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ORIGINAL ARTICLE

Anticancer Activity of Ethyl Acetate Extract from *Lethariella cladonioides in vitro* and *in vivo*

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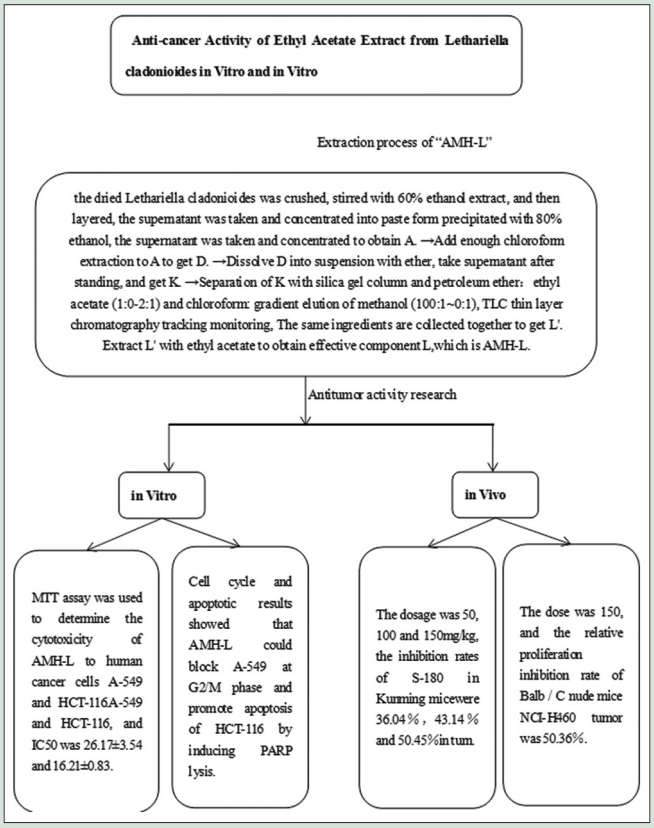
ABSTRACT

Background: *Lethariella cladonioides*, also known as Red Snow Tea and Golden Silk Tea, mainly grows in Tibet, Sichuan and Yunnan of China. Its medicinal value has been gradually discovered. **Objectives:** This study mainly evaluated the anti-cancer effect of AMH-L extracted from *L. cladonioides in vitro* and *in vivo* and to explore its anticancer mechanism. **Materials and Methods:** MTT assay was used to determine the cytotoxicity of AMH-L to human cancer cells A-549 and HCT-116 *in vitro.* Sarcoma S-180 and human lung cancer NCL-H460 cells were implanted into Kunming mice and Balb/C nude mice, respectively, anti-cancer activity *in vivo* was assessed by recording body weight, organ coefficient and tumor inhibition rate. Flow cytometry and western blotting were used to preliminarily explore the anticancer mechanism. **Results:** AMH-L, the active component of Red Snow Tea, had strong anticancer activity against A-549 and HCT-116, and IC_{50} was 26.17 ± 3.54 and 16.21 ± 0.83. When the dosage was 50, 100 and 150 mg/kg, the inhibition rates of S-180 in Kunming mice were 36.04%, 43.14%, and 50.45% in turn, respectively. At the end of the experiment, the dose was 150, and the relative proliferation inhibition rate of Balb/C nude mice NCI-H460 tumor was 50.36%. Cell cycle and apoptotic results showed that AMH-L could block A-549 at G2/M phase and promote apoptosis of HCT-116 by inducing poly (ADP-ribose) polymerase lysis. **Conclusion:** *L. cladonioides* extract AMH-L exhibits strong anticancer activity *in vivo* and *in vitro* and can be used as a potential anticancer drug for further study.

Key words: Anticancer drug, apoptosis, cell cycle, cleaved poly (ADP-ribose) polymerase, lichen

SUMMARY

- *Lethariella cladonioides* (also known as Red Snow Tea, Golden Silk Tea, Latin name: *Lethariella cladonioides*) is a lichen plant growing on the plateau. It is used as a hypolipidemic and cholesterol-lowering health product. In recent years, the medicinal value of *Lethariella cladonioides* has been gradually discovered. Ethyl acetate extract AMH-L (Antimutagen-He) was obtained from *Lethariella cladonioides* by ethanol precipitation, chloroform, ether and ethyl acetate extraction. In this experiment, the cytotoxicity of ethyl acetate extract AMH-L from *Lethariella cladonioides* was evaluated *in vitro* by HCT-116 and A-549 cells. *In vivo* experiments, Kunming mice and Balb/C nude mice were inoculated with sarcoma S-180 and NCI-H460 respectively to evaluate the anti-tumor effect of AMH-L. Finally, the mechanism of AMH-L on A-549 and HCT-116 cells was detected by flow cytometry and Western blot. MTT results show that the IC_{50} of AMH-L for A-549 and HCT-116 is 26.17 ± 3.54, 16.21 ± 0.83. In order to further explore the inhibitory mechanism of A-549 and HCT-116, we also studied the cell cycle and apoptosis, and We found that AMH-L can block A-549 cells in the G2/M phase, thereby inhibiting the growth and proliferation of tumor cells. However, for HCT-116, G0/G1 phase, S phase and G2/M phase cells decreased with the increase of the concentration of HCT-116. Especially in G0/G1 phase, AMH-L may inhibit the proliferation of HCT-116 by promoting cell apoptosis. Annexin V/propylene iodide staining and apoptotic results also showed the cleavage of poly (ADP-ribose) polymerase in HCT-116 cells, (ADP-ribose) polymerase is the cleavage substrate of caspase, the core member of apoptosis, increased (ADP-ribose) polymerase cleavage suggests that AMH-L induces apoptosis of HCT-116, but Caspase-3 in HCT-116 was not activated, it is suggested that the activation of (ADP-ribose) polymerase in HCT-116 is caused by other signaling pathways besides Caspase-3, but it is not clear which signaling molecule is at work in this study, this experiment excluded the effects of AMH-L on Caspase-3 and PTEN signaling pathways in A-549, HCT-116 cells.
- AMH-L significantly inhibits the growth of sarcoma S-180 *in vivo* for NCI-H460 tumors in balb/c nude mice and the volume growth trend was slower than that in the control group, but there was no significant difference between the two groups. At the end of administration, the tumor quality significantly reduced the inhibition rate of 49.44% compared with the control group, no significant difference was found in the main organ coefficients.



Abbreviations Used: AMH: Ethyl Acetate Extract from *Lethariella cladonioides*.

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INTRODUCTION

More than 18,100,000 new cancers occurred worldwide, with more than 9,600,000 deaths.[1] Increasing incidence of cancer, especially in developing countries.[2] By 2035, 14 million new cancer cases are predicted, accounting for nearly 60% of the global cancer incidence.[3] Because cancer cells have special biological characteristics, it is still a difficult task for scientists to find safe and effective anticancer drugs. Screening new anticancer drugs from biological resources is considered to be the most cost-effective and rapid method for discovering anticancer drugs.[4-6] Many bioactive extracts and compounds are being evaluated as promising anticancer agents in the world.

Lethariella cladonioides (also known as Luxinxue Tea, Golden Silk Tea, English name: *L. cladonioides*), The active ingredients extracted from black snow tea can strongly inhibit the mutagenesis of many carcinogens.[7-9] significantly inhibits carcinogen-induced growth of animal tumors,[10-12] The main anticancer bioactive component AMH-L was extracted from this *L. cladonioides* by further photochemistry separation and anti-cancer bioactivity tracing. In this experiment, we studied its anticancer activity *in vitro* and *in vivo*.

MATERIALS AND METHODS

AMH-L

Active Component AMH-L of *L. cladonioides*, the dried *L. cladonioides* was crushed, stirred with 60% ethanol extract, and then layered, the supernatant was taken and concentrated into paste form precipitated with 80% ethanol, the supernatant was taken and concentrated to obtain A. Add enough chloroform extraction to A to get D. Dissolve D into suspension with ether, take supernatant after standing, and get K. Separate K with a silica gel column, the eluent is petroleum ether and ethyl acetate (1:0-2:1), then chloroform and methanol (100:1-0:1). The same ingredients are collected together to get L. Extract L' with ethyl acetate to obtain effective component L, which is AMH-L.

Cancer cell line

Cancer cells A-549, HCT-116, NCI-H460, and S-180 were donated by the Third Affiliated Hospital of Kunming Medical University (Yunnan cancer institute).

Animals

Kunming mice, male, 18–22 g, purchased from the Laboratory Animal Center of Kunming Medical University; Balb/C nude mice, specific pathogen free (SPF) class, male, 18–20 g, purchased from Beijing Weitong Lihua Laboratory Animal Technology Co., Ltd. Raising in SPF Animal Laboratory of Kunming Medical University, the animals were randomly grouped according to their body weight.

Instrument

Ultra-low temperature refrigerator Forma995 (Thermo Electronics Co., Ltd.); Thermo Forma Series II carbon dioxide incubator (Thermo Fisher Scientific, USA); Thermo Multiskan Spectrum microplate reader (Thermo Fisher); Ultra-clean workbench (Shanghai Bo Xun Industrial Co., Ltd.); BD FACSCanto II flow cytometer (BD company).

Experimental procedures

Experimental 1: Anti-cancer activity of AMH-L in vitro

Adjust the A-549 and HCT-116 cells in the logarithmic growth phase to a density of 1×10^5 cells/ml, inoculate the cells in 96-well plates, add 100 μ cell suspension to each well, and place at 37°C, 5% CO_2 incubator, Add different concentrations (6.25, 12.50, 25.00, and 50.00 μg/ml) of AMH-L drugs after 24 h, Negative control group added the same dose of DMSO (1 μl/ml). After 72 h of incubation, 200 μl of new culture medium containing 10% MTT (5 mg/ml) was added to each pore. After 4 h, the culture medium was discarded and 150 μu of DMSO was added to each pore. After 10 min of shaking in the dark, the absorbance (OD) of each well was measured at a wavelength of 570 nm and the cell inhibition rate IR% was calculated.

Inhibition Rate (IR%) = $(OD_{DMSO}\text{-}OD_{AMH\text{-}L})/OD_{AMH\text{-}L}*100$

Experiment 2: Anti-cancer effect of AMH-L on sarcoma S-180 of Kunming mice in vivo

S-180 tumor cells were subcultured twice in mouse abdominal cavity, execute mice, the abdomen was disinfected and the ascites were removed aseptically. Diluted with sterile saline, 0.1 ml suspension (about $5–6 \times 10^6$ tumor cells) was injected into the left forelimb of 48 healthy male Kunming mice to form a subcutaneous tumor model. Forty-eight mice successfully inoculated with tumors were randomly divided into four groups according to their body weight, i.e., drug administration group (50, 100, and 150 mg/kg) and solvent control group. Intraperitoneal injection, once every two days, dose of 0.1ml/10 g. The animals were executed the next day after the fifth administration, and the tumors were taken out and weighed to calculate the tumor inhibition rate.

IR (%) = (1-mean tumor weight of the treatment group/mean tumor weight of the negative control group) ×100.

Experiment 3: Anticancer effect of AMH-L on male balb/c nude mice

After adapting 16 Balb/C nude mice to feeding for 5 days, about $5–6 \times 10^6$ NCL-H460 cells were inoculated subcutaneously on the right side of each nude mice. Twelve days after inoculation, the tumor volume (TV) was about 200 mm^3. According to the size of the tumors, the mice carrying the tumors were randomly divided into two groups: experimental group 150 mg/kg and solvent control group, with 8 mice in each group. Intraperitoneal injection, once every two days, dose of 0.1ml/10 g. TV and weight of mice were measured every 4 days. The mice were executed 30 days after the first administration. The weight of mice and the mass of main organs were measured to calculate the organ coefficient (OE).

TV = $a \times b^2/2$; ("a" Tumor long diameter, "b" Tumor short diameter).[13]

IR (%) = (1-mean tumor weight of the treatment group/mean tumor weight of the negative control group) ×100.

OE = Organ weight/Body weight ×100.

Experiment 4: Effect of AMH-L on cell cycle of A-549 and HCT-116

The concentration of AMH-L was 5.0, 10.0 and 20.0 μg/ml in the experimental group, DMSO in the solvent control group and 5-Fu (20 μM) in the positive control group. The suspension cells and adherent cells of A-549 and HCT-116 were collected together. PBS was washed and fixed overnight with 70% ethanol, propylene iodide staining solution was used to stain for 30 min at 488 nm, and flow cytometry was used to detect.

Experiment 5: Effects of AMH-L, an ethyl acetate extract from Lethariella cladonioides, on caspase-3, poly (ADP-ribose) polymerase and PTEN

Treatment of four cancer cells A-549, HCT-116 with different concentrations of AMH-L. After discarding the culture medium and washing with cold PBS for three times, add 0.3 ml low salt cracking buffer to each pore and shake the plate in the cold chamber for 30 min. Collect cells into Eppendorf (EP) tube and shake them violently in cold chamber for 15 min. After centrifuging for 5 min with 13.2K rpm at 4°C, the supernatant was transferred to other EP tubes. Check protein concentration and adjust to the same level in each cell line. Samples were stored at −20°C and

detected by Western blotting for cleaved caspase-3, cleaved (ADP-ribose) polymerase (PARP), phosphorylated PTEN, and GAPDH.

Statistical analysis

The experimental values are expressed as mean ± Standard deviation, statistical analysis was performed using SPSS 17.0 software (IBM Corporation, Armonk, NY, USA)., *in vitro* experiments using two independent samples *t*-test (bilateral), and the rank sum test was not consistent with the normal distribution, $^*P < 0.05$ was statistically significant.

RESULTS

Experiment 1: Inhibition of cancer cell proliferation by ethyl acetate extract AMH-L of *Lethariella cladonioides*

In vitro cytotoxicity of AMH-L to A-549 and HCT-116 is shown in Figure 1. AMH-L had strong anticancer activity against A-549 and HCT-116, IC_{50} was 26.17 ± 3.54 and 16.21 ± 0.83, respectively. There was significant difference between AMH-L and negative group ($P < 0.05$). Within a certain range, the inhibitory rate increased with the increase of AMH-L concentration, which showed obvious selectivity to cancer cell types, and HCT-116 cells were sensitive.

Experiment 2: Anticancer effect of AMH-L, an ethyl acetate extract of Black Snow Tea, on mouse sarcoma S-180 *in vivo*

In the course of the experiment, the weight of the mice was measured as an indicator of the effect of AMH-L on the sarcoma S-180 in mice. As shown in Figure 2a, there was no significant difference in body weight between AMH-L group and negative control group. As shown in Figure 2b, at the end of the experiment, the tumor quality of mice in the administration group was significantly different from that of the control group. As shown in Table 1, with the increase of drug concentration, the inhibition rate of tumors in mice increased. The inhibition rates of tumors in the drug group (50, 100, 150 mg/kg) were 36.04%, 43.14% and 50.45% respectively.

Experiment 3: Anticancer effect of *Lethariella cladonioides* extract AMH-L on nude mice inoculated with NCI-H460 balb/c

At the end of the experiment, tumors, liver, spleen, kidney and testis were separated, weighed and OEs were calculated. The results are shown in Table 2. As shown in Figure 3a, mice in the experimental group showed weight loss after administration and slow weight gain after the 10th day of administration; but, at the end of the experiment, the weight of mice was still lower than that of the control group. Figure 3b is the change of TV in mice during experimental detection. Figure 3c The tumor quality of mice in the experimental group was obviously controlled compared with that of the control group. At the end of the experiment, AMH-L (150 mg/kg) inhibited NCL-H460 tumors in nude mice, and the inhibition rate of IR was 49.44%.

Experiment 4: Effect of ethyl black tea extract AMH-L on cell cycle of A-549 and HCT-116

Flow cytometry was used to analyze the effects of different concentrations of AMH-L (5, 10, 20 μm/ml) on the cell cycle of A-549 cells. 24 h after AMH-L was applied to A-549 cells, the decrease of A-549 cells in G0/G1 phase occurred in a dose-dependent manner. From 47.9% to 1.0% in the control group, and from 13.4% to 28.5% in the G2/M phase. These results indicate that AMH-L affects the division cycle of A-549 cells and blocks them in G2/M phase. With the increase of AMH-L concentration, HCT-116 cells in G0/G1 phase, S phase and G2/M phase decreased, especially in G0/G1 phase. It may be that AMH-L inhibits the proliferation of HCT-116 by promoting cell apoptosis. Respectively [Figure 4a and b].

Table 1: Inhibitory rate of different concentration of AMH-L on S-180 tumors in mice (%)

Group	Dose (mg/kg)	Inhibition rate (%)
AMH-L	50	36.04*
AMH-L	100	43.14*
AMH-L	150	50.45*

Data are shown as mean+SD ($n = 12$)* versus control (DMSO), $^*P < 0.05$

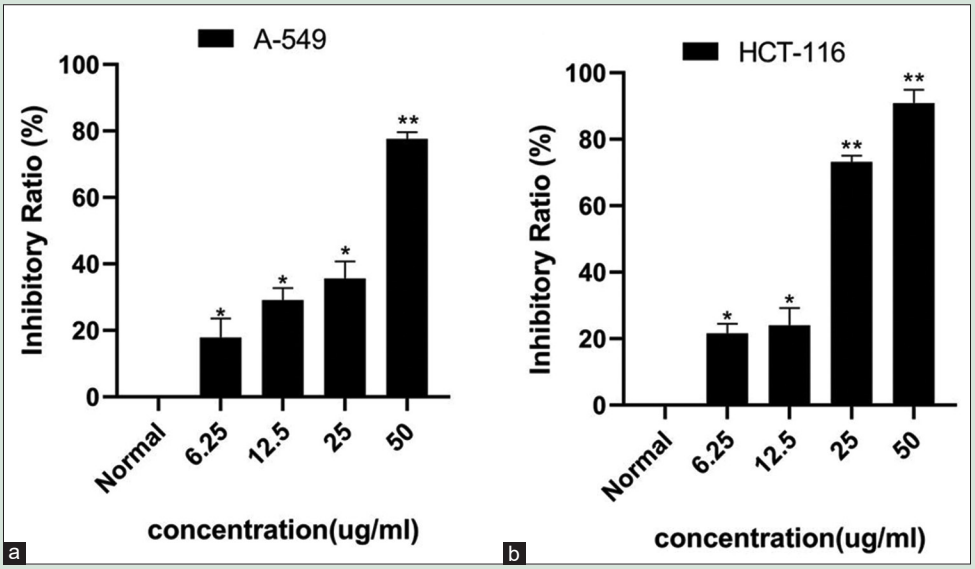

Figure 1: The effect of AMH-L on tumor cells *in vitro*. (a) Inhibition of A-549 cells; (b) Inhibition of HCT-116 cells *versus control(DMSO), $^*P < 0.05$,$^{**}P<0.01$

Experiment 5: Effect of ethyl acetate extract of *Lethariella cladonioides* on caspase-3, poly (ADP-ribose) polymerase and PTEN

In order to clarify the mechanism of AMH-L inhibiting the growth of cancer cells, the expression of caspase-3, PARP and PTEN was further detected, staurosporine was used for positive control and DMSO for negative control, GAPDH is considered to be a constitutive expression of housekeeping proteins and is often used as a control for internal control of protein levels in experiments. GAPDH imprinting was the same in each cell line, i.e. the level of protein loaded in each cell line was the same. In this experiment [Figure 5], caspase-3 was not detected in both negative and positive control groups and AMH-L group, suggesting that neither Staurosporine nor AMH-L could induce the lysis of caspase-3. Compared with the negative control group, AMH-L can induce the splitting of PARP in HCT-116 cells, but not the expression of PARP in

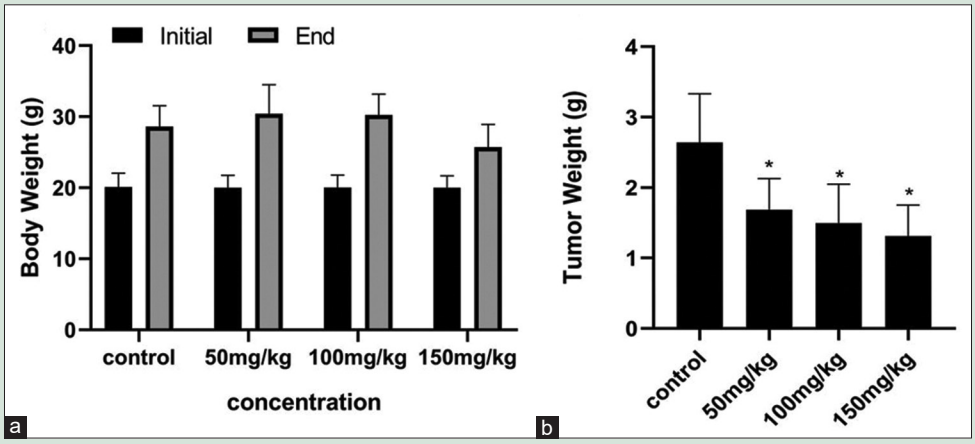

Figure 2: The effect of AMH-L on S-180 cells *in vivo*. (a) Effects of different concentrations of drugs on body weight of mice. (b) Effects of different concentrations of drugs on the quality of tumors in mice *$P < 0.05$, $n = 12$

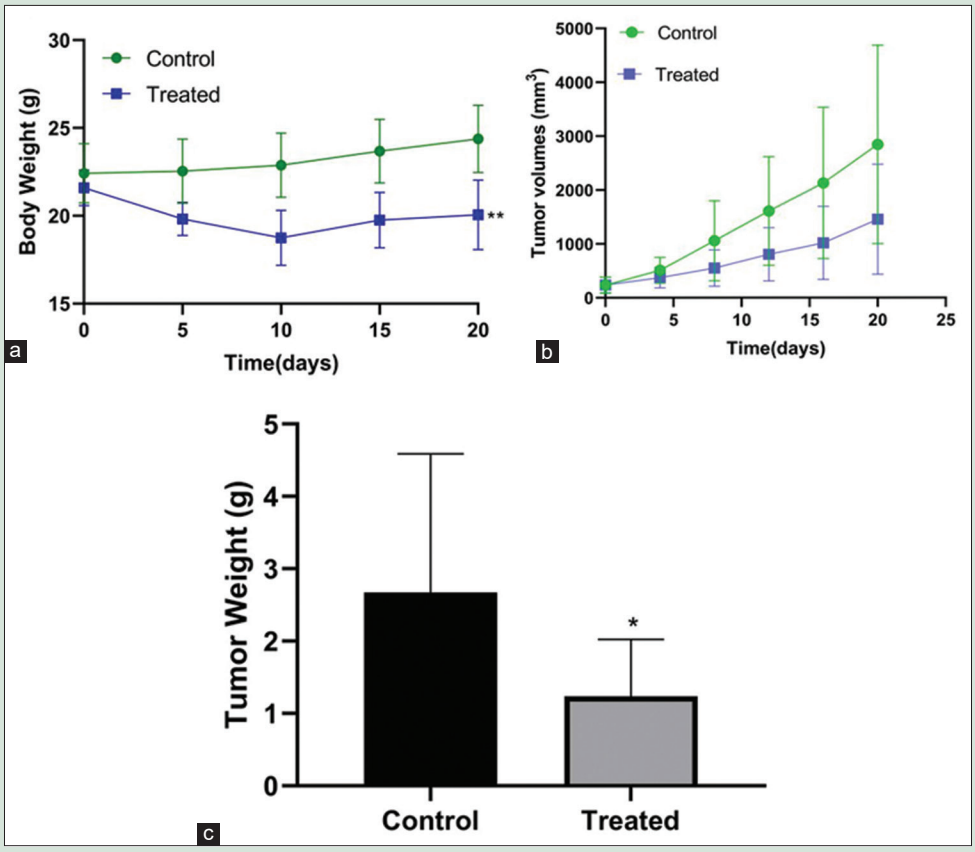

Figure 3: The effect of AMH-L on the tumorigenicity of NCI-H460 cells *in vivo*. (a) Body weight at each measurement of the experimental group and the control group. (b) Tumor volume at each measurement of the experimental group and the control group. (c) Tumor quality of the administration group and the control group at the end of the experiment, *$P < 0.05$, **$P<0.01$, $n = 8$

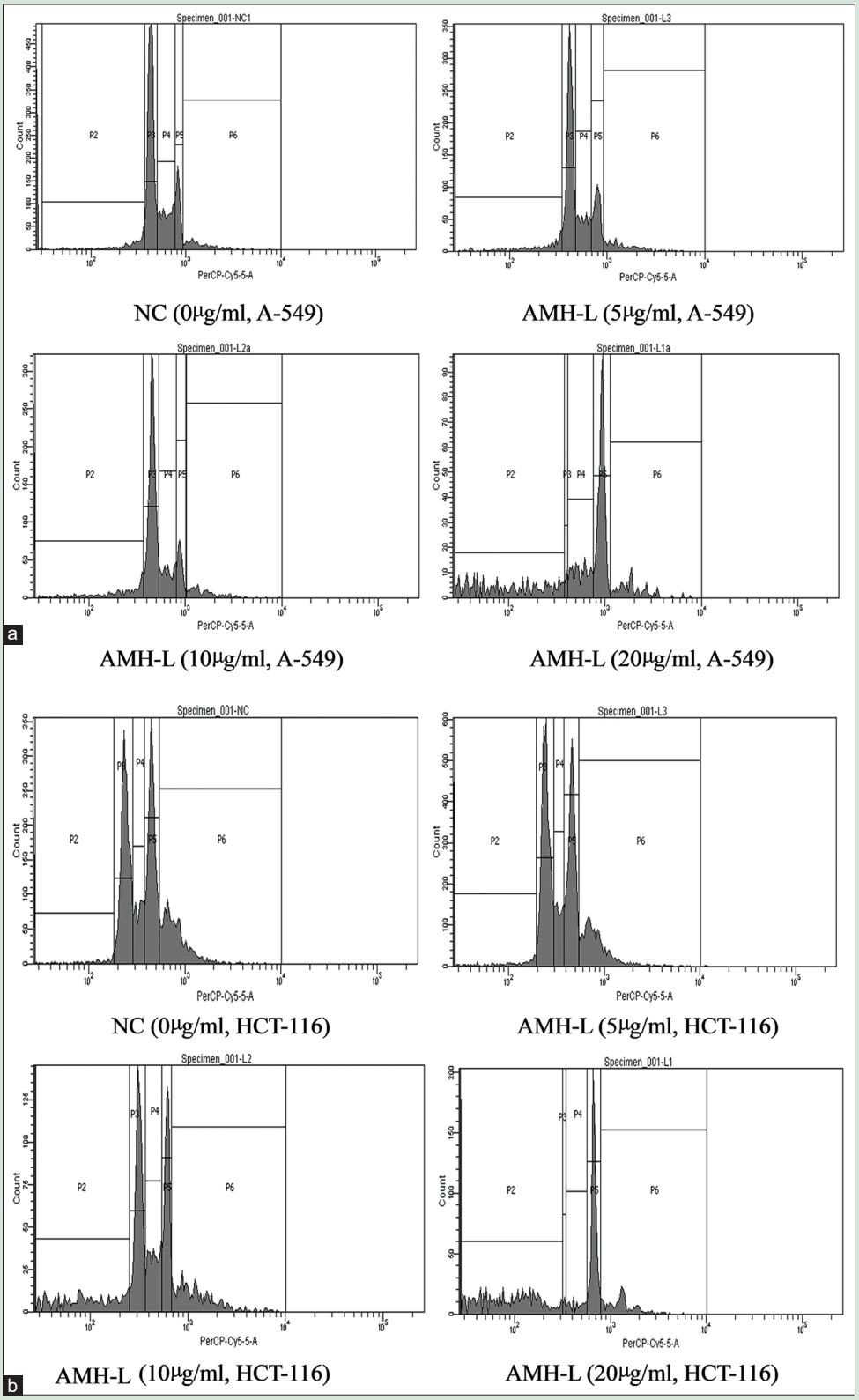

Figure 4: Effect of AMH-L on cell cycle. (a) AMH-L (5, 10, 20 μm/ml) was applied to A-549 cells for 24 hours. (b) AMH-L (5, 10, 20 μm/ml) was applied to HCT cells for 24 h

A-549 cells, indicating that the mechanism of AMH-L inhibiting the proliferation of the two cells is different.

In addition, there was no significant difference in the expression of PTEN between negative and positive control groups and AMH-L drug group, and PTEN could be lysed. This experiment showed that AMH-L, an active component of *L. cladonioides*, inhibited the proliferation of HCT-116 and induced the cracking of PARP. However, the inhibition of A-549 proliferation is not related to

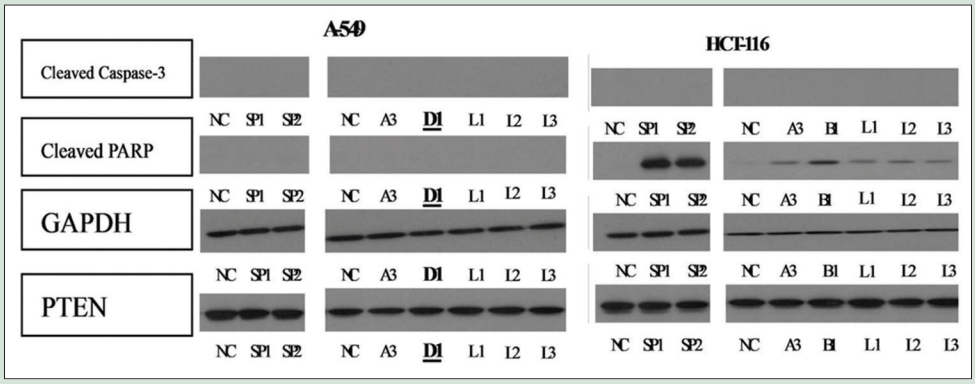

Figure 5: Effects of AMH-L on cell cycle arrest-related proteins in A549 and HCT-116 cells, cells were treated with AMH-L at a specified concentration for 24 h. Western blotting was used to detect the expression of cell cycle arrest-related proteins Caspase-3, poly (ADP-ribose) polymerase and PTEN. NC refers to negative control, SP1, SP2 are Staurosporine, L1, L2, L3 are AMH-L5u, 10u, 20u, A3, D1 are other drugs

Table 2: Effect of AMH-L on major organs in mice

Groups	Liver (%)	Spleen (%)	Kidney (%)	Testis (%)
Control	7.530±0.783	1.619±0.381	1.926±0.148	0.944±0.047
AMH-L (150 mg/kg)	7.863±0.712	1.711±0.484	2.091±0.334	0.805±0.139

Data represent mean±SD (n = 8), $^{*}P < 0.05$, $^{**}P < 0.01$

caspase-3, PARP and PTEN, and its inhibition mechanism needs to be further explored.

DISCUSSION

There are more than 175,000 lichens in the world. They are complex organisms composed of fungi and photosynthetical spouses.[14] Lichen-derived bioactive compounds offer great promise for biopharmaceutical applications, and some lichens have proven effective in anti-cancer research.[15-17] *L. cladonioides* (also known as Luxinxue Tea, Golden Silk Tea) is Larch and fir dried-up trees distributed in the snowy plateau bryophyte belt above 4000 m in Haibo. Black Snow Tea is extracted by drying, alcohol precipitation, chloroform, ether and ethyl acetate to obtain anti-tumor active components. This research group is named AMH-L. Sonolic acid is the main anti-bacterial and anti-inflammatory component of *L. cladonioides* and has strong cytotoxicity. Usnic acid is the main antibacterial and anti-inflammatory component of *L. cladonioides* and has a strong cytotoxic effect. Usnic acid is the main antibacterial and anti-inflammatory component of red snow tea and has a strong cytotoxic effect.[18-20] Our results of high performance liquid chromatography and TLC showed that there was no sonolic acid in AMH-L and the cell morphology treated with AMH-L was different from that treated with sonolic acid. Therefore, it is necessary to identify the anticancer bioactive compounds in the active component AMH-L. In this study, MTT results showed that AMH-L, an ethyl acetate extract from *L. cladonioides*, had strong anticancer activity against A-549 and HCT-116. IC_{50} was 26.17 ± 3.54, 16.21 ± 0.83. *In vivo* anticancer activity of sarcoma S-180 and human lung cancer NCI-H460 transplanted into Kunming mice and balb/c nude mice was studied. The results showed that AMH-L could effectively inhibit the growth of S-180 sarcoma in Kunming mice. After the experiment, there was no significant difference in body weight between the experimental group and the control group, but the quality of the tumor was significantly reduced compared with the control group [Figure 2]. For S-180 sarcoma, the inhibition rate was 50.45% when the dosage was 150 mg/kg. For NCI-H460 tumors in balb/c nude mice, from the recorded data, the volume of AMH-L (150 mg/kg) in the experimental group increased slowly on the 4th day of administration compared with that in the control group. However, there was no significant difference compared with the control group. The tumor quality at the end of the administration was significantly reduced compared with the control group [Figure 3c]. The tumor inhibition rate was 49.44%. In order to further study the mechanism of AMH-L inhibiting tumor growth, we studied the cell cycle and apoptosis of A-549 and HCT-116 cells. The results showed that [Figure 4] AMH-L inhibited cell proliferation by blocking A-549 cells at G2/M phase and interfering with normal cell division cycle, in HCT-116 cells, G0/G1 phase, S phase and G2/M phase decreased, especially in G0/G1 phase. The result may be to promote cell apoptosis, meanwhile, Western blotting [Figure 5] showed that AMH-L could promote apoptosis by inducing PARP lysis in HCT-116 cells, PARP, a DNA repair enzyme, is the cleavage substrate of caspase, the core member of apoptosis, and plays an important role in the repair of DMA and apoptosis, PARP can be sheared by many kinds of Caspase *in vitro*. It is the main shear target of Caspase-3 *in vivo*. It is also commonly considered as an indicator of Caspase-3 activation.[21,22] Decomposition of PARP results in a decrease in the level of PARP in the cell, which acts similarly to a PARP inhibitor. Cleavage of PARP promotes cell breakdown and acts as a marker for cells undergoing apoptosis.[23] Caspase-3 is considered to be one of the key performers of apoptosis[24] As a tumor suppressor,[25,26] PTEN is involved in a variety of human cancers. However, Caspase-3 was not activated in this result, it can be seen that PARP cleavage induced by AMH-L in HCT-116 cells is not caused by Caspase-3. Caspase-3 and PTEN were not involved in the apoptosis of HCT-116 and A-549 cells, especially the apoptotic mechanism of A-549 cells need further exploration.

CONCLUSION

Our study shows that AMH-L, an ethyl acetate extract of *L. cladonioides*, has strong antitumor activity *in vitro* and *in vivo*, its mechanism needs further study, therapeutic effect needs further improvement, and current studies suggest that it may be a potential anticancer drug.

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Conflicts of interest

There are no conflicts of interest.

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