tetramethylsilane (TMS) as the internal standard. A Gilson GX-281 preparative HPLC equipped with a UV/Vis-156 detector was employed for the separation and purification of chemical constituents of *C. nitidissima*. The other instruments used for cytotoxic activity assay included a Thermo Forma CO_2 incubator, a Nikon Eclipse Ti inverted microscope, a Dynex Spectra MR microplate reader and a clean bench. Cell Counting Kit-8 (CCK-8) was purchased from Nan Jing Tong Ren Chemical Research Institute (Nanjing, Jiangsu Province, China). Except for chromatographically pure acetonitrile, all the chemicals used were analytical grade.

2.3. Extraction and Isolation

The fresh leaves of *C. nitidissima* (one-year-old, 20 kg) were air-dried in the shade for approximately two weeks, and then grounded into powder. These plant materials were extracted three times with 95% ethanol (3×20 L, 2 h each time) under reflux. The combined ethanol extracts were evaporated under reduced pressure to yield a dark green residue, and then suspended in water (1 L) to remove chlorophyll by using petroleum ether extraction (3×1 L), following extraction with n-butyl alcohol twice (2×1 L) to obtain n-butyl alcohol extract. An adsorption column packed with Diaion HP-20 macroporous resin was performed to isolate the n-butyl alcohol extracts, and traced by 0.5% anisaldehyde-sulphuric acid chromogenic agent to obtain fractions by eluting with 60% and 95% ethanol, respectively. Then five fractions (**A**-**E**) were collected. 1.58 g of Fraction **E** was taken for further separation, after purification on repeated column chromatography via silica gel, afforded seven triterpenes (62 mg of **1**, 23 mg of **2**, 18 mg of **3,** 15 mg of **4**, 11 mg of **5**, 44 mg of **6** and 32 mg of **7**).

Fraction **C** (833 mg) was subjected to preparative HPLC on a Phenomenex C_{18} column (250×21.20 mm, 5 μm) at wavelength of 203 nm , using a mixture of acetonitrile (A) and water (B) as mobile phase in a gradient system (25%A at 0-5 min, 40%A at 5-35 min, 50%A at 35-60 min), and successively obtained a mixture and three saponins (16 mg of **9**, 28 mg of **10**, 25 mg of **11**), and the mixture was separated by repeated preparative HPLC to afford 32 mg of **8** and 27 mg of **12**. There are seven triterpenes and five saponins were isolated from the leaves of *C. nitidissima*.

2.4. Determination of Total Saponins in Leaves of C. nitidissima

The total content of saponins in leaves of *C. nitidissima* was measured using a coloration method proposed by Baccou [15]. Briefly, 5 g of plant materials were reflux extracted for 6 h by using a Soxhlet extractor with 150 mL ethyl acetate, then the solution was concentrated to 20 mL. 2 mL of the concentrated solution was reacted for 20 min using 1 mL of 0.5 % anisaldehyde solution in ethyl acetate and 1 mL of concentrated sulphuric acid at 60℃ in the test-tube, and then cooled for 10 min to room temperature in a water-bath. The absorbance of this reaction solution was measured at 430 nm. The calibration curves were established by using oleanolic acid as a standard.

2.5. Cell Culture and Scratch Assay

Bel-7402 and SMMC-7721 cells were maintained in high glucose DMEM (Gibco, USA) that supplemented with 10% FBS (Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. All cells were incubated at 37℃ in a humidified atmosphere containing 5% CO_2. The spreading and migration of these cells were assessed *in vitro* using a scratch wound assay [16]. The cells were seeded at 2.5×10^4 cells /well in 96-well plates in 10% FBS-supplemented DMEM and incubated for 24 h to nearly confluent cell monolayers. Then, a linear wound was created in the monolayer by drawing with a sterile 100 μL plastic pipette tip. The wounded monolayers were washed twice to remove cellular debris using phosphate buffer saline (PBS). 1% FBS-supplemented DMEM containing the samples (0-200 μg/mL) was added and incubated for 0, 24 h and 48 h at the conditions described above. Then, the cells were photographed for migration assessment.

2.6. Cell Proliferation and Cytotoxicity Assay

Cell viability on Bel-7402 and SMMC-7721 cells were measured using CCK-8 assay. The cells were dispensed in 96-well plates at a concentration of 8×10^3 cells/well. After overnight incubation, they were treated with various concentrations of samples and incubated for 24 h, 48 h at 37 °C. Two hours before the end of incubation, 20 μL of CCK-8 was added to each well. The cell inhibition rate was calculated according to the absorbance value at 590 nm measured using a microplate reader. Each experiment was performed in triplicate.

2.7. Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Statistical evaluation was analyzed by one-way ANOVA using SPSS software (version 17.0, SPSS Inc., Chicago, USA). The value of $p<0.05$ between the treated groups and the control was considered to be statistically significant.

3. Results and Discussion

The previous studies demonstrated that there are multiple chemical constituents in *C. nitidissima*, including polyphenols [5], flavonoids [9,17], terpenes [10], saponins [11,18] and *etc*. Several investigations have shown that *C. nitidissima* leaves are rich in polyphenols [3,5] and flavonoids [14,19-21]. However, there is no literature to report the total content of saponins so far. To determine the total saponins content, a 0.5% anisaldehyde-sulphuric acid solution was used as a chromogenic agent, and oleanolic acid as a standard. In this work, the total saponins content exhibited distinct diversity that varied with the augment of leafage. The new *C. nitidissima* leaves possess the highest

total saponins content (6.21±0. 15%), and the content is 1.98±0.12% and 2.15±0.18% for one and two-year-old leaves, respectively. These results indicate that *C. nitidissima* leaves are rich in saponins.

3.1. Chemistry

There are twelve triterpenes and saponins were isolated from the leaves of *C. nitidissima* (Figure **1**). NMR, IR, and MS techniques were used for the structure elucidation. By comparing the spectral data reported in works of literature, they were identified to be β-daucosterol (**1**)[22], α-spinasterol β-D-glucoside (**2**)[23], lupeol (**3**)[24,25], 3β-acetoxy-20-lupanol (**4**)[26], 3β,6α,13β-trihydroxyolean-7-one (**5**)[27], oleanolic acid (**6**)[28], oleanolic acid 3-acetate (**7**)[29], ginsenoside Rg_1(**8**)[30], ginsenoside F_5 (**9**)[31], ginsenoside F_1 (**10**)[32], ginsenoside R_d (**11**)[33] and (3β,6α,12β)-3,6,12-trihydroxydammar-24-en-20-yl-2-O-β-D-glucopyranosyl-(2→1)-O-β-D-glucopyranosyl-(2→1)-O-α-L-Rhamnopyranoside (**12**). Especially, compound **12** is a new dammarane triterpenoid which has not been reported. It is worth noting that compounds **8-12** are five dammarane tetracyclic triterpenoids derived from 20(*S*)-protopanaxatriols, and they are firstly isolated from *C. nitidissima* plant.

Compound **12**, obtained as white powder, the data of ^{1}H NMR (600 MHz, Pyridine-d_5) and ^{13}C NMR (150 MHz, Pyridine-d_5) listed in Table 1, negative HRESIMS: *m/z* 945.5423 $[M-H]^+$ (calculated for $C_{48}H_{82}O_{18}$, 945.54284, Figure **1S**). The ^{13}C NMR and DEPT spectrum of compound **12** displayed forty-eight signals corresponding to nine methyls, ten methylenes, twenty-three methines and six quaternary carbon atoms. Acid hydrolysis produced two kinds of sugars, which were D-glucose and L-rhamnose identified by TLC analysis, and there are three anomeric proton at δ_H 5.19 ppm (1H, d, *J*=7.9), 5.27 ppm (1H, d, *J*=7.6), 6.52 ppm (1H, s), and δc 18.8 ppm (CH_3) of characteristic signal L-rhamnose. Then eighteen signals of ^{13}C NMR chemical shift were assigned to the sugar moieties, and thirty carbon signals were assigned to the aglycone part. Comparing the NMR data with literature[34,35], ginsenoside Rg_1(**8**) and ginsenoside F_5(**9**), the downfield ^{13}C NMR chemical shift at δ_C 83.3 ppm (C-20) suggested that compound **12** was a 20(*S*)-protopanaxatriol derivative with glycosidic linkages at C-20. The anomeric protons signals at δ_H 5.19 ppm (1H, d, *J*=7.9, Glc-1'), 5.27 ppm (1H, d, *J*=7.6, Glc-1"), 6.52 ppm (1H, s, Rha-1'") displayed correlations with anomeric carbon signals at δ_C 98.3 ppm, 101.9 ppm and δ_C 101.9 ppm in HSQC, and showed correlations with δ_C 83.3 ppm (C-20), 75.2 ppm (C-Glc-2'), 78.6 ppm (C-Glc-2") in HMBC, respectively, indicating that the sequences of sugar residues were the Glc-1' link to C-20, Glc-1" link to C-Glc-2', and Rha-1'" link to C-Glc-2". The ^{1}H and ^{13}C NMR data of the monosaccharide residues were assigned starting from the readily identifiable anomeric protons by means of the HSQC, ^{1}H-^{1}H COSY, and HMBC spectra. The β-anomeric configurations of the D-glucoses were determined by $J_{H1',H2'}$ and $J_{H1",H2"}$ coupling constants at 7-8 Hz. The ^{1}H-^{1}H COSY and selected HMBC (H→C) correlations are shown in Figure **2**. To further confirm the structure of compound **12**, the possible fragmentation pathway is presented in Figure **2S**. Therefore, compound **12** was determined to be 3β,6α,12β)-3,6,12-trihydroxydammar-24-en-20-yl-2-O-β-D-glucopyranosyl-(2→1)-O-β-D-glucopyranosyl-(2→1)-O-α-L-rhamnopyranoside.

3.2. Cytotoxic Activity

The invasion and metastasis of cancer are one of the main reasons that affect patients' health, even survival time. In the present study, the effects of the extracts and compound **12** from *C. nitidissima* leaves on Bel-7402 and SMMC-7721 cells migration were assayed using scratch wound assay at a concentration of 50 μg/mL. As shown in Figure **3**, compound **12** can effectively inhibit Bel-7402 and SMMC-7721 cells migration when compared to the control. Inhibition of the extracts on Bel-7402 cells migration was also observed, but less influence on SMMC-7721 cells. Furthermore, the cytotoxic effects of the extract and compound **12** were evaluated against Bel-7402 and SMMC-7721 cells. After treatment of 24 h and 48 h with 12.5-200 μg/mL of samples, cell viabilities were measured by CCK-8 assay. As shown in Figure **4**, the extract and compound **12** were found to exert proliferation inhibitory effects on two tumor cell lines in a dose-dependent manner. Compound **12** can reduce cell viability in Bel-7402 and SMMC-7721 cells, and the inhibitory rate of cell viability at 200 μg/mL reached about 30% in Bel-7402 cells, and about 21% in SMMC-7721 cells when treated 48 h. The

extracts of *C. nitidissima* leaves also exhibited cytotoxic effect against Bel-7402 cells with an inhibitory rate of about 26% at 200 μg/mL, but showed less inhibition effect on SMMC-7721 cells.

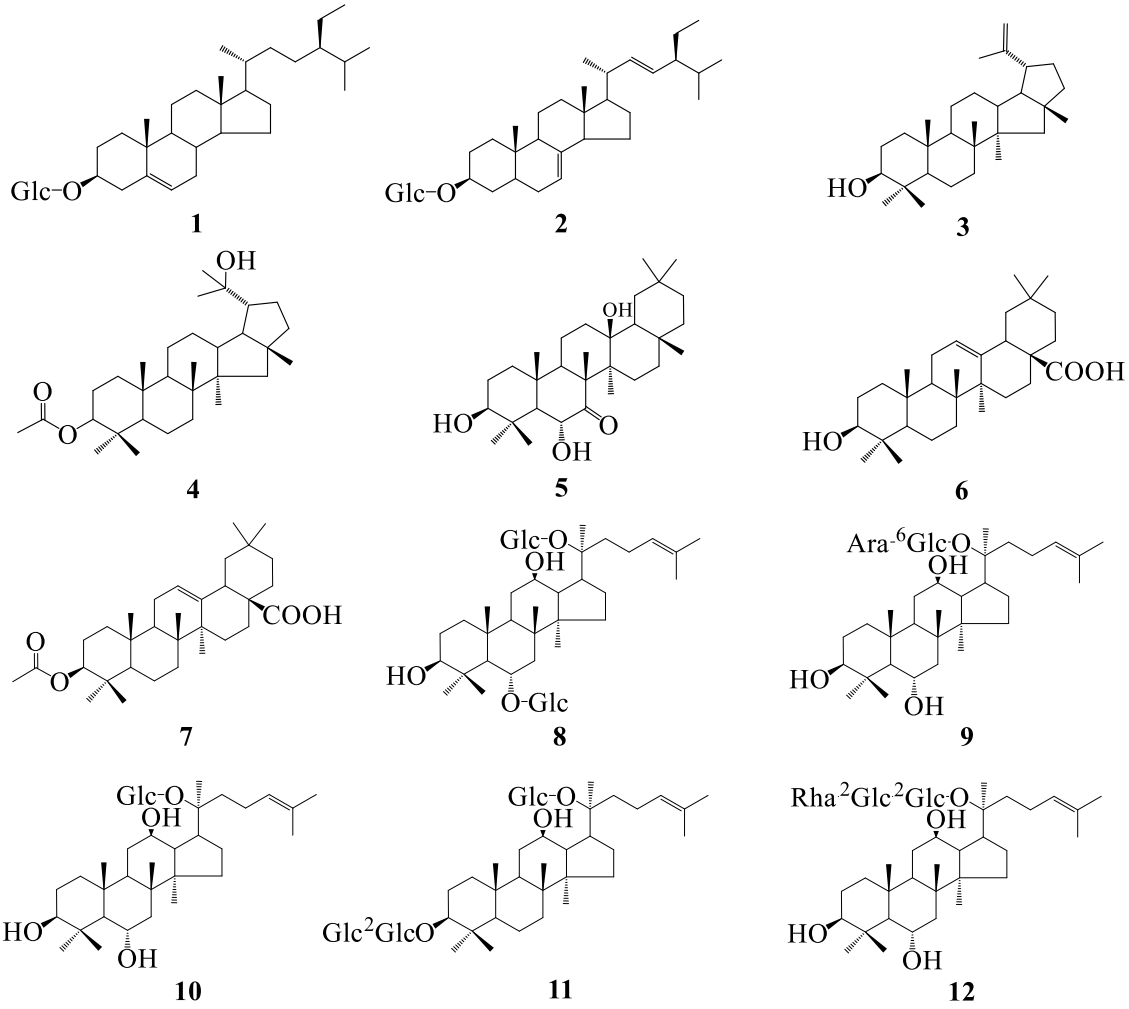


Figure 1. Chemical structures of compounds **1-12** isolated from *C. nitidissima*.

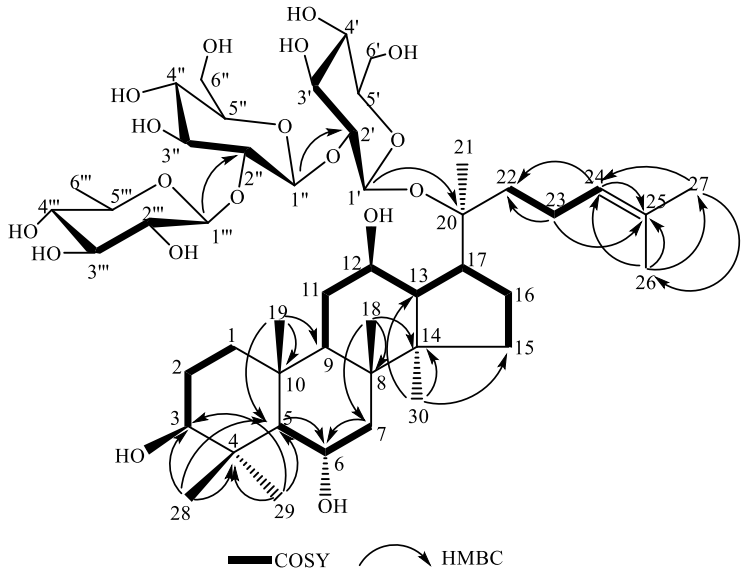


Figure 2. Key ^{1}H-^{1}H COSY and HMBC correlations of compound **12**

Table 1. ^{1}H and ^{13}C NMR data for compound **12** (at 600 MHz in pyridine-d_5, δ in ppm, J in Hz)

Triterpenes and saponins from leaves of *Camellia nitidissima*

Aglycon Moiety Position	**δ_H mult., (*J* Hz)**	**δc mult.**	**Sugar Moiety Position**	**δ_H mult. (*J* Hz)**	**δc mult.**
1	1.68 m, 0.92 m	39.4 CH_2	Glc-1'	5.19 *d* (7.9)	98.3 CH
2	1.88 m, 1.69 m	27.8 CH_2	Glc-2'	4.00 [b]	75.2 CH
3	3.49 m-*like*	78.4 CH	Glc-3'	4.20 [b]	79.5 CH
4		40.0 C	Glc-4'	4.23 m-*like*	72.6 CH
5	1.39 *d* (9.7)	60.8 CH	Glc-5'	3.99 m	78.4 CH
6	4.70 [b]	74.6 CH	Glc-6'	4.51 [b], 4.36 m	63.1 CH_2
7	2.26 m, 1.97 m	46.0 CH_2	Glc-1"	5.27 *d* (7.6)	101.9 CH
8		41.2 C	Glc-2"	4.38 [b]	78.6 CH
9	1.52 m	49.6 CH	Glc-3"	4.38 [b]	79.3 CH
10		39.7 C	Glc-4"	4.18 m	71.6 CH
11	2.08 m, 1.48 m	31.0 CH_2	Glc-5"	4.01 m	78.3 CH
12	4.18 m	70.2 CH	Glc-6"	4.51 [b], 4.36 m	62.9 CH_2
13	1.94 m	49.1 CH	Rha-1"'	6.52 s	101.9 CH
14		51.4 C	Rha-2"'	4.82 brs	72.4 CH
15	2.07 m, 0.86 m	30.7 CH_2	Rha-3"'	4.69 m	72.3 CH
16	1.80 m, 1.26 m	26.7 CH_2	Rha-4"'	4.36 [b]	74.2 CH
17	2.50 [b]	51.7 CH	Rha-5"'	4.97 *dd* (9.6, 6.1)	69.5 CH
18	1.19 s	17.8 CH_3	Rha-6"'	1.79 *d* (6.1)	18.8 CH_3
19	0.97 s	17.5 CH_3	3'-OH	7.48 brs	
20		83.3 C	4'-OH	6.63 s	
21	1.62 s	22.3 CH_3	6'-OH	6.16 s	
22	2.38 m, 1.77 m	36.0 CH_2	3"-OH	7.33 s	
23	2.49 m, 2.23 m	23.2 CH_2	4"-OH	7.27 s	
24	5.27 [b]	126.0 CH	6"-OH	6.06 s	
25		130.9 C	2"'-OH	7.19 [c]	
26	1.61 s	25.8 CH_3	3"'-OH	6.66 s	
27	1.64 s	17.7 CH_3	4"'-OH	7.43 brs	
28	2.12 s	32.2 CH_3			
29	1.37 s	17.2 CH_3			
30	0.95 s	17.3 CH_3			
3-OH	5.76 *d* (5.5)				
6-OH	6.33 s				
12-OH	5.60 s				

[a] The assignments were based on DEPT, HSQC, ^{1}H-^{1}H COSY and HMBC experimental data.
[b] Overlapped with other signals.
[c] Overlapped with the solvent peak of pyridine-d_5.

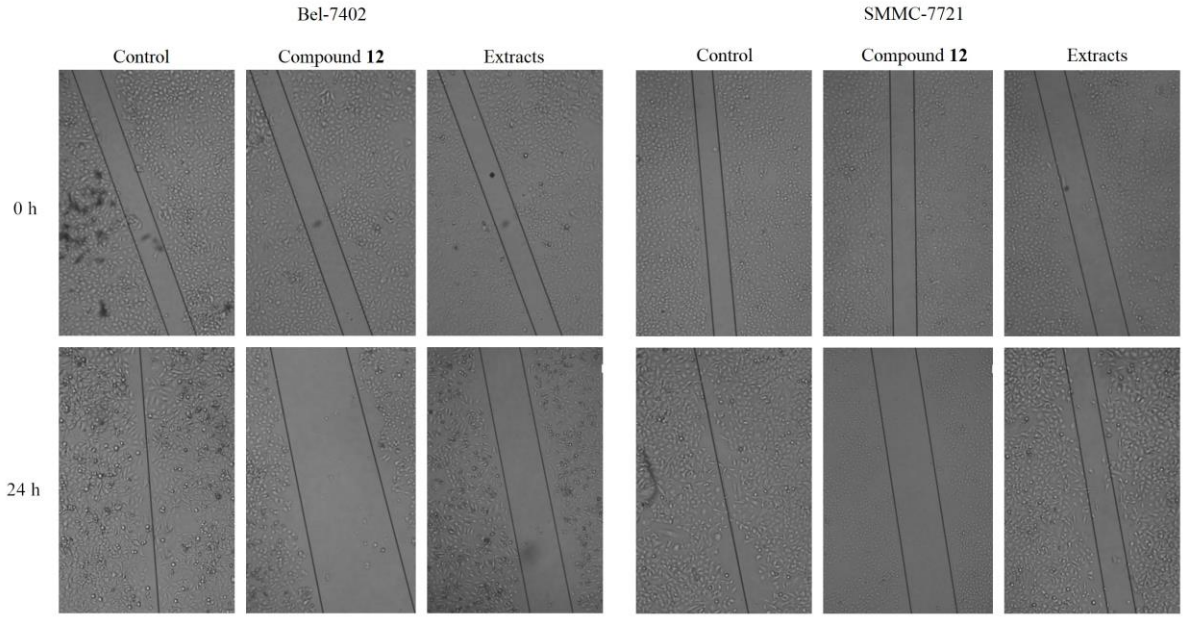
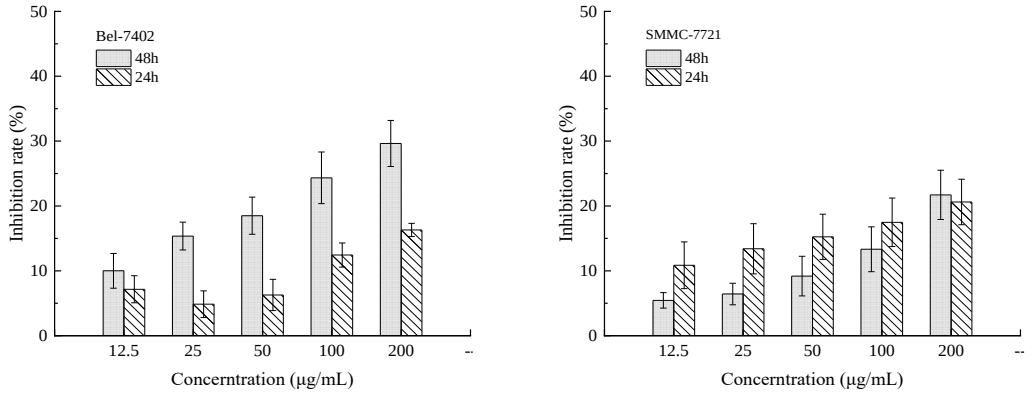
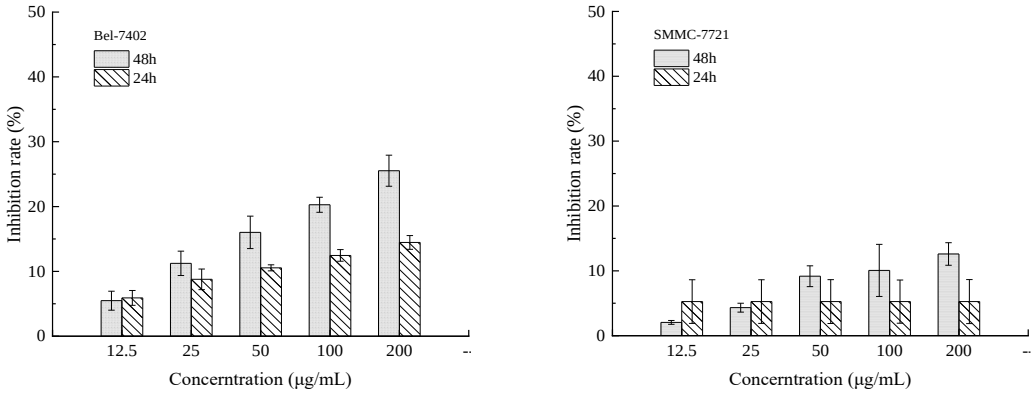


Figure 3. Micrographs of Bel-7402 and SMMC-7721 cells migration after treatment with 50 μg/mL extract and compound **12** for 0 h and 24 h. Cells treated without any samples were used as the control.



A



B

Figure 4. Proliferation inhibitory effect of the extracts (A) and compound **12** (B) obtained from *C. nitidissima* leaves on Bel-7402 and SMMC-7721 cells.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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