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NO Inhibitory, Farnesoid X Receptor, and Cytotoxic Activities of Phytochemical Composition Isolated from *Aglaia perviridis*

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Abstract: To search for novel and bioactive agents from the genus *Aglaia*, phytochemical investigation on the leaves and twigs of *A. perviridis* was performed, leading to the isolation of twenty-six compounds. Their structure was determined mainly by the NMR, ESI-MS spectra data, and by comparison with previously described compounds. Compounds **6**–**8**, **11**–**13**, **16, 18–20,** and **22**–**26** were firstly reported from *A. perviridis*. Besides, the NO inhibitory, Farnesoid X receptor, and cytotoxic activities of selected compounds were firstly evaluated. 1*α*H,5*α*H-guaia-6-ene-4*β*,10*β*-diol (**7**), 2'-hydroxy-4,4',6'-trimethoxychalcone (**22**), and 3-hydroxy-5,7,4'-trimethoxyflavone (**23**) showed moderate nitric oxide inhibitory activity with IC_{50} values of 27.44±1.70, 22.29±1.53, and 19.29±3.96 μM, respectively; piscidinol A (**11**) and hispidol B (**12**) firstly showed moderate agonistic activities on Farnesoid X receptor with the EC_{50} values of 6.19±1.08 and 8.61±0.32 μM, respectively; Among these compounds, lochmolin F (**6**) exhibited the strongest cytotoxic effect on SiHa cells with the IC_{50} value of 19.58±0.21 μM. Further flow cytometry and western blot analysis demonstrated that lochmolin F (**6**) could induce cell apoptosis and S cell cycle arrest in the SiHa cells. These results first emphasized the potential of lochmolin F (**6**) as lead compound for developing anti-cancer drugs.

Keywords: *Aglaia perviridis*; phytochemical composition; anti-inflammatory; FXR; cytotoxic activities.

1. Introduction

The genus *Aglaia* is the largest genus of the angiosperm plant family Meliaceae, which comprises more than 120 species that are mainly distributed in the tropical and sub-tropical rainforest areas of

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Southeast Asia and the Pacific region [1], of which seven species and one variant grow in China [2], Some *Aglaia* species have been traditionally used as folk medicines in China for treating fever, cough, diarrhoea, and contused wounds [3]. To date, various amides, triterpenoids (e.g., cycloartanes, dammaranes, and tirucallanes), and flavaglines (e.g., cyclopenta[b]benzofurans, cyclopenta[bc]benzopyrans, and benzo[b]oxepines) have been isolated from this genus [4-9]. In particular, some of the flavagline derivatives showing interesting antiviral [10, 11], antifungal [12], antineoplastic [13], cytotoxic [14, 15], and insecticidal [16, 17] activities have been found. Potential anti-inflammatory activity was also observed for some triterpenes [18, 19]. In continuation of the discovery of novel and bioactive natural products from plants of *Aglaia* species, the chemical constituents of the species *A. perviridis*, a wild shrub indigenous to Yunnan Province of China were investigated. Their structure was determined mainly by the NMR, ESI-MS spectra data, and by comparison with previously described compounds. Selected isolated compounds were firstly evaluated for their Nitric oxide (NO) inhibitory, Farnesoid X receptor (FXR), and cytotoxic activities against four cancer lines (the A549, SiHa, 5-8F, and HepG2 cell lines). In addition, the action mechanism of lochmolin F (**6**) inhibiting SiHa cell proliferation was further studied. Herein, we report the isolation, structural elucidation, and activity evaluation of selected the isolates.

2. Materials and Methods

2.1. Chemicals and Reagents

The NMR spectra were obtained on Bruker Avance DRX500 and Bruker Avance III HD 600 spectrometer (Bruker corporation, Karlsruhe, German), using tetramethylsilane as an internal standard. ESIMS were carried out on a Shimadzu UPLC-IT-TOF mass spectrometer (Shimadzu Corporation, Japan). Semi-preparative HPLC was performed on a Waters 600 pump system with a 2996 photodiode array detector by using a YMC-Pack ODS-A column (300 × 10 mm, S-5 μm). Column chromatography was run over silica gel (200−300 mesh, Qingdao Marine Chemical Factory, Qingdao, China). Sephadex LH-20 gel (40−70 μm, Amersham Pharmacia Biotech AB, Uppsala, Sweden). Spots were detected firstly on TLC (Qingdao Marine Chemical Factory) under 254 nm UV light and then by spraying with 10% H_2SO_4 in ethanol for plate heating. All solvents used were of analytical grade (Shanghai Chemical Reagents Co. Ltd), and all solvents used for HPLC were of chromatographic grade (J & K Scientific Ltd.). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega Corporation (Madison, Wisconsin, USA). Annexin V-FITC apoptosis detection kit was purchased from Yeasen Biotechnology (Shanghai) Co., Ltd. Antibodies against cyclin A2, CDK2, cyclin D1, and GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA) (all used at 1:1000 dilutions). Griess Reagent was purchased from Promega Corporation (Madison, Wisconsin, USA). Hoechst 33342 was purchased from Beyotime Biotechnology (Shanghai).

2.2. Plant Material

The twigs and leaves of *A. perviridis* were collected from Menglun town (101◦25′E, 21◦41′N), Mengla Country, Yunnan Province, People's Republic of China at an altitude of 570 m in May 2016, and they were

identified by Professsor Youkai Xu from Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences (XTBG). A voucher specimen (HITBC-028497) is deposited in the herbarium at XTBG.

2.3. Plant Extraction

The air-dried powdered twigs and leaves (5 kg) of *A. perviridis* were extracted with 95% EtOH (3×30 L, 7 days each time) at room temperature. After evaporation of the solvent under reduced pressure, the residue (365 g) was suspended in H_2O (1 L) and partitioned successively with petroleum ether (3 × 1.0 L) and EtOAc (3 × 1.0 L). The petroleum ether extract (112 g) was subjected to silica gel column chromatography (CC) eluted with $CHCl_3$/MeOH (50:1-1:1, v/v) to afford four fractions (P-1–P-4). Fraction P-1 (20 g) was repeatedly subjected to silica gel CC eluted with $CHCl_3$/Me_2CO (50:1–2:1, v/v), to yield **9** (8 mg) and **10** (15 mg). Fraction P-3 (15 g) was separated by Sephadex LH-20 gel CC eluted with MeOH/H_2O gradient (1:1–4:1, v/v) to afford subfractions P-3a–P-3d. Fraction P-3a (3 g) was purified by semipreparative HPLC (MeCN/H_2O, 70:30, v/v, 3 mL/min) to yield **11** (9 mg), **12** (24 mg), and **13** (60 mg). Fraction P-3b (3 g) was purified by semipreparative HPLC (MeCN/H_2O, 52:48, v/v, 3 mL/min) to yield **6** (4 mg), **7** (6 mg), and **8** (3 mg). Fraction P-3d (4 g) was further purified by semipreparative HPLC (MeCN/H_2O, 60:40, v/v, 3 mL/min) to yield **1** (16 mg), **2** (5 mg), **3** (5 mg), **4** (4 mg), and **5** (5 mg). The ethyl acetate extract (105 g) was chromatographed over a microporous resin, eluted with gradient mixtures of MeOH/H_2O (20:80, 40:60, 60:40, 80:20, and 100:0, v/v) to afford five major fractions (E-1−E-5). Fraction E-3 (60 g) was separated by silica gel CC eluted with $CHCl_3$/MeOH (50:1–1:1, v/v) to yield three major fractions E-3a–E-3c (11.0, 7.2, and 8.0 g, respectively). Fraction E-3a (11.0 g) was first chromatographed on a Sephadex LH-20 gel column eluted with MeOH and then purified with semipreparative HPLC (MeCN:H_2O, 69:31, 3 mL/min) to yield **14** (7 mg), **15** (9 mg), **19** (12 mg), and **20** (4 mg). Fraction E-3c (8.0 g) was separated by silica gel CC eluted with $CHCl_3$/EtOAc (20:1–1:1, v/v), followed by Sephadex LH-20 gel purification eluted with MeOH, to afford **16** (3 mg), **17** (4 mg), and **18** (4 mg). Fraction E-4 (35 g) was subjected to silica gel CC, to yield two subfractions E-4a−E-4b. Fraction E-4a (2.0 g) was purified by semipreparative HPLC (MeCN/H_2O, 60:40, v/v, 3 mL/min) to afford **21** (4 mg), **22** (12 mg), **23** (50 mg), and **24** (4 mg). Fraction E-4b (16.0 g) was purified by semipreparative HPLC (MeCN/H_2O, 55:45, v/v, 3 mL/min) to afford **25** (3 mg), **26** (3 mg), respectively. All of the compounds met the criteria of ≧ 95% purity, as determined by NMR analysis.

2.4. Cell Culture

A549 (lung carcinoma), SiHa (cervical carcinoma), 5-8F (nasopharyngeal carcinoma), HepG2 (hepatocellular carcinoma), and HUCEC (human cervical epithelial cells) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The RAW264.7 macrophages were obtained from Kunming Institute of Zoology, Chinese Academy of Sciences). All cells were cultured in DMEM medium supplemented with 10% FBS, containing 100 units/mL penicillin and 100 μg/mL streptomycin (1% PS) at 37 °C in the presence of 5% CO_2.

2.5. Assay for Inhibition Ability toward LPS-induced NO Production and Cytotoxicity Testing

The RAW264.7 macrophages were seeded in 96-well plates with 1×10^4 cells/well and allowed to adhere for 12 h at 37 °C in a humidified atmosphere containing 5% CO_2. Then, the RAW264.7 macrophages were treated with tested compounds and 1 μg/mL lipopolysaccharide (LPS) for 24 h. NG-monomethyl L-arginine (Sigma) was used as a positive control. An equal volume of DMSO was used as vehicle control. The cell viability was determined by MTS assay before NO production assay, and the NO production was measured by the accumulation of nitrite in the culture supernatants using the Griess Reagent System at OD 550 as previously reported [20].

2.6. Cell Viability Assay

The obtained compounds were tested for their cytotoxic activity against the A549, SiHa, HUCEC, 5-8F, and HepG2 cancer cell lines using the MTS method. Briefly, 100 μL adherent cells were seeded into each well (5×10^3 cells/well) of 96-well cell culture plates and allowed to adhere for 18 h before testing drug addiction. Each tumor cell line was exposed to a test compound at concentrations of 0, 2.5, 5, 10, 20, and 40 μM in DMSO in triplicate for 24 h or 48 h, with cisplatin as the positive control. An equal volume of DMSO was used as vehicle control. After 24 h or 48 h incubation, the medium was removed. Subsequently, 100 μL medium and 20 μL MTS solution was added to each well, which was incubated for another 4 h to give a formazan product. The OD value of each well was measured at 490 nm using a Biorad 680 instrument.

2.7. Cell Cycle Analysis

For analyzing the cell cycle distribution, flow cytometry was performed as described previously [21]. In brief, SiHa cells (4×10^5 cells/well) were plated at 6-well plates and challenged on compounds for 24 h and 48 h, respectively. Next, the cells were collected, washed twice with cold PBS, and fixed in 70% cold ethanol at 4 °C overnight. After being washed with chilled PBS, the cells were incubated with RNase A (0.5 mg/mL) at 37 °C for 1 h and then stained with PI (final concentration of 10 μg/mL) at room temperature in the dark for 15 min. Afterward, the cell cycle analysis was carried out on a flow cytometer (FACS Calibur, BD Biosciences, San Jose, USA).

2.8. Apoptosis Detection

SiHa cells were seeded in 12-well plates at a density of 2×10^5 cells/well and incubated with compounds for 24 h and 48 h, respectively. After treatment, the cells were harvested and stained with the Annexin V-FITC apoptosis detection kit according to the manufacturer's instructions. Then, stained cells were analyzed by a flow cytometer.

2.9. FXR Coactivator Interaction Assay

The FXR coactivator interaction assay was performed in 384-well solid-bottom plates. A mixture of 5 nM purified, GST-tagged, human FXR LBD protein and 5 nM biotinylated coactivator steroid receptor coactivator-1 peptide (CPSSHSSLTERHKILHRLLQEGSPS, Anaspec), 25 μg/mL anti-GST acceptor (PerkinElmer), and streptavidin-conjugated AlphaScreen beads were added into the assay buffer containing 50 mM Tris (pH = 8.0), 50 mM KCl, 0.1% BSA, and 2 mM DTT with test compounds in dose-response. The plates were incubated for 2 h at room temperature and read on an Envision (PerkinElmer) at 570 nm. The model assessing fit is a sigmoidal dose–response (variable slope). Each compound is tested in three independent experiments with the EC_{50} value shown as mean ± SD. Control assays are performed with obeticholic acid [22].

2.10. Statistical Analysis

All data were presented as mean ± standard deviation. Three independent experiments were conducted for *in vitro* study. IC_{50} values (μM) from cell viability assay were calculated from dose-response curve-fitting *via* nonlinear regression analysis on GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). Significant differences between experimental and vehicle groups were evaluated with a one-way analysis of variance followed by Tukey's test. $p < 0.05$ or < 0.01 were considered statistically or highly statistically significant, respectively.

3. Results and Discussion

3.1. Isolation and Identification of Compounds

The 95% ethanol extract of the twigs and leaves of *A. perviridis* was subjected to silica gel column chromatography, Sephadex LH-20, and semi-preparative reversed-phase HPLC to obtain 26 secondary metabolites, pyramidatine (**1**) [23], perviridamide (**2**) [24], 4-hydroxy-pyramidatine (**3**) [24], silvestrol (**4**) [13], episilvestrol (**5**) [13], lochmolin F (**6**) [25], 1*α*H,5*α*H-guaia-6-ene-4*β*,10*β*-diol (**7**) [26], 15-hydroxy-*α*-cadinol (**8**) [27], cabraleahydroxylactone (**9**) [28], cabraleadiol (**10**) [29], piscidinol A (**11**) [30], hispidol B (**12**) [31], heteroclitalactone M (**13**) [32], 2*β*,2*β*-dihydroxy-5*α*-pregn-17(20)-(*Z*)-en-16-one (**14**) [33], lansisterone E (**15**) [34], *β*-sitosterol 3-*O*-*β*-glucoside (**16**) [35], (+) eudesmin (**17**) [36], (–) eudesmin (**18**) [37], forsythenin (**19**) [38], epipinoresinol (**20**) [39], naringenin trimethyl ether (**21**) [40], 2'-hydroxy-4,4',6'-trimethoxychalcone (**22**) [41], 3-hydroxy-5,7,4'-trimethoxyflavone (**23**) [42], 5-hydroxy-7,4'-dimethoxyflavanone (**24**) [43], ethyl asterrate (**25**) [44], matairesinol (**26**) [45]. Their structures were elucidated by NMR, ESI-MS spectra data as well as comparison with those reported in the literature. Compounds **6**–**8**, **11**–**13**, **16, 18–20**, and **22**–**26** were firstly reported from the species.

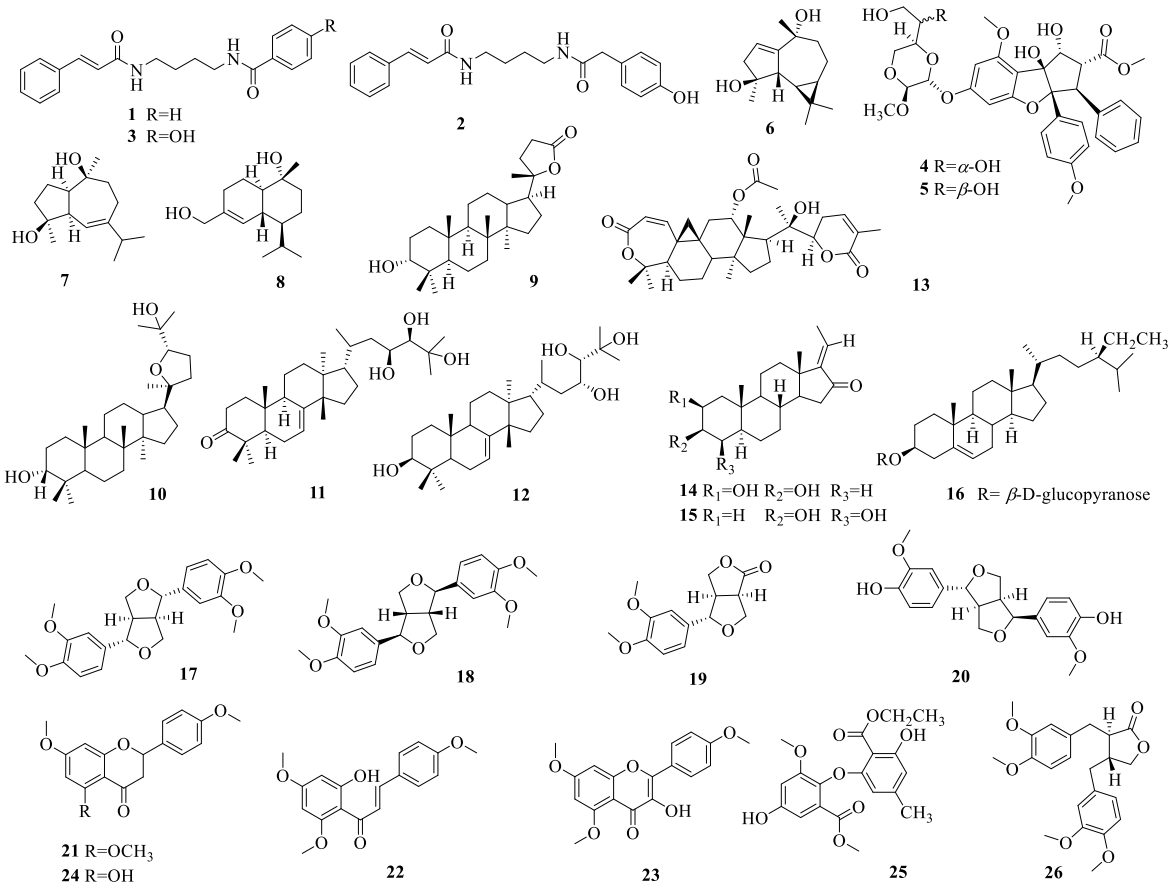

Figure 1. The structure of compounds **1**−**26**

3.2. NO Inhibitory Activity of Compounds

To discover effective NO inhibitors, selected compounds were evaluated for their NO inhibitory activity on LPS-induced RAW264.7 cell lines. Firstly, the cell viability of selected compounds was determined by MTS method [46]. The results indicated that except compounds **1**−**6**, **11**, **12**, and **14**−**16**, other compounds do not effect on RAW264.7 cell viability at the concentration of 50 μM, so they were further evaluated for their NO inhibitory activity in the LPS-induced RAW264.7 cell lines using a Griess Reagent System. Compounds **7**, **22**, and **23** showed moderate NO inhibitory activity with IC_{50} values of 27.44±1.70, 22.29±1.53, and 19.29±3.96 μM, respectively. Compounds **8**, **13**, and **21** exhibited weak NO inhibitory activity with IC_{50} values of 37.96±1.58, 46.70±2.38, and 33.71±2.79 μM, respectively (Table 1). Previous studies have reported that compound **7** exhibited cytotoxicities against the three human cancer cell lines H460, MCF-7, and PC-3 with IC_{50} values of 56.7 ± 1.4 μM, 76.7 ± 1.4 μM, and 69.0 ± 1.9 μM, respectively [26]; compound **8** exhibited inhibitory effects on NO production of BV-2 microglia activated by LPS with IC_{50} values of 19.89 ± 0.83 μM [47], which is consistent with the result in this study; compound **21** showed significant molluscicidal activity with LC_{50} values of 3.9 μg/mL [48]; compound **22** exhibited the strongest antioxidant properties with values of 42.23, 1497.22, and 781.53 mg TE/g against ABTS, CUPRAC, and FRAP assays, respectively [49], and antifungal activity [50], compound **23** were found to be strong DPPH free radical scavengers with IC_{50} values of 119 μM and showed strong inhibition activity towards EBV activation in Raji cells [51]. This study is the first to report the NO inhibitory activity

of these compounds, providing more candidate compounds for the development of anti-inflammatory drugs.

Table 1. Effect of selected compounds on LPS-induced NO production in RAW264.7 cells[a]

Compound	IC_{50}/μM	Compound	IC_{50}/μM
7	27.44±1.70	**19**	N.T.
8	37.96±1.58	**20**	>50
9	>50	**21**	33.71±2.79
10	>50	**22**	22.29±1.53
13	46.70±2.38	**23**	19.29±3.96
17	>50	**24**	>50
18	>50	**25**	>50
NG-monomethyl L-arginine [b]	14.17±1.08	**26**	>50

[a] Data are represented as mean ± SEM (*n = 3*); N.T.: Not tested. [b] Positive controls.

3.3. FXR Activity Evaluations for selected Compounds

FXR plays a key role in bile acid homeostasis, inflammation, fibrosis, and metabolism of lipids and glucose and becomes a promising therapeutic target for nonalcoholic steatohepatitis (NASH) or other FXR-dependent diseases [22]. To discover novel FXR agonists, selected compounds were evaluated for their FXR bioactivity. The reported FXR agonist obeticholic acid was selected as the positive control. As shown in Table 2, the results indicated that compounds **11** and **12** showed moderate agonistic activities on FXR with the EC_{50} value of 6.19±1.08 and 8.61±0.32 μM, respectively. Previous studies have mainly reported the antitumor activity of compounds **11** and **12** against various tested cancer cell lines, such as KB, MCF-7, KB-C2, P-388, RT112, HCT-116, and M231 [52-56]. They were triucallane-class triterpenoids, which structure was different from reported bile acid-derived FXR agonists that bring severe pruritus and an elevated risk of cardiovascular disease for patients [57, 58]. Thus, further structural modifications to compounds **11** and **12** were expected to develop a non-bile acid FXR agonist.

Table 2. The Agonistic Activities on Farnesoid X receptor of selected compounds[a]

Compound	EC_{50} (μM)	Compound	EC_{50} (μM)
1	>20	**13**	N.T.
2−**6**	N.T.	**14**	>20
7	>20	**15**	>20
8	>20	**16**−**24**	N.T.
9	>20	**25**	>20
10	>20	**26**	N.T
11	6.19±1.08	obeticholic acid [b]	0.32±0.01
12	8.61±0.32		

[a] Data are represented as mean ± SEM (*n = 3*); N.T.: Not tested. [b] Positive controls.

3.4. Evaluation of Cytotoxicity Activities of Selected Compounds

Except for compounds **4** and **5**, other compounds were evaluated for their cytotoxicity against four cancer cell lines (the A549, SiHa, 5-8F, and HepG2 cell lines) by the MTS method [46]. Only compounds **6** and **11** showed cytotoxicity against SiHa cell lines with the IC_{50} value of 19.58±0.21 and 29.74±0.08 μM, respectively, and compounds **6** and **11** showed cytotoxicity against 5-8F cell lines with the IC_{50} value of 26.51±0.42 and 20.89±0.16 μM, respectively (Table 3). Among these compounds, compound **6** exhibited the strongest cytotoxic effect on SiHa cells. To date, compound **6** has been reported to be isolated only from *Sinularia lochmodes* and has no cytotoxic activity against tumor cell lines HeLa, SK-Hep1 and B-16, as well as anti-inflammatory activity against the accumulation of pro-inflammatory iNOS and COX-2 proteins in RAW264.7 macrophage cells [25]. Piscidinol A (**11**) was reported to exhibit moderate cytotoxicity against various human cancer cell lines PC3, BGC-823, KE-97, KB, Huh-7, jurkat, and MCF-3 (IC_{50} = 11.8, 67.5, 70.0, 35.4, 40.3, and 25.8 μM, respectively) [52, 53]. Thus, the action mechanism of compound **6** inhibiting SiHa cell proliferation was further studied.

Table 3. Cytotoxic activities of selected compounds against tumour cell lines (IC_{50} μM)[a]

Compound	A549	SiHa	5-8F	HepG2
1–3	>40	>40	>40	>40
4–5	N.T.	N.T.	N.T.	N.T.
6	>40	19.58±0.21	26.51±0.42	>40
7–10	>40	>40	>40	>40
11	>40	29.74±0.08	20.89±0.16	>40
12–26	>40	>40	>40	>40
Cisplatin[b]	8.5±0.22	22.31±0.03	3.0±0.43	12.52±0.18

[a] Data are represented as mean ± SEM (*n = 3*); N.T.: Not tested. [b] Positive controls.

*3.5. The Action Mechanism of Lochmolin F (**6**) Inhibiting SiHa Cell Proliferation*

*3.5.1. Lochmolin F (**6**) Inhibited Cell proliferation in SiHa Cell Lines*

As shown in Figure 2, cell viability was significantly decreased in a time- and dose-dependent manner in SiHa cell lines. After a 48 h incubation with 0, 2.5, 5, 10, 20, and 40 μM lochmolin F (**6**), cell survival decreased to 68.18%, 45.10%, 37.71%, 34.54%, and 36.27% in the SiHa cells compared to negative control. However, compound **6** showed no cytotoxicity against normal human cervical epithelial cells HUCEC at the concentration of 40 μM. Thus, the lochmolin F (**6**) might have the ability to inhibit SiHa cell proliferation selectively.

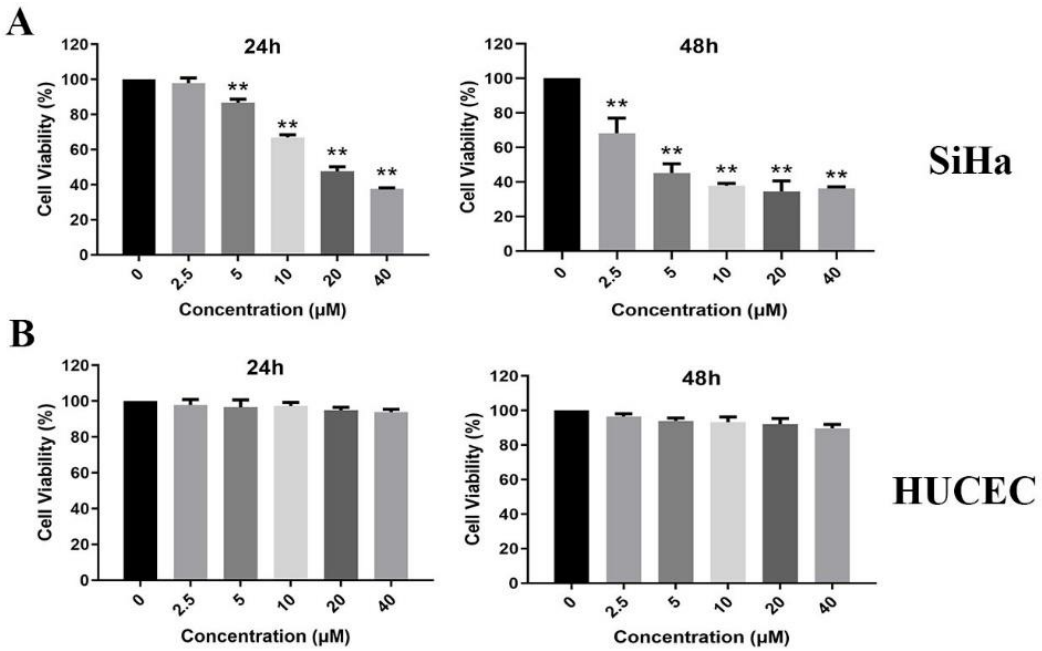

Figure 2. Lochmolin F (**6**) inhibited cell proliferation in SiHa and HUCEC cell lines after treatment with different concentrations (0, 2.5, 5, 10, 20, and 40 μM) of lochmolin F (**6**) for 24 h and 48 h. The data are presented as the mean ± SD (*n = 3*) and were analyzed using one-way ANOVA followed by Tukey's post hoc test. *$P<0.05$ and **$P<0.01$ versus the Control group.

*3.5.2. Lochmolin F (**6**) Induced Apoptosis in SiHa Cell Lines*

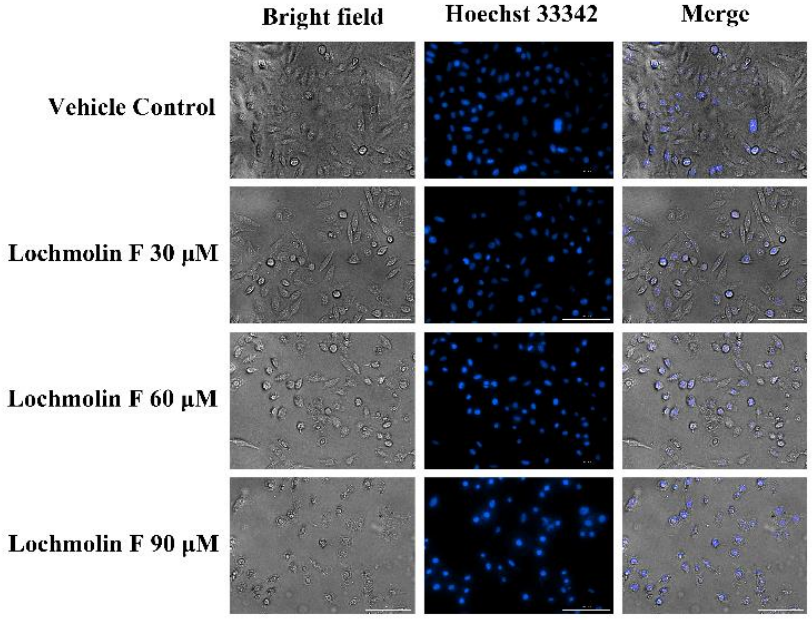

Figure 3. Cell morphology of SiHa cell lines was captured with a microscope after treatment with control (DMSO) or the indicated concentrations (30, 60, 90 μM) of lochmolin F (**6**) for 48 h. Scale bar, 100 μm.

To investigate the effect of lochmolin F (**6**) on the cell morphology of SiHa cell lines, cell lines treated with different concentrations (0, 30, 60, 90 μM) of lochmolin F (**6**) for 48 h were stained with the cell-permeable DNA dye Hoechst 33342. The nuclear morphological changes of cells were examined by fluorescent microscopy. As shown in Figure 3, the nucleus shrinks, and apoptotic bodies are formed. It was shown that the number of apoptotic bodies increased in a dose-dependent manner following the treatment of the cells with lochmolin F (**6**).

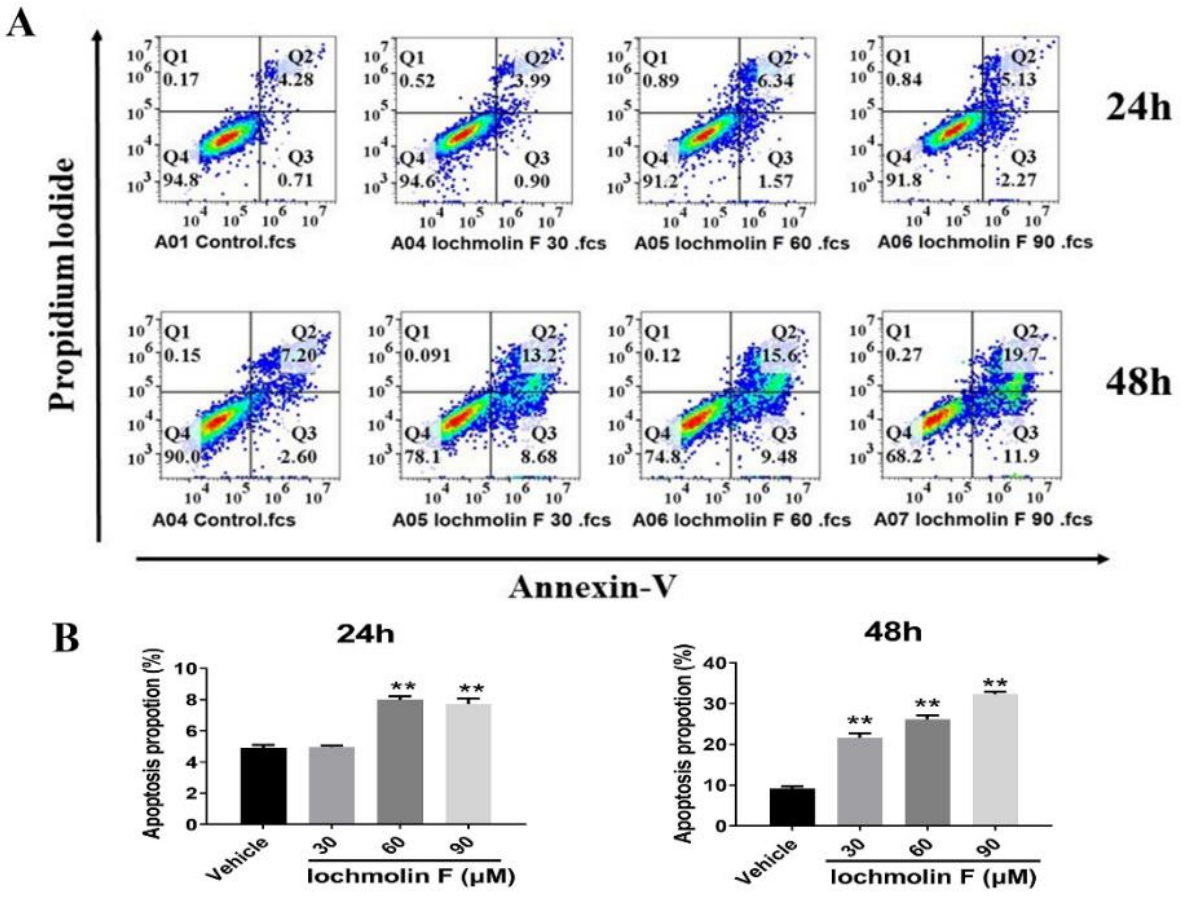

Figure 4. Lochmolin F (**6**) induced apoptosis in SiHa cell lines. (A) SiHa cell lines were treated with control (DMSO) or the indicated concentrations (30, 60, 90 μM) of lochmolin F (**6**) for 24 h and 48 h, followed by Flow cytometry analysis to determine the apoptosis proportion. (B) Late-stage apoptosis rate (Q2) and early-stage apoptosis (Q3) of cells were quantified. The data are presented as the mean ± SD (*n=3*) and were analyzed using one-way ANOVA followed by Tukey's post hoc test. **P<0.05* and ***P<0.01* versus the Control group.

To further determine whether lochmolin F (**6**) induces SiHa cells apoptosis, cells treated with different concentrations (0, 30, 60, 90 μM) of lochmolin F (**6**) for 24 h and 48 h were double-stained with Annexin V/PI and then subjected to the flow cytometry. As shown in Figure 4A–B, the number of apoptotic cells in the treated SiHa cells increased in a time- and dose-dependent manner.

*3.5.3. Lochmolin F (**6**) Induced S Cell Cycle Arrest in SiHa Cell Lines*

To evaluate whether lochmolin F (**6**) could cause cell cycle arrest, we used flow cytometry analysis. The percentage of S phase cells in SiHa cultures was increased after treatment with different concentrations (0, 30, 60, 90 μM) of lochmolin F (**6**) for 24 h and 48 h (Figures 5A and 5B). We confirmed these results by detecting the expression levels of cyclin A2, cyclin D1, and CDK2 proteins, all of which are essential for the S phase [59]. After treatment with different concentrations (0, 30, 60, 90 μM) of lochmolin F (**6**) for 24 h, the expression levels of cyclin A2 and CDK2 proteins were decreased significantly (Figure 5C and 5D). Together, these data indicate that lochmolin F (**6**) induced SiHa cells to arrest at the S phase by inhibiting the expression levels of cyclin A2 and CDK2.

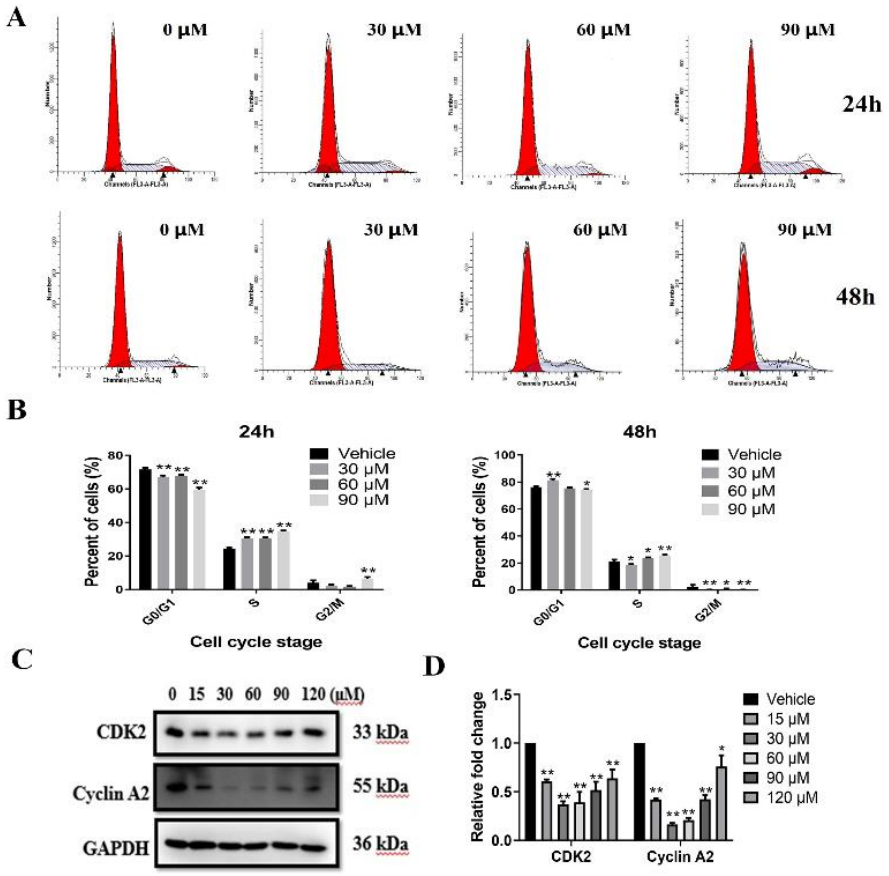

Figure 5. Lochmolin F (**6**) induced cell cycle arrest in SiHa cells. (A) SiHa cells were cultured with control (DMSO) or the indicated concentrations (30, 60, 90 μM) of lochmolin F (**6**) for 24 h and 48 h and analyzed by flow cytometry to assess the cell cycle arrest. (B) The number of SiHa cells in different cell cycle phases was quantified following flow cytometry. The data are presented as the mean ± SD (*n=3*) and were analyzed using one-way ANOVA followed by Tukey's post hoc test. **P<0.05* and ***P<0.01* versus the Control group. (C) Western blot was performed to analyze the expression changes of proteins CDK2 and Cyclin A2. The data are presented as the mean ± SD (*n=3*) and were analyzed using one-way ANOVA followed by Tukey's post hoc test. **P<0.05* and ***P<0.01* versus the Control group.

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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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