The Antitumor Activity of *Meconopsis Horridula Hook*, a Traditional Tibetan Medical Plant, in Murine Leukemia L1210 Cells

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Key Words
*M. horridula* • L1210 cells • Apoptosis • Cell cycle arrest • ROS

Abstract

**Background:** *Meconopsis horridula Hook* (*M. horridula*) has been used as a traditional Tibetan medicine to relieve heat and pain as well as mobilize static blood, and it is recognized as a good treatment for bruises. This study is the first trial to evaluate the tumor inhibitory activity of *M. horridula* extract and its underlying mechanism in the hope of providing evidence to support the anticancer function of *M. horridula*. **Methods and Results:** *M. horridula* extract was cytotoxic to L1210 cells in a dose- and time-dependent manner. SEM (scanning electron microscope) observation revealed obvious morphological changes in L1210 cells after *M. horridula* treatment. Flow cytometry analysis demonstrated that the extract dose-dependently induced early apoptosis. Additional apoptosis parameters, such as alterations in nuclear morphology and DNA damage, were also observed. Furthermore, *M. horridula* treatment induced G2/M arrest. *M. horridula* treatment significantly increased reactive oxygen species (ROS) production, suggesting that ROS are a key factor in *M. horridula*-induced apoptosis. Volatile constituent detection found 15 abundant chemicals in *M. horridula*, which may contribute to its anticancer effect. **Conclusion:** In conclusion, *M. horridula* extract induced L1210 cell apoptosis and inhibited proliferation through G2/M phase arrest, and ROS were involved in the process.
Introduction

Leukemia and lymphoma are a group of heterogeneous neoplastic white blood cell disorders characterized by uncontrolled proliferation and blocked differentiation of hematopoietic cells [1]. According to the WHO, leukemia and lymhapsomas are the fifth leading cause of cancer death worldwide [2]. The current conventional treatment for leukemia involves radiotherapy, chemotherapy, hormonal therapy, immune therapy and symptomatic and supportive therapy. However, conventional chemotherapy drugs, such as 5-fluorouracil, doxorubicin and Paclitaxel, produce severe side effect for patients. Therefore, there is an increasing interest in therapy with drugs of plant origin [3]. Natural products such as traditional Chinese herbal medicines provide a promising approach for cancer treatment because of their low toxicity against normal cells and potential effectiveness against cancer cells [4].

Tibetan medicine is a component of the most important ethnic drugs in traditional Chinese medicine, and it is very popular worldwide, especially in India and Europe. Meconopsis, an endangered genus of ornamental flowers, belongs to the Papaveraceae family. It has been used as the medicine for hundreds of years in Tibet [5]. The Meconopsis genus comprises approximately 50 species, and 43 species are primarily distributed throughout the Qinghai-Tibetan Plateau (QPT) and the neighboring mountains, except a few that inhabit Europe [6, 7]. The Meconopsis species is attractive for scholars because of its beautiful flowers and medicinal functions. Some Meconopsis species have reported anti-inflammatory and antioxidant effects [8, 9].

Meconopsis horridula is a perennial herb with sharp spines on its leaves and stems and is one of the most widely distributed species of the Meconopsis genus. It is scattered on grassy or rocky slopes at altitudes of 3000-4900 m in southwestern China [10]. For hundreds of years, M. horridula has been used as a traditional Tibetan medicine to clear away heat, relieve pain, mobilize static blood, and to treat bruises [11]. Modern pharmacological studies have demonstrated that M. horridula has sedative, anti-inflammatory and anti-Shigella activities [12]. M. horridula is usually constituted in herbal combination remedies for treating osteomyelitis and liver disease, such as Bawei Qinpi Pills, Ershiwwuei Luronghao ills, and Ganluling Pills. In addition, alkaloids, flavonoids, and phenylpropanoids are reportedly abundant in Meconopsis species, including M. horridula [13, 14]. Several of these compounds have been shown to exert anticancer functions, suggesting that M. horridula may also have anti-cancer effects. We previously examined 4 Meconopsis species, including Meconopsis racemosa, Meconopsis integrifolia, Meconopsis punicea and Meconopsis horridula, to test their anti-cancer effects by MTT assays, and the data suggested that M. horridula was the most efficient (data not shown). This study is the first attempt to evaluate the tumor inhibition activity of M. horridula extract and its underlying mechanism.

Apoptosis is programmed cell death characterized by DNA degradation, chromatin condensation, and nuclear fragmentation [15], and it plays an important role in tissue homeostasis and development [16]. Apoptosis deregulation is important in cancer initiation and progression [15, 17]. Therefore, cancer cell apoptosis induction has long been regarded an important target in cancer treatment.

Reactive oxygen species (ROS) is a key apoptosis induction factor. Many natural products have been reported to inhibit cancer cell proliferation by increasing intracellular ROS level. For instance, Fomitopsis pinicola chloroform extract induced SW-480 cell apoptosis by increasing ROS accumulation [18]. Acidic PAP-3 (a polysaccharide) has also been shown to exert anti-cancer effects through the ROS-mediated mitochondrial apoptotic pathway [19]. In this study, we examined the pro-apoptotic effects of M. horridula extract and the role of ROS.

The weakly immunogenic L1210 cell line is similar to the human leukemia condition and is a typical cell modal to study the anti-cancer effects of several natural products [20]. In our study, we used L1210 cells to evaluate the anti-cancer effects of M. horridula extract.
**Materials and Methods**

*Preparation of *M. horridula* ethanol extracts*

The fresh *M. horridula* plants were collected in Yushu County, Tibetan Autonomous Prefecture of Yushu Qinghai Province in August 2011 (altitude 4639 m). The plant species identifications were authenticated by Associate Prof Jun-Hua Du at School of Life and Geographical Sciences, Qinghai Normal University.

Dried *M. horridula* powder was homogenized in 95% ethanol at 45°C, using ultrasonic-assisted extraction (UAE) three times. The ethanol extract was obtained from vacuum filter and then the supernatant was collected and concentrated with a rotary evaporator (RE-2000 A; Belong, Shanghai, China). The ethanol fraction was homogenized in 70% ethanol and the supernatant was filtered using 0.22 μm filters.

*GC-MS instrument and analytical conditions*

To investigate the composition of *M. horridula* extract, GC–MS analysis was performed with a 7890A GC/5975C MS system (Agilent, USA) fitted with a fused silica capillary column (HP-5 MS, 30 m × 25 mm ID, 0.25 μm film thickness; Agilent J&W Scientific, Folsom, CA, USA). One microliter of the sample was analyzed, with a split ratio of 10:1 (v/v). The temperature rise program had an initial temperature of 50 °C for 2 min, which was raised 10 °C/min to 200 °C and then maintained for 2 min; raised to 250 °C at 5 °C/min and then maintained for 6 min; and raised to 280 °C at 10 °C/min and isothermally maintained for 5 min. The injector temperature was set to 280 °C and the interface temperature was 150 °C. The Helium MS source was used as the carrier gas at a flow rate of 1 ml/min. The ionization source temperature was 250 °C. Mass spectrometry was determined by the full-scan method, ranging from 50 to 600 (m/z). Metabolites were identified by comparison with the NIST Mass Spectral Search Program 2008 database (version 2.0, FairCom Co., Columbia, MO, USA).

*Cell cultures*

Murine leukemia L1210 cells were purchased from the American type culture collection (CCL-219 TM, ATCC). Cells were cultured under standard conditions in DMEM medium (Sigma-Aldrich) supplemented with 10% (v/v) 10% heat-inactivated horse serum (Gibco/Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin-streptomycin solution (Hyclone, Logan, UT, USA) and 1 mM L-glutamine (Sigma-Aldrich), routinely subcultured every day, and incubated at 37 °C in humidified atmosphere with a 5% CO₂ incubator.

*Cell viability assay*

The effect of *M. horridula* extract on L1210 cell viability was determined with the conventional MTT (Sigma, St Louis, USA) reduction assay [18]. Briefly, 100 μL L1210 cell suspension was placed in a 96-well culture plate at a density of 1×10⁵ cells/mL and incubated with various concentrations of *M. horridula* (0, 30, 60, 90 and 120 μg/mL) extract for distinct time points (24 h and 48 h). The treated cells were incubated with MTT solution, and the formazan crystals were dissolved in 100 μL 10% SDS, 5% isobutanol and 0.01 M HCL solution. Absorbance at 570 nm was recorded with a microplate reader (ELX800; BIO-TEK, Winooski, VT, USA). The cell viability of treated samples was obtained by comparison with the untreated control.

PBMCs (peripheral blood mononuclear cells) were used to evaluate the cytotoxicity of *M. horridula* extract on normal cells. PBMCs were isolated using a lymphocyte separation kit (Applygen Technologies Inc., Beijing, China) according to the manufacturer’s protocol. Briefly, separation liquid was immediately added to blood collected from a mouse, and the sample was centrifuged at 800 g for 30 min. The middle fraction was collected as PBMCs. PBMCs were treated with different dosages of *M. horridula* extract (0, 100, 200 and 300 μg/ml, or 70% ethanol as the solvent control) for 24 or 48 h. PBMC viability was assessed using a trypan blue exclusion test.

*Scanning electron microscopy (SEM) observation*

After 24 h following various concentrations of *M. horridula* extract (0, 60, 90 and 120 μg/ml), cells in each group were fixed by 2.5% glutaraldehyde, washed by PBS, dehydrated by graded alcohol, displaced, dried at the critical point, gold evaporated, and finally observed under a SEM (S-3400N, Hitachi, Tokyo, Japan).
Hoechst 33342 staining

Hoechst 33342 (HO) is a fluorescent probe that binds to double-stranded DNA and represents changes in nuclear morphology. To examine whether *M. horridula* treatment induced nuclear changes in L1210 cells, HO staining was performed. After *M. horridula* extract (0, 60, 90, 120 μg/ml) treatment for 12, 24 or 48 h, cells were stained with 10 μM HO for 15 min at room temperature. The stained cells were washed three times with PBS and observed by fluorescence microscopy with standard excitation filters (Nikon E-600, Japan).

Apoptosis detection by flow cytometry

Apoptotic cells were quantified with an Annexin V–FITC Apoptosis Detection Kit (Invitrogen, USA) according to the manufacturer’s protocol. Briefly, 1×10^5 cells/ml were treated with various concentrations (0, 60, 90 and 120 μg/mL) of *M. horridula* extract for 12 h at 37°C. The cells were then harvested and re-suspended in binding buffer. They were stained with 10 μl Annexin V–FITC and 5 μL PI for 15 min at room temperature in the dark. The apoptotic index was immediately determined by flow cytometry (Guava easy Cyte 8HT, Millipore, Billerica, MA, USA).

Cell cycle analysis

The ratio of cells in the G1, S, and G2/M phases of the cell cycle was determined by their DNA content. Cells at 1×10^5 cells/ml were treated with various concentrations (0, 60, 90, 120 μg/mL) of *M. horridula* extract for 72 h. Cells were harvested and washed twice with cold PBS and fixed with 70% ice-cold ethanol at 4°C overnight. The fixed cells were washed twice with cold PBS and incubated with 100 μg/ml RNase A (Sigma, St. Louis, USA) for 30 min at 37°C, then stained with 50 μg/ml propidium iodide (PI; Sigma, St. Louis, USA) for 30 min in the dark and analyzed by flow cytometry (Millipore, USA).

DNA fragmentation assay

Krysko et al. [21] described an easy and quantitative way to analyze DNA fragmentation which is based on flow cytometric detection of DNA hypoploidy after adding PI to the dying cells and permeabilizing them by freeze-thawing. The size of DNA fragments appeared as a hypoploid DNA histogram. To investigate the effect of *M. horridula* extract on DNA damage of L1210 cells, oligonucleosomal DNA fragmentation by flow fluorocytometry was performed. Cells were treated with various concentrations (0, 60, 90, 120 μg/mL) of *M. horridula* extract for 12 and 24 h, then they were stained with 5 μg/ml PI and freeze-thawed for 30 s. Cells were immediately analyzed by flow cytometry.

Intracellular reactive oxygen species detection

The intracellular ROS level was evaluated by the oxidative conversion of 2′, 7′-dichlorofluorescein-diacetate (DCFH-DA) to fluorescent 2′, 7′-dichlorofluorescein (DCF) [18]. L1210 cells were treated with the mentioned concentrations of *M. horridula* extract for 2 and 6 h. The treated cells were harvested, washed twice with PBS, re-suspended in 500 μl of 10 μM DCFH-DA (Molecular Probes Inc., Invitrogen, CA, USA) and incubated at 37°C for 30 min in the dark. The samples were then immediately detected by flow cytometry. Histograms were analyzed using FCS Express V3.

Statistical analysis

All the experiments were performed in triplicate, and data were expressed as means ± SD. IC<sub>50</sub> values were calculated by regression analysis. The data were subjected to an analysis of Duncan’s multiple range tests (SPSS, version 18.0). A significant difference was judged to exist at a level of *p < 0.05 and **p < 0.01.

Results

GC-MS analysis for *M. horridula* extract

We used GC-MS assay to analyze the volatile constituents of *M. horridula* extract. We considered compounds to be “identified” when their mass spectral fit values were at the default value of 90% or above. As shown in Table 1, we identified a total of 15 constituents in the extract.
Cytotoxicity of M. horridula

As shown in Fig. 1A, L1210 cell viability was 81.9 ± 3.6% (*p < 0.05) after treatment with 60 µg/mL M. horridula for 24 h. As the dose increased from 90 to 120 µg/mL, cell viability decreased from 56.6 ± 1.3% (**p < 0.01) to 67.8 ± 3.3% (**p < 0.01). We calculated the IC_{50} to be 90.5 µg/mL at 24 h. The cell viability decreased more sharply at 48 h, and the IC_{50} value was 79.5 µg/mL. In contrast, M. horridula extract did not have cytotoxic effects on PBMCs (p > 0.05) at the used concentrations, suggesting that M. horridula has some selective cell killing activity (Fig. 1B).

SEM observation

We observed the effect of M. horridula extract on L1210 cell morphology by SEM (Fig. 2). In the control group, cells appeared normal with a round shape, intact membrane and...
abundant microvilli. Compared to the control, cells treated with 60 µg/ml *M. horridula* were slightly changed with decreased microvilli on the cell surface. However, 90 and 120 µg/ml *M. horridula* extract induced significant deformities. There was obvious structural damage on the cell surface with few microvilli remaining.

*M. horridula*-induced nuclear damage

The nuclear damage after different doses of *M. horridula* extract treatment was evaluated by HO staining. As shown in Fig. 3, the nuclear HO staining in control cells was slightly blue and homogeneous. However, cells treated with *M. horridula* show significant morphological changes in a time-dependent manner, such as condensed chromatin and fragmented punctuate blue nuclear fluorescence. After 12 h of *M. horridula* incubation, the treated cells showed no obvious changes in cell morphology and nuclei comparing to the control. As the incubation time increased to 24 and 48 h, bright blue nuclear HO fluorescence was observed in all treated cells and displays a dose-dependent manner.

*M. horridula* extract induced apoptosis

To determine whether *M. horridula* extract induced L1210 cell apoptosis, we performed Annexin V–FITC/PI double staining. As shown in Fig. 4, *M. horridula* extract induced L1210 cell apoptosis in a dose-dependent manner after 12 h incubation. The early apoptosis ratio
Fan et al.: M. Horridula Triggers L1210 Cells Apoptosis and G2/M Phase Arrest

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(low right) increased from 13.57% to 37.73% as the concentration increased from 60 to 120 µg/ml, while the apoptosis ratio in control group was only 2.07%. The results indicate that M. horridula induced early L1210 cell apoptosis at 12 h after extract addition.

Effect of M. horridula extract on cell cycle distribution

Cell cycle arrest has long been regarded as a cancer treatment target. To determine whether the growth-inhibitory effect of M. horridula extract on L1210 cells was associated with cell cycle arrest, we analyzed the distribution of cells in different cell cycle phases by measuring intracellular DNA content. As shown in Fig. 5, M. horridula extract (0, 60, 90, 120 µg/mL) treatment for 72 h increased the percentage of cells in the G2/M phase from 5.95% to 21.09%, while the proportion of cells in S phase decreased from 64.08% to 47.53% and the percentage of cells in G1 remained the same. The results indicate that M. horridula extract induced L1210 cell G2/M phase arrest in a dose-dependent manner (**p<0.01).

M. horridula extract induced DNA fragmentation

We performed PI staining with flow cytometry to evaluate M. horridula-induced DNA damage in L1210 cells. As shown in Fig. 6, 12 h treatment of 60 µg/mL extract induced a 3.67-fold increase in DNA damage, but increased concentrations did not induce more severe damage. At 24 h, DNA damage level sharply increased to 14.02-fold more than control upon 90 µg/mL M. horridula extract treatment. As the concentration increased to 120 µg/mL, the DNA damage level did not further increase (**p<0.01).

M. horridula treatment caused ROS accumulation

We analyzed intracellular ROS production by flow cytometry with DCF staining. As shown in Fig. 7, M. horridula extract treatment increased intracellular ROS levels. At 2 h, M.
horridula extract (120 µg/mL) treatment significantly increased ROS accumulation (41.8%) compared to control cells (4.73%). When the incubation time increased to 6 h, the percentage of cells with bright DCF fluorescence significantly increased in each treatment group. The percentages of the three treated groups (60, 90 and 120 µg/mL) were 35.5%, 33.8% and 57.67%, respectively, compared to 8.07% in the control sample.

Discussion

Traditional Tibetan medicine is based upon Indian medicine and traditional Chinese medicine. It continues to be practiced in Tibet, India, Nepal, Bhutan, Ladakh, Siberia, China and Mongolia, and it has been more recently practiced in parts of Europe and North America. With the rapid development of natural medicine, an increasing number of researchers have devoted themselves to studying Tibetan medicine, especially its important member- the Meconopsis species. In the present study, we tested the anticancer activity of M. horridula (belongs to the Meconopsis species) extract in vitro using the L1210 cell line.

Reports have suggested that M. horridula contains four compounds, including luteolin, apigenin, hydnoocarpin and β-sitosterol [22]. However, we also previously detected quercetin, protopine, dicentrine and nonacosanol in M. horridula extract (data not shown).
analyses of *M. horridula* have primarily focused on flavonoids compounds. However, botanical medicines are composed of complicated constituents, such as flavonoids, saponin and volatile compounds. Therefore, we analyzed the volatile constituents of *M. horridula* extract to identify more functional compounds in *M. horridula*. We identified 15 abundant chemicals in *M. horridula* extract, including Octadecadienoic acid (38.08%), Octadecadienal (17.79%), γ-sitosterol (7.37%), n-Hexadecanoic acid (7.95%). Many of these chemicals have been reported to have anticancer effects. For instance, n-Hexadecanoic acid has anti-tumor activity against human leukemic cells and murine cells [23, 24]. Essential oil composed primarily of n-Hexadecanoic acid and octadecanoic acid had significant cytotoxicity against oral cancer (KB), breast cancer (MCF-7) and small cell lung cancer (NCI-H187) [25]. Steroidal compounds showed potent growth inhibition against cancer cells [26]. Taken together, we hypothesized that the specific efficacy of such components in *M. horridula* results in significant anti-tumor effects. To confirm our hypothesis, we carefully investigated cell viability, ROS production, apoptosis and cell cycle arrest after *M. horridula* treatment.

We chose to use L1210 cells to evaluate the anticancer activity of *M. horridula* extract. Our results indicated that *M. horridula* extract decreased L1210 cell survival in a dose- and time-dependent manner, while having little effect on normal PBMCs, suggesting that *M. horridula* extract may exert a selective killing effect. SEM observation showed obvious alterations on L1210 cell morphology after *M. horridula* treatment.

Apoptosis is a hallmark for the appraisal of potential agents for cancer prevention, and a wide variety of natural products have been known to interfere with cell proliferation or induce apoptosis [27]. The morphological characteristics of apoptosis include membrane blebbing, cell shrinkage, chromatin condensation and apoptotic body formation [28]. The results in Fig. 4 suggest that *M. horridula* primarily triggers early apoptosis of L1210 cells in a time- and dose-dependent manner. Furthermore, the presence of condensed chromatin and fragmented punctate blue nuclear fluorescence in L1210 cells confirmed apoptosis induction. DNA fragmentation is a canonical biochemical apoptotic feature. Many plants extracts have been shown to induce DNA damage [18]. Here, we found that *M. horridula* induced-apoptosis may be related to DNA damage. DNA damage often involves several signaling pathways, which can also result in cell cycle arrest and eventual apoptosis [29]. DNA damage activates P53 to induce G1 and G2/M phase arrest and apoptosis [30]. Our results suggest that *M. horridula* induces irreversible DNA damage to cause cell cycle arrest and apoptosis.

Excessive amounts of ROS can cause DNA damage, cell cycle arrest and apoptosis [31]. Although all aerobic cells are equipped with protective enzymatic and non-enzymatic antioxidants, increased oxidative stress may overwhelm the protective mechanisms, leading to cell injury [32]. Many natural compounds, such as flavonoids and triterpenoids, have been reported to increase oxidative stress by increasing intracellular ROS levels [33]. Thus, ROS are considered an important factor of natural anticancer agents. In the current study, we found that ROS accumulation preceded other changes, including DNA damage, G2 phase arrest and apoptosis, after *M. horridula* incubation, suggesting that ROS accumulation is an early event in *M. horridula*-induced cell apoptosis.

Mitochondria accumulate calcium ion upon cytosolic Ca\(^{2+}\) increase, leading to mitochondrial membrane depolarization [34]. Calcium ion uptake into mitochondria stimulates the tricarboxylate cycle, resulting in reduction of pyridine nucleotides, which may couple cell and metabolic oxidative stress activity [35]. Increased calcium release then induces further mitochondrial depolarization and ROS production [35]. It has been reported that a major constituent of *Scutellaria* wogonin induced prolonged elevation of intracellular Ca\(^{2+}\) levels in tumor cells, leading to ROS accumulation and eventual apoptosis [36]. In the current study, we observed that oxidative stress and cell apoptotic factors were increased in *M. horridula*-treated cells, suggesting that they have been caused by release of Ca\(^{2+}\). However, further research is required.

In conclusion, we demonstrated that *M. horridula* extract inhibited L1210 cell proliferation. This inhibitory effect was related to G2/M phase arrest and cell apoptosis,
and ROS likely play an important role in the process. Our findings suggest that *M. horridula* extract induces L1210 cell apoptosis by increasing oxidative stress without damaging normal PBMC cells, suggesting that *M. horridula* may be a safe natural product. Further research on the tumor inhibition activities of *M. horridula* is required. Deep investigation into potential therapeutic targets and the compound in *M. horridula* that contributed to the anticancer activity should be carefully explored.

**Abbreviations**

Meconopsis horridula Hook (M. horridula); 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT); Propidium iodide (PI); 2', 7'-dichlorodihydrofluoresceindiacetate (H2DCF-DA); Hoechst 33342 (HO), Scanning electron microscope (SEM); Gas Chromatography-Mass Spectrometer (GC-MS); Reactive oxygen species (ROS); Peripheral blood mononuclear cells (PBMCs); Heart and Umbilical Vein Endothelial (HUVE). Fetal bovine serum (FBS).

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**Disclosure Statement**

The authors declare no conflict of interest.

**References**