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Phosphorylation and anti-tumor activity of exopolysaccharide from *Lachnum* YM120

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ABSTRACT

Phosphorylated polysaccharide PLEP-1a, with the PO_4^{3-} content of 6.39%, was prepared from LEP-1a by phosphorylation. IR, ^{13}C NMR and ^{31}P NMR results of PLEP-1a showed that the original basic structure of the polysaccharide was not changed, and the $-H_2PO_3$ group was linked at C_6 of LEP-1a. The results of anti-tumor experiments *in vivo* showed that 100 mg/kg and 400 mg/kg of LEP-1a could significantly improve the food consumption, body weight, tumor inhibition rate and thymus index of S180 sarcoma mice, and increase the levels of SOD, IL-2 and TNF-α in mice blood serum, indicating that LEP-1a had an excellent anti-tumor activity. Furthermore, PLEP-1a had a significantly enhanced inhibitory effect on S180 sarcoma mice than LEP-1a, suggesting that phosphorylation is an effective way of improving the biological activity of LEP-1a.

1. Introduction

Malignancy is a common clinical disease, which is also one of the major causes of human death (Ren et al., 2010). Malignancy is primarily treated by radiotherapy, chemotherapy and other methods. Acute side effects of radiotherapy can occasionally be life threatening, it may affect patient compliance in addition to making them vulnerable to the adoption of alternative forms of treatment which promise cure (Yarney et al., 2013). The frequently used chemotherapy drugs are mainly synthetic substances, which can kill tumor cells and simultaneously have a certain degree of killing effect on normal cells (Huang, Hou, & Zhu, 2004; Huang, Zhang, Zou, & Ji, 2004; Yarney et al., 2013). In addition, chemotherapy will cause relatively serious side effects such as loss of appetite and weight, body weakness, and immunosuppression in patients (Mikó et al., 2005). Therefore, exploring and developing natural anti-tumor drugs without toxic side effects are of great significance for human health protection.

Fungal polysaccharides are secondary metabolites of fungi, as immunomodulating agents, they have significant anti-tumor effects (Chen & Lu, 2002; Shen & Tao, 2003). For example, lentinan with a main chain structure of β-1,3-glucan has significant anti-tumor and immune-promoting effects (Li & Wang, 2002). Polyporus polysaccharide, which is also a β-1,3-glucan, performed potent anti-tumor effect by regulating the immune system through improving the activity of inducible nitric oxide synthase (iNOS), increasing the expression level of mRNA and stimulating the synthesis of NO by macrophages (Huang, Hou, et al., 2004; Huang, Zhang, et al., 2004). It has been reported that biological activities of polysaccharides depend on their structure (Zhang, Qiu, Mo, Wang, & Wu, 2008). Modification of polysaccharides such as sulfation, phosphorylation, acetylation, alkylation, sulfonylation and carboxymethylation can improve original activities or acquire new activities (Ge, Zhang, & Sun, 2008; Wang, Li, Guo, & Hu, 2002). Phosphorylation of polysaccharide is a kind of covalent modification, during which the hydroxyl group on the branched chain is substituted by phosphate radical (Andreas, Tom, Lone, René, & Søren, 2002). Yuan et al. (2005) found that *in vitro* antioxidant activity of phosphorylated carrageenan oligosaccharides increased obviously. Liu, Wan, Lin, and Lu (2011) also reported that the anti-herpesvirus activity of polysaccharide from *Polygonatum cytonema* was increased significantly after phosphorylation. However, study about the anti-tumor activity of the phosphorylated polysaccharide has not been reported.

Lachnum sp. is saprophytic fungi, which can produce a large number of polysaccharides with hypoglycemic, antioxidative, anti-aging activities under submerged culture conditions. In recent years, we have found that homogeneous components of polysaccharides produced by *Lachnum* YM278 and YM261 were both glucans linked by β-1,3-D-glucopyranoside (Ye et al., 2011, 2012). However, modification and activity change after modification of the polysaccharides are not investigated. Recently, we have found that the homogeneous component of exopolysaccharide from *Lachnum* YM120 (LEP-1a) was also a glucan linked by β-1,3-D-glucopyranoside. And this study is intended to modify LEP-1a by phosphorylation and

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evaluate the anti-tumor activities of LEP-1a before and after phosphorylation.

2. Materials and methods

2.1. Materials and reagents

LEP-1a is a homogeneous component of exopolysaccharide purified from *Lachnum* YM120, which are preserved in Microbial Resource and Application Research Center of Hefei University of Technology.

Superoxide dismutase (SOD), catalase (CAT), mice interleukin-2 (IL-2) ELISA and tumor necrosis factor-α (TNF-α) ELISA kits were all purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China); cyclophosphamide was purchased from Hualian Pharmaceutical Co., Ltd. (Shanghai, China); other reagents were all analytical reagents, which were purchased from Shanghai Zhenqi Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Experimental animals

Ninety Kunming mice (45 male and 45 female), with the body weight of 20 ± 2 g, were purchased from Experimental Animal Center of Anhui Medical University, Hefei, China (Certificate number: Experimental Animal Standard No. 1 of Anhui Hospitals). The animals were kept at 23 ± 2 °C with the humidity of 55 ± 5%, and cultured in a 14 h:10 h light–dark cycle.

S180 sarcoma cells were provided by Institute of Functional Nano & Soft Materials Laboratory of University of Science and Technology of China.

2.3. Phosphorylation reaction

Phosphorylation was performed according to the method described by Zhang et al. (2008) with minor modification. Phosphorylation reagents (8.57 g sodium tripolyphosphate and 1.43 g sodium trimetaphosphate) were dissolved in 100 ml double-distilled water and then 1 g LEP-1a was added. The reaction was performed at 80 °C for 5 h after the pH was adjusted to 9 by $NaHCO_3$, then the solution was precipitated with three volumes of 95% ethanol for 24 h and freeze dried. The obtained sample was re-dissolved in water, dialyzed in dialysis bag (with molecular weight cutoff of 13,000 Da) with distilled water for 24 h, and freeze dried to get the phosphorylated polysaccharide (PLEP-1a).

2.4. Determination of PO_4^{3-} in PLEP-1a

Standard curve of PO_4^{3-} was established according to the method of Sun, Pan, Zeng, and Cao (2011). 5 ml of PO_4^{3-} standard solution at different concentrations (0–20 μg/ml) was added into different colorimeter tubes firstly, then 3 ml of 0.1 M tris buffer solution (pH 7.0) and 3 ml of constant phosphorus reagent were added. The mixtures were placed in a 45 °C water bath for 30 min and the absorbance value was measured at 580 nm, respectively. The standard curve of PO_4^{3-} was drawn with the PO_4^{3-} concentrations as abscissa and absorbance values as ordinates.

PO_4^{3-} content of PLEP-1a was determined by the molybdenum blue colorimetric method (Sun et al., 2011). 0.1 g of PLEP-1a was placed in a beaker and added with 1 ml H_2SO_4 and 1 ml HNO_3. Then the mixture was heated to smoke, cooled and 1 ml of 30% H_2O_2 was added, and then slowly heated. The above steps were repeated until the solution no longer smoke. Then 1 ml of 6 M HCl was added to the beaker and the mixture was heated until the acid was completely decomposed. The absorbance value of the solution was measured according to the method used in standard curve establishment and the PO_4^{3-} content was calculated according to the regression equation of the standard curve.

2.5. Infrared spectra

IR spectra of LEP-1a and PLEP-1a were performed by Nexus670 Fourier transform-infrared spectroscopy (Thermo Nicolet, USA) with the scan range of 4000–400 cm^{-1} (Kumara, Jooa, Choib, Kooc, & Chang, 2004).

2.6. NMR spectra

^{13}C NMR of LEP-1a and PLEP-1a were performed using Bruker AV-500 NMR spectrometer (Bruker, Germany). And ^{31}P NMR of PLEP-1a was also detected by the mentioned NMR spectrometer. Chemical shifts were given in ppm.

2.7. Anti-tumor activity

2.7.1. Establishment of S180 sarcoma mice model

Mice S180 sarcoma cells were cultured in complete medium (DMEM high glucose medium containing 10% fetal bovine serum) at 37 °C for 3 d. The concentration of the cells was adjusted to 2×10^6/ml. Twenty Kunming mice were intraperitoneally injected with 0.5 ml cell suspension for tumor cell proliferation *in vivo*, respectively. After breeding for 7 d, ascites of the mice under good growth status were gathered and centrifuged at 3000 rpm for 3 min, and the cells were diluted with sterile saline to a concentration of 10^7/ml. The S180 tumor cells were inoculated into the Kunming mice by subcutaneous injection at the right oxter at a dose of 0.2 ml per mice. After breeding for 4 d, the mice with sarcoma in diameter about 0.5 cm were considered as established successful models (Song, Lu, & Li, 2012).

2.7.2. Grouping and treatment

Sixty model mice were randomly divided into 6 groups: model control group (0.9% saline), positive control group (25 mg/kg cyclophosphamide), low-dose LEP-1a group (100 mg/kg), high-dose LEP-1a group (400 mg/kg), low-dose PLEP-1a group (100 mg/kg) and high-dose PLEP-1a group (400 mg/kg). Ten healthy mice were used as the blank control group. Each mouse was weighed respectively, and then given 0.2 ml drug by gavage for 10 d. Daily food consumptions were recorded and the average values were calculated.

2.7.3. Determination of tumor inhibition rate and immune organ indices

The second day after last gavage, mice were weighed and killed by dislocation of the neck. The tumors were separated and weighed, the inhibitory rates were calculated. Spleens and thymuses were taken and weighed, and organ indices were calculated.

$$\text{Inhibitory rate}\ (\%) = \frac{\text{Tumor weight of the mouse in model control group} - \text{Tumor weight of the mouse in treatment group}}{\text{Tumor weight of the mouse in model control group}} \times 100.$$

$$\text{Organ index}\ (\%) = \frac{\text{Organ weight}}{\text{Body weight}} \times 100.$$

2.7.4. Measurement of SOD, IL-2 and TNF-α

The blood was collected by eye enucleation and the serum was separated by centrifugation at 4000 rpm for 10 min at 4 °C.

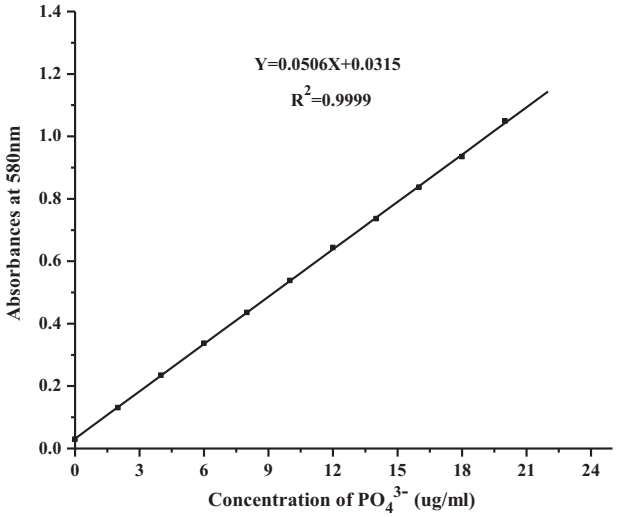

Fig. 1. The standard curve of PO_4^{3-}.

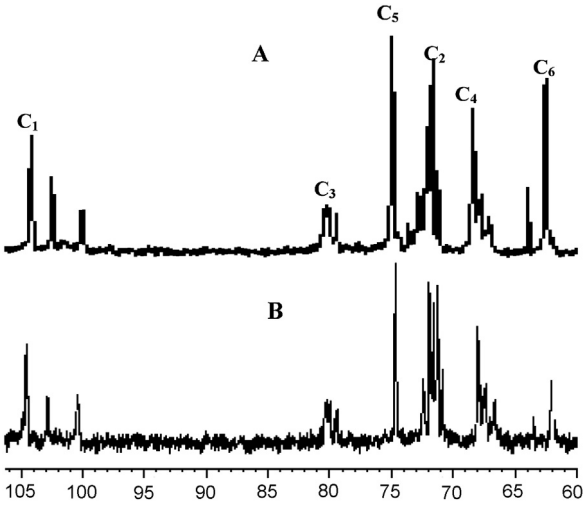

Fig. 3. ^{13}C NMR spectra of LEP-1a and PLEP-1a at 27 °C.

Determinations of SOD, IL-2 and TNF-α were performed in accordance with the kit instructions at room temperature (25 °C).

2.8. Statistical analysis

All data were presented as mean ± SD after statistical analysis, *t*-test of intergroup difference was processed with software SPSS13.0. Differences were considered statistically significant when $P < 0.05$ and very statistically significant when $P < 0.01$.

3. Results and discussion

3.1. Content of PO_4^{3-} in PLEP-1a

Fig. 1 showed the standard curve of PO_4^{3-} and the PO_4^{3-} concentrations were on the abscissa and the absorbance values at 580 nm were on the ordinate. The regression equation of $y = 0.0506x + 0.0315$, $R^2 = 0.9999$ was obtained. The content of PO_4^{3-} in PLEP-1a was calculated to be 6.39%.

3.2. Infrared spectra analysis

Fig. 2B showed two characteristic absorption peaks of PLEP-1a, the one at 1257.208 cm^{-1} was caused by the stretching vibration of P═O and the other one at 986.844 cm^{-1} was caused by the stretching vibration of the P—O—C (Yuan et al., 2005). Beyond that, parameters such as the absorption waveform, the wave number, the absorption peak intensity and the peak width in Fig. 2B were all basically similar to those in Fig. 2A. These results suggested that they both LEP-1a and PLEP-1a had the typical characteristic absorption peaks of β-D-1,3-glucopyranoside (Ye et al., 2011), and indicating that the original basic structure of LEP-1a was not changed after phosphorylation.

3.3. NMR spectra analysis

Compared with LEP-1a (Fig. 3A), the chemical shifts of C_1, C_2, C_3, C_4 and C_5 in PLEP-1a (Fig. 3B) were not changed significantly, while the signal peak at C_6 (δ 62.189) in PLEP-1a was significantly reduced, which was caused by the combination of —OH at C_6 with $—H_2PO_3$ group (Liu et al., 2011). The peak at 4.45 ppm (Fig. 4) was the signal peak of P in $—H_2PO_3$ group, indicating that LEP-1a was

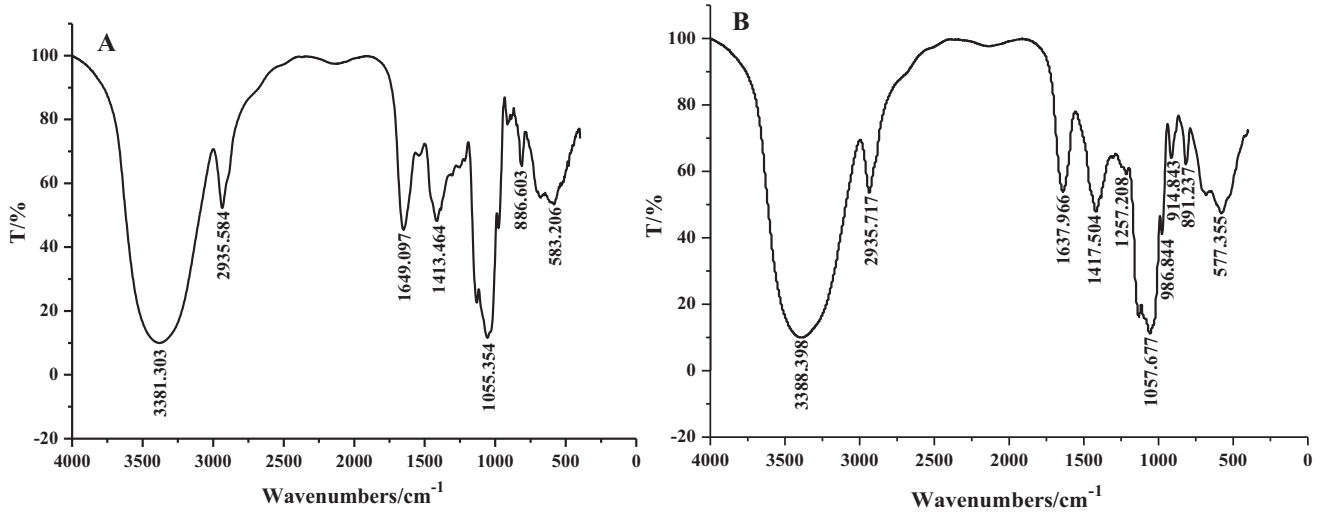

Fig. 2. Infrared spectra of LEP-1a and PLEP-1a.

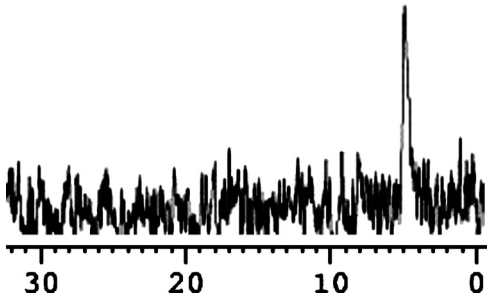

Fig. 4. ^{31}P NMR spectrogram of PLEP-1a at 27 °C.

modified successfully (Suflet, Nicolescu, Popescu, & Chitanu, 2011). In summary, the basic structure of β-D-1,3-glucopyranoside of LEP-1a was not changed after phosphorylation, and the $-H_2PO_3$ group was linked at C_6 of the LEP-1a.

3.4. Effects of LEP-1a and PLEP-1a on S180 sarcoma in mice

Compared with the model group, food consumption of mice in LEP-1a and PLEP-1a groups were all increased in varying degrees (Table 1), whereas the food consumption of mice in positive control group was decreased by 26.06% ($P < 0.01$). Weight gain rates of mice in LEP-1a and PLEP-1a groups were significantly lower than those in model group, but had no significant difference with those in normal group, indicating that LEP-1a and PLEP-1a could significantly inhibit the growth of tumor in mice without inhibitory effects on the growth of weight. Weight growth rates of mice in positive control group were significantly reduced, which indicated that cyclophosphamide (chemotherapy drug) not only inhibited the growth of tumor in mice, but also inhibited the weight of mice. Compared with the mice in LEP-1a and PLEP-1a groups, phenomena including sluggish, dull fur, piloerection and activity reduce were observed in the mice in positive control group. These manifestations might be due to the decline in immune function caused by chemotherapy drug (Chen, Lin, Deng, & Kuang, 2008), showing that cyclophosphamide had significant side effects on S180 sarcoma mice.

Both 100 mg/kg and 400 mg/kg doses of LEP-1a showed significant inhibitory effects on tumors with a dose–effect relationship (Table 1). Tumor inhibitory rates of PLEP-1a were higher than those of LEP-1a at the same concentration, indicating that tumor inhibitory effects of PLEP-1a on S180 sarcoma mice were more significant than those of LEP-1a.

Thymus and spleen are important immune organs of animals. They achieve the purpose of tumor suppression through participation in immune responses, regulation of cellular immune and promotion of tumor cells apoptosis (Miao, Dong, Ji, Ma, & Liu, 2012). Compared with the model group, the thymus indices of mice in LEP-1a group were elevated in different degrees, which probably because LEP-1a could promote the generation of T-lymphocytes (Yoshino et al., 1992). It was worth noting that the thymus indices of mice in PLEP-1a group were all higher than those in LEP-1a group. Additionally, the spleen indices of mice in LEP-1a and PLEP-1a groups were not significantly different from those in model group, which were consistent with the reports of Yang, Zhang, Wang, and Wang (2007). The thymus indices and spleen indices of the mice in positive control group were all reduced (Table 1), indicating that cyclophosphamide caused a decline in immune function to a certain extent (Yang et al., 2007).

Free radicals are widespread in organisms, and when at low concentration they are necessary for the body to keep healthy. Free radicals have the ability to regulate the signal transmission between cells, participate in cell immunity and induce cell proliferation (Thannickal & Fanburg, 2000). However, excessive free radicals can cause serious damages to the body through lipid peroxidation, participate in the occurrence and result in deterioration of canceration (Gao & Zou, 2005). SOD is a free radicals clearing agent, can clear the excessive free radicals timely to maintain the physiological balance, prevent the damages to the body and alleviate the degree of canceration (Eapen et al., 1998). IL-2, also known as T-cell growth factor, achieves the purpose of tumor suppression by promoting mitosis of lymphocytes, enhancing the killing effect of killer cells and assisting the formation of antibody (Gao et al., 2005). TNF-α is a potential cytokine which can directly inhibit and kill tumor cells, and induce the apoptosis of tumor cells (Chen, Cao, Wang, & Ye, 2012).

Compared with the model group, SOD, IL-2 and TNF-α of mice in LEP-1a, PLEP-1a and positive control groups were all increased to varying degrees, and which were all close to those of mice in normal group (Table 2). The inhibitory effects of PLEP-1a on S180 sarcoma mice were more significant than those of LEP-1a, suggesting that phosphorylation improved the anti-tumor activities of the polysaccharide.

The above results showed that LEP-1a had remarkable anti-tumor activity and the anti-tumor activity could be significantly enhanced after phosphorylation. Anti-tumor activity of cyclophosphamide (chemotherapy drug) performed slightly stronger than LEP-1a and PLEP-1a. However, compared with the mice in LEP-1a and PLEP-1a groups, the food consumption, body weight, thymus index and spleen index of mice in the positive control group were

Table 2
Effects on SOD, IL-2 and TNF-α of S180 sarcoma mice.

Groups	SOD (U/ml)	IL-2 (pg/ml)	TNF-α (pg/ml)
Normal group	119.04 ± 1.99	10.57 ± 1.90	47.13 ± 1.44
Model control group	106.56 ± 4.84	5.05 ± 1.01	44.63 ± 1.81
LEP-1a (100 mg/kg)	122.08 ± 3.53[a]	5.67 ± 1.33	46.36 ± 2.09
PLEP-1a (100 mg/kg)	114.23 ± 2.86	7.56 ± 1.06[a]	46.99 ± 1.31
LEP-1a (400 mg/kg)	115.27 ± 3.16	7.93 ± 1.47[a]	48.04 ± 1.77
PLEP-1a (400 mg/kg)	117.21 ± 2.36	8.68 ± 1.41[a]	50.49 ± 1.89[b]
Positive control group	121.87 ± 4.15[a]	10.62 ± 1.82[b]	49.94 ± 1.78[a]

[a] $P < 0.05$, significantly different from model control group.
[b] $P < 0.01$, highly significantly different from model control group.

Table 1
Effects on food consumption, weight gain rate, tumor inhibition rate and organ index of mice with S180 sarcoma.

Groups	Food consumption (g/mouse/d)	Weight gain rate (%)	Tumor inhibition rate (%)	Thymus index (mg/10 g)	Spleen index (mg/10 g)
Normal group	3.32 ± 0.31	4.80 ± 0.43		37.83 ± 5.22	46.24 ± 5.7
Model control group	2.84 ± 0.28	9.61 ± 0.64		33.96 ± 5.99	44.93 ± 7.88
LEP-1a (100 mg/kg)	2.94 ± 0.23	5.49 ± 0.58[a]	53.02	34.39 ± 5.81	45.95 ± 5.05
PLEP-1a (100 mg/kg)	2.99 ± 0.29	5.81 ± 0.47[a]	66.1	36.12 ± 4.89	47.08 ± 6.65
LEP-1a (400 mg/kg)	3.05 ± 0.32	4.22 ± 0.59[a]	60.85	41.29 ± 5.66[a]	46.2 ± 5.89
PLEP-1a (400 mg/kg)	3.12 ± 0.18[a]	4.49 ± 0.38[a]	68.57	43.11 ± 5.51[a]	47.41 ± 6.85
Positive control group	2.10 ± 0.37[b]	3.35 ± 0.25[b]	69.04	33.55 ± 6.45	40.4 ± 3.78

[a] $P < 0.05$, significantly different from model control group.
[b] $P < 0.01$, highly significantly different from model control group.

significantly reduced, and the conditions of the mice were abnormal. These results showed that cyclophosphamide had significant side effects which were not observed in LEP-1a and PLEP-1a.

4. Conclusion

Phosphorylated polysaccharide PLEP-1a was prepared from LEP-1a, of which the PO_4^{3-} content was 6.39%. IR, ^{13}C NMR and ^{31}P NMR analysis results showed that the basic structure of the β-D-1,3-glucopyranoside was not changed and $-H_2PO_3$ group was connected to C_6 of LEP-1a. The results of *in vivo* anti-tumor experiment showed that LEP-1a could significantly increase the food consumption, body weight, tumor inhibition rate and thymus index of the S180 sarcoma mice and effectively elevate the level of SOD, IL-2 and TNF-α, indicating that LEP-1a had significant anti-tumor activity. The anti-tumor activity of PLEP-1a was significantly enhanced compared with LEP-1a. In addition, LEP-1a and PLEP-1a had no significant side effects. Therefore, we presumed that LEP-1a and PLEP-1a could be used as novel, natural, potential anti-tumor drugs in tumor therapy.

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