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Anti-cervical cancer and anti-metastatic effects of sub-fraction 1a of *Solanum nigrum* L.

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In the present study, the crude polyssaccharides from *Solanum nigrum* L. was fractioned by DEAE-cellulose column chromatography, and was further purified by Sephadex-100 column chromatography. We investigated the anti-cervical cancer activity and anti-metastasis effects of the sub-fraction 1a of polysaccharides from *Solanum nigrum* L. (SNL-P1a) in tumor-bearing mice models, and explored the probable mechanism underlying the pharmacological activity of the polysaccharide. Mice were grouped into the model control, positive control and SNL-P1a of low and high dose treatment groups. After experiment ended, the tumor growth and the lung metastasis inhibition rates were calculated respectively. Furthermore, the serum antioxidant enzyme activities in mice were measured by spectrophotometer method. Results showed that SNL-P1a inhibited the growth and the metastasis of cervical cancer significantly. Moreover, SNL-P1a treatment increased serum antioxidant enzyme activity and LDH activity. These results suggested that the antioxidant activity of SNL-P1a might be beneficial to the cervical cancer therapy.

Keywords: Polysaccharides, *Solanum nigrum* L., Anti-cervical cancer, Anti-metastasis, Antioxidant, LDH

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Cancer of the uterine cervix is the second leading cause of death from cancer among women worldwide and uterine cervix tumor is also the most prevalent gynecological tumor in China. Usually, treatment for cervical cancer is a combined approach, including surgery, radiotherapy and chemotherapy, and it depends upon the histological type and the stage of cancer[1].

During the past three decades, many polysaccharides and polysaccharide-protein complexes have been isolated from fungi including mushrooms, yeasts, algae, lichens and plants. The biological activities of these polysaccharides have attracted more attention recently in biochemical and medical fields because of their immunomodulatory and antitumor effects[2].

Reactive oxygen species (ROS) can induce oxidative damage to DNA, lipids and proteins and result in the failure of cellular functions, through which tumors, inflammation, shock, atherosclerosis, diabetes, and ischemia occur[3]. Biological antioxidants are natural compounds which can prevent the uncontrolled formation of free radicals and activated oxygen species, or inhibit their reaction with biological structures. These compounds include antioxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GR) and non-enzymatic antioxidants, such as glutathione (GSH), vitamin C and vitamin E[4,5]. Accordingly, natural products and some synthetic drugs with antioxidant potential may have beneficial effects on the overall disease processes[6].

Metastasis of cancer cells is a major cause of cancer-related mortality. The development of cancer metastasis consists of multiple steps, in which cancer cells migrate from the primary tumor site, invade surrounding tissues, move through the blood or lymphatic system to distant tissues, extravagate from the vasculature, and eventually proliferate to form secondary tumors at new sites. Hence, inhibition of any of these processes could be an effective antitumor approach[7].

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Solanum nigrum L. (SNL) is an herbal plant that commonly grows in temperate climate zones. It has been used as a traditional folk medicine because of its diuretic and antipyretic effects. In the traditional Chinese medicine, it has been used for centuries to cure inflammation, edema, mastitis and hepatic cancer[8,9]. In our previous study, we also found that the crude polysaccharides isolated from SNL (SNL-P) and its purified sub-fraction 1a (SNL-P1a) could inhibit the growth of uterine cervical carcinoma (U14) and modulate tumor-bearing mice immune system[10~12]. However, there is still no report on whether the antitumor and anti-metastasis activity of it is correlated with its antioxidants effects on the body.

In the present study, we prepared SNL-P1a by column chromatography, and further evaluate the inhibition effects of SNL-P1a on the growth of U14 cervical cancer and lung metastasis. In addition, we demonstrated that the SNL-P1a could exert its tumor inhibition and metastasis suppression effects by enhancing the antioxidant activity in serum in mice models.

Methodology

Materials

Cyclophosphamide was purchased from Pude Pharmacy Inc. (Shanxi province, China); DEAE-Cellulose and Sephadex G-100 were purchased from Sigma; silica gel plates (Silica gel GF254) were purchased from Qingdao Haiyang Chemical Co.; Kits used for analyzing SOD, malondialdehyde (MDA), GSH-Px, T-AOC, alkaline phosphatase (AKP) and lactic dehydrogenase (LDH) were obtained from Nanjing Jiancheng Biology Engineering Research Inc. (Jiangsu province, China). All other chemicals used in experiments were of analytical reagent grade.

Preparation of SNL-P1a

Solanum nigrum L. was collected in October 2014 from Taihang Mountain in Hebei province, China. The whole plant was dried in the shade and authenticated by Dr JC Zhao at Hebei Normal University, China, where the herbarium voucher has been kept (E407). The method used for preparation of SNL-P1a was the same as we previously reported[12]. The quantity used in the experiments was based on the dry weight of SNL-P1a and the dose used in the experiment (25 mg/kg and 50 mg/kg) was based on our preliminary test.

Thin-layer chromatography (TLC) and infrared spectra of SNL-P1a

TCL was performed on a silica gel plate. An aliquot of each sample was spotted onto the silica gel plate with a developing solvent system of chloroform/methanol (10:1, v/v) or petroleum ether/ethyl acetate (2:1, v/v). The spots were visualized by spraying the plates with spraying the plates with spraying solutions of 1 % solution of phenylamine-diphenylamine-phosphate in water.

The IR spectrum of SNL-P1a was determined, using a Fourier-transform infrared spectrophotometer (FTIR, Nicolet, USA) equipped with an OMNIC work station. SNL-P1a was ground with KBr powder (spectroscopic grade) and then pressed into a 1mm pellet for FTIR measurement in the frequency range 4000-500 cm^{-1} .

Cell line and animals

Uterine cervical carcinoma (U14) cell line was obtained from Institute of Medical Material, Chinese Academy of Medical Sciences. Balb/c mice were provided by the Experimental Animal Center of Xiehe Medical University. They were maintained under temperature-controlled room at 20±2 °C, and kept in groups of 10 animals per cage, provided with a standard pellet diet and water. All experiments were carried out using 6-8-week old mice weighting 20.0±2.0 g. The animals were treated according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and their experimental use was approved by the Animal Ethics Committee of Yanshan University (Ethics number: YD2015004).

Effect of SNL-P1a on tumor growth

Animals were randomly divided into test groups consisting of 10 mice per group. Under the sterile condition, 7-10 days old ascites was collected from the mice and was diluted to about 1×10^7 cell/mL with sterile physiological saline. 0.2 mL of diluted ascites was injected into the left axilla s.c of each mouse (day 0). After 24 h of inoculation, SNL-P1a was supplied daily by intraperitoneal injection at doses of 25 and 50 mg/kg. The group administered with vehicle alone (sterile physiological saline, i.p.) was taken as control treatment, and the group treated with Cyclophosphamide (CTX, 25 mg/kg, i.p.) was considered as the standard reference drug. All groups were continuously treated for 13 days and during the experiment time; all of the animals were fed with a standard pellet diet and water ad libitum. The maximum diameter (*a*) and minimum diameter (*b*) of transplanted tumors were measured after tumor formation, body weight and tumor volumes were monitored every two days.

The tumor volume was expressed according to the following formula:

$$V = (a \times b^2)/2$$

where, V stands for the volume of the measured tumor, a is the maximum and b is the minimum diameter of the measured tumor [13].

Effect of SNL-P1a on lung metastasis of U14 cells

The experimental metastasis assay was carried out by the method described previously (Welch 1997). Animals were randomly divided into 4 test groups consisting of 10 mice per group. Under the sterile condition, 7-10 days old ascites was collected from the mice and was diluted to about 5×10^5 cell/mL with sterile physiological saline. 0.1mL cell suspensions were injected into mice via tail vein. The groups of negative control, CTX positive control, SNL-P1a (L) and SNL-P1a (H) were administrated continually as above for 13 days. The mice were sacrificed on day 14 after the injection of U14 cells, and the number of metastatic colonies on the lung surface was counted under microscopic observation, the lung metastasis inhibition rate was calculated using the following formula:

$$\text{Inhibition rate} = (Nc-Nt)/Nc\times100\%$$

where, Nc stands for the mean number of metastatic colonies of control group, Nt stands for the mean number of metastatic colonies of treated group[14].

Preparation of serum samples in tumor bearing mice

On the 14th day, the mice of all groups were sacrificed and blood of treated and control mice was collected in heparinized tubes and plasma was separated. The blood was immediately homogenized in 0.1M Tris–HCl, *p*H 7.4, plasma homogenate was used for various analyses.

Modulatory effect of SNL-P1a on serum antioxidant enzymes activities in tumor-bearing mice

Activities of serum SOD, CAT, GSH-Px and T-AOC activities in control, CTX and SNL-P1a treated mice were measured according to the recommended methods of reagent kits, respectively.

Effect of SNL-P1a on serum MDA level

Activities of serum MDA levels in control, CTX and SNL-P1a of low and high dose treatment mice were measured according to the recommended method of reagent kit, and the color reaction was measured at 532 nm.

Effect of SNL-P1a on serum LDH and AKP activities of tumor-bearing mice

Activities of serum LDH and AKP in control, CTX and SNL-P1a treated mice were measured according to the recommended method of reagent kits, respectively.

Effect of SNL-P1a on liver and kidney in tumor bearing mice

To further assess the toxicity of SNL-P1a, the liver and kidney in SNL-P1a (50 mg/kg) treated mice were excised and fixed in 4 % formalin, embedded in paraffin, and cut in 4 μm sections for histology study.

Statistical analysis

The data obtained were analyzed statistically by One-way ANOVA method in Graph Pad Prism5 statistical software. Significance of any differences between groups was evaluated using Student' *t*-test. All values in tables and figures were expressed as mean: ± S.D.

Results

Preparation and characterization of SNL-P1a

SNL-P1a isolation and purification were based on our previously published work[12]. Water-soluble polysaccharides was obtained by water extraction and ethanol precipitation, and then subjected to DEAE-cellulose ion exchange chromatography with NaCl elution resulting in SNL-P1, SNL-P2 and SNL-P3 peaks. The SNL-P1 was the major fraction of the three, so it was further separated into SNL-P1a, SNL-P1b and SNL-P1c by gel permeation chromatography. SNL-P1a was identified to be a homogeneous polysaccharide component and the retention time was 7.771 min by HPLC. In addition, the monosaccharide composition of SNL-P1a was analyzed by TLC and revealed the presence of glucose, rhamnose, arabinose and xylose in SNL-P1a. According to the IR spectrum, the purified SNL-P1a displayed an intense O-H vibration absorption peak near 3435 cm^{-1}, and a weak C–H peak at around 2905 cm^{-1}, which showed that SNL-P1a is a carbohydrate. The relatively strong absorption peak at around 1650~1550 cm^{-1} indicated the amino existence in SNL-P1a. The absorbance of polysaccharides in the range 1000–1200 cm^{-1} was the pyranoid ring C–O–C and C–O–H link-band positions. The absorption peak at around 1083 cm^{-1} indicated the existence of β (1-3) glucosidic bond.

Effect of SNL-P1a on transplanted tumor growth

To evaluate the antitumor activity of SNL-P1a *in vivo*, we created mouse cervical carcinoma model by s.c. injection of U14 cells into mice. After tumor formation, we monitored the tumor growth by accumulating their volumes. As shown in Fig. 1, the tumor volumes in control, CTX, SNL-P1a (L) and SNL-P1a (H) treatment groups all increased with the increment of inoculation time. Among them, tumors in control mice grew the fastest and tumor volume reached 1986.4±107.9 mm^3 on 14^{th} day after inoculation. The growth of tumors in 25 mg/kg and 50 mg/kg SNL-P1a treatment groups slowed down, and tumor volumes reached 1256.3±56.9 mm^3 and 987.4±56.6 mm^3 respectively on 14^{th} day after inoculation, the growth inhibition rate was 36.75±3.17 % and 50.29±3.69 %, respectively. In CTX treatment group, the tumor grew the most slowly, and its tumor growth inhibition rate reached 80.20±4.82 %.

Effect of SNL-P1a on lung metastasis of U14 cells

After notified that SNL-P1a inhibited the growth of U14 tumor, we further evaluated the anti-metastatic activity of SNL-P1a by *in vivo* experimental lung metastasis assay, in which U14 cells were injected into tail veins of Balb/c mice. The result showed that intraperitoneal administration of SNL-P1a (25 mg/kg or 50 mg/kg) from day 1 to day 13 after tumor injection resulted in significant decrease in the number of metastatic nodules on the lung surface compared to negative control group ($p < 0.05$, $p < 0.01$) (Fig. 2). Body weights of mice were slightly reduced during SNL-P1a-treatment, but they recovered to normal levels at the end of the experiment (data not shown).

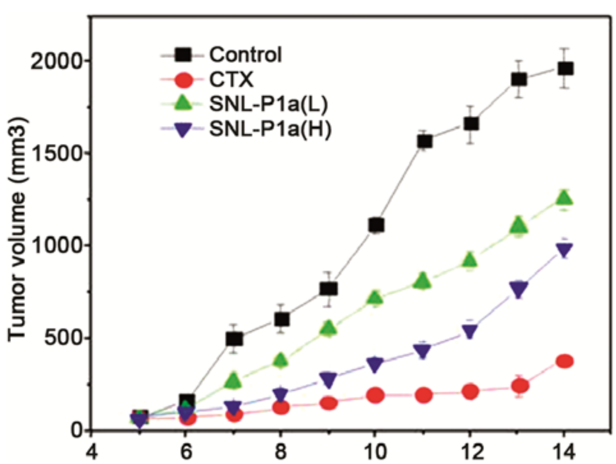

Fig. 1—Effect of SNL-P1a on tumor volume growth of mice

Modulatory effect of SNL-P1a on serum antioxidant enzymes activities in tumor-bearing mice

Previous reports demonstrated that one of the important roles in the pathogenesis of cancers is free radical reactions induced by reactive oxygen species (ROS). To determine SNL-P1a effect on serum ROS activity, we examined SNL-P1a effect on the activities of SOD, CAT, GSH-Px and T-AOC in tumor-bearing mice. Table 1 showed the effect of SNL-P1a on serum SOD, T-AOC, CAT and GSH-Px activities in tumor-bearing mice. Compared with the model control group, the administration of both CTX and SNL-P1a of high dose significantly increased serum SOD, CAT, GSH-Px and T-AOC activities in tumor-bearing mice ($p < 0.05$, $p < 0.01$). Low dose

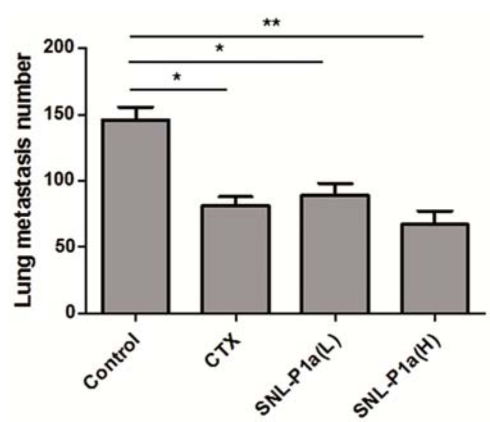

Fig. 2—Effect of SNL-P1a treatment on U14 cervical cancer pulmonary metastasis in mice. **and * represents significant differences compared with the control, $p < 0.01$ and $p < 0.05$, respectively.

Table 1—Effect of SNL-P1a on serum activities of SOD, CAT, GSH-Px and T-AOC in tumor-bearing mice ($\bar{x}$ ±S.D, n=10)

Groups	Treatment (mg/kg)	SOD (U/mL)	CAT (U/mL)	GSH-Px (U/mL)	T-AOC (U/mL)
Control	Vehicle	128.13±8.72	1.45±0.09	13.21±1.72	2.36±0.61
CTX	25	169.49±9.77 **	3.66±0.10 **	19.77±1.98 *	9.59±1.32**
SNL-P1a (L)	25	144.32±5.48	2.49±0.17 **	16.66±1.65	9.18±1.97 **
SNL-P1a (H)	50	163.39±7.57 **	2.99±0.13 **	19.47±2.01 *	12.31±4.44 *

**and * represents significant differences compared with the control, $p < 0.01$ and $p < 0.05$, respectively.

of SNL-P1a could also increase the serum CAT and T-AOC activities significantly ($p < 0.01$), but the increment effects of this component on serum SOD and GSH-Px activities were not significant.

Effect of SNL-P1a on serum MDA level in tumor-bearing mice

Fig. 3 showed the effect of SNL-P1a on serum MDA levels in tumor-bearing mice. Compared with the model control group, the MDA level in SNL-P1a of low and high dose treatment groups were all decreased significantly, CTX treatment also reduced the MDA in cervical cancer mice, but did not show significant difference compared with the model control group.

Effect of SNL-P1a on serum LDH and AKP activities in tumor-bearing mice

The release of LDH is a well-known method for the quantification of cell damage. As shown in Table 2, compared with the model control group, the activity of LDH in CTX and SNL-P1a of low and high dose treatment groups both deceased significantly ($p < 0.01$, $p < 0.01$). Our results showed that the activity reduction of serum LDH in SNL-P1a treatment mice might be correlated with the inhibition effect of this component on tumor growth.

Table 2—Effects of SNL-P1a on serum AKP and LDH activities of tumor-bearing mice ($\bar{x}$ ±S.D., n=10).

Groups	Treatment (mg/kg)	AKP (U/L)	LDH (U/L)
Control	vehicle	109.36±15.78	6158.40±43.36
CTX	25	87.58±20.93	5488.29±51.39 **
SNL-P1a(L)	25	117.76±17.31	5750.89±26.71**
SNL-P1a(H)	50	132.90±44.43	5147.77±41.12 **

** represents significant differences compared with the control, $p < 0.01$.

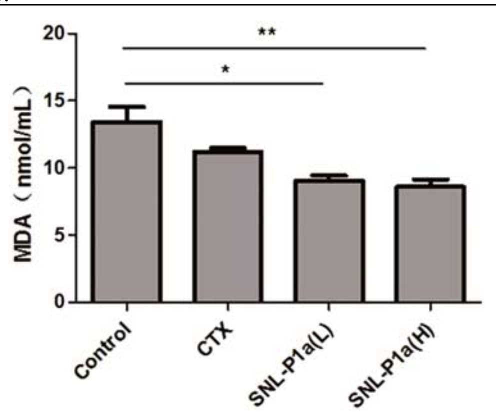

Fig. 3—Effect of SNL-P1a on serum MDA level in tumor-bearing mice. **and * represents significant differences compared with the control, $p < 0.01$ and $p < 0.05$, respectively.

AKP is a phosphate hydrolase, which enables the transfer of inorganic phosphates to an acceptor substrate[15,16]. Many studies report that AKP dephosphorylates proteins involved in cell growth and differentiation, apoptosis and cell migration[17,18]. Abnormal expression of AKP isoenzymes has been found in malignant tissues, being often established as a useful prognostic indicator[19,20]. As shown in Table 2, compared with the model control group, serum AKP activity in tumor-bearing mice of low and high dose SNL-P1a and CTX treatment group did not change significantly ($p > 0.05$).

Effect of SNL-P1a on liver and kidney

To determine if SNL-P1a had any side effects on liver and kidney, a pathological examination of liver and kidney tissues was conducted. There was no obvious pathological change in the liver and kidney tissues of SNL-P1a (50 mg/kg) treated mice. The sections of liver and kidney showed that the central vein and hepatic lobule were distinct; hepatocellular disposed compact and orderly, glomerular and renal tubular were also evident (Fig. 4).

Discussion

Polysaccharides are important components of plants, fungi, yeast, algae and lichens, and have attracted more and more attention in the biochemical and medical areas due to their immunomodulatory and antitumor effects[21]. SNL-P1a is a novel polysaccharide isolated from *Solanum nigrum* L. and could significantly suppress the growth of U14

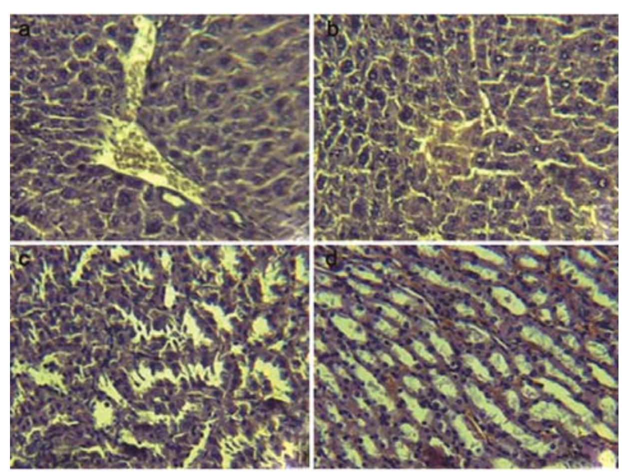

Fig. 4—Pathology sections of liver (A, B) and kidney (C, D) of SNL-P1a (50 mg/kg b.w.) treated mice (HE ×100), which showed that central vein and hepatic lobule were distinct, hepatocellular disposed compact and orderly, glomerular and renal tubular were also evident.

cervical cancer and modulate the immune system in tumor-bearing mice[12]. The present study further assessed its serum antioxidant enzymes modulatory effects in tumor-bearing mice and analyzed the relationship between the antitumor effect, anti-metastasis effect and its antioxidation activity, and suggested a potential therapeutic role of polysaccharides isolated from plants in the treatment of cervical cancer.

Oxidation is essential to many organisms for the production of energy to fuel biological processes. However, ROS are often over-produced under pathological conditions, resulting in oxidative stress[22,23]. There is a natural dynamic balance between the amount of free radicals generated and the antioxidant defense system, which protects the body against the pathogenesis involved in some degenerative diseases.

In our study, we can see that SNL-P1a treatment could up-regulated the serum SOD activity in tumor-bearing mice. It has been previously reported that administration of polysaccharides from some plants could prevent GSH depletion and lipid peroxidation, and increase SOD activity in the tissues of rats with the cancer[24,25]. Such antioxidant activity could be explained by the fact that SNL-P1a stimulated synthesis of enzymes involved in free radical production, creating a powerful redox cycle that allows for continuous cytoprotection against oxidative stress. Compared with model control group, SNL-P1a treatment dose-dependently significantly enhanced the serum SOD, CAT and GSH-Px activities in tumor-bearing mice. The activities of SOD, CAT and GSH-Px are known to serve as protective responses to eliminate reactive radicals[26]. The observation that the seum levels of SOD, CAT and GSH-Px were up-regulated to a higher level in tumor-bearing mice indicates that the tissues restored to its normal activity by the protection of SNL-P1a. This fact was further substantiated by the decrease in the level of MDA upon SNL-P1a administration.

T-AOC reflects the capacity of non-enzymatic antioxidant defense system. Therefore, measurement of serum T-AOC could give a more precise indication of the relationship between antioxidants and the tumor growth inhibition in cervical cancer bearing model mice[27]. Our results showed that SNL-P1a treatment caused a dramatic increment in serum T-AOC activities, which further indicated that the tumor growth inhibition effect of SNL-P1a treatment had certain relationship with its antioxidant activity.

The MDA value is measured as biomarkers of lipid peroxidation. Lipid peroxidation is a very sensitive biomarker of oxidative stress in detecting the antioxidant effects of SNL-P1a. In this study, compared with model control group, treatment with SNL-P1a resulted in a reduction level of serum lipid peroxidation, demonstrating the protective effects of SNL-P1a to the cervical cancer-bearing mice.

Cancer cells produce and retain LDH to maintain growth of the tumor and the increased serum LDH level is well known as a common characteristic in humans and animals with malignant tumors[28], and it is believed that this elevation is caused by enzyme leakage from dead cancer cells occurring during high tumoral cell turnover[29]. Our results showed that SNL-P1a treatment could down-regulate the serum LDH level compared with that of the model control group, which could be explained since SNL-P1a inhibited the growth of tumor cells and further reduced the leakage of LDH from tumor cells.

AKP is a kind of phosphate ester, and exists widely in human body tissue and body fluids. Researches had shown that primary liver cancer could lead to the increment of alkaline phosphatase activity, and the level of serum AKP had reference value on bone metastases diagnosis[30,31]. In our study, we have observed that AKP activity in SNL-P1a treatment group remains unchanged, which maybe because that our short treatment with SNL-P1a to the mice did not affect the AKP level in time.

Intraperitoneal administration of SNL-P1a resulted in significant reduction of metastatic nodules of the lung surface compared to the untreated control group. Although the exact mechanism of action of SNL-P1a in the in vivo experimental tumor-bearing and metastasis models are still unclear, host anti-oxidation strengthen by SNL-P1a may be partly responsible for the suppression of U14 growth and metastasis.

Conclusion

The doses of SNL-P1a such as 25 and 50 mg/kg used in this study could not only significantly inhibit the growth of transplanted tumor and lung metastasis in mice, but also improve the antioxidant enzymes such as SOD, CAT, GSH-Px and T-AOC in the serum activity, and reduce oxidative stress of cervical cancer mice. These results suggested that the antioxidant activity of SNL-P1a might be beneficial to the cervical cancer therapy. Further studies regarding their anticancer activities of other cell lines, and their mechanisms are necessary.

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