



# Dose-Dependent and Time-Dependent Antitumor Activity of *Pleurotus ostreatus* Polysaccharides in Vitro

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**Abstract:** Elucidation of the *in vitro* antitumor effects of *Pleurotus ostreatus* polysaccharides is very important for their future clinical application. In this paper, we use Sarcoma-180 (S180) cells to test the antitumor activity of the mushroom polysaccharides. The S180 cells in freshly prepared mouse ascites were directly treated by water-soluble polysaccharides from mycelium of *Pleurotus ostreatus* and immediately detected by MTT [3-(4,5)-dimethylthiazol-2-yl]-5-(3,4-dimethyl-2-pyridyl)-tetrazolium bromide method. The results can be directly visualized with naked eyes through the color changes. The *in vitro* antitumor activity of *P. ostreatus* polysaccharides on S180 cells are dose-dependently and time-dependently. *P. ostreatus* strains p11, p23, P44, p105 and p176 showed significant inhibition to S180 cell activity under high polysaccharides concentration (1000µg/ml ~ 1250µg/ml), and there are non-significant effects under the middle concentration (750µg/ml). On the contrary, the S180 cell activity were significantly enhanced under low concentration (250µg/mL-500µg/ml). The effects of polysaccharides generally increased during 4-8 treat hours, but remained stable more than 7 hours. However, the strains P23, P44 and P105 enhanced the S180 cell activity for short time processing (4-5h), but inhibited the cell activity for long time processing (6-8h). Among the tested mushroom strains, P44 exhibited the highest inhibition rate (68.4%) under high concentration of 1250µg/ml and treat time of 7-8 hours, P23 had the highest enhancement (119.5%) under low concentration of 250µg/ml and treat time of 8 hours. The antitumor activity of *P. ostreatus* polysaccharides was dependent on its concentration and treat time, indicating the complicated antitumor mechanism of mushroom polysaccharides and the strict study should be conducted before their clinical application.

**Keywords:** *Pleurotus ostreatus*, Polysaccharides, MTT, S180 Cell, Antitumor Activity

## 1. Introduction

Cancer treatment has been a major domestic and international medical problem. The traditional tumor excision surgery, radiotherapy, and chemotherapy all have certain harm to human body function. The small side effects and good curative effects of edible fungal polysaccharides make it possible to be ones of the best natural antitumor medicines [1-3]. Clarification of the antitumor mechanism of edible fungi's polysaccharides is the premise of its clinical application. Research on the *in vitro* inhibition of edible fungal polysaccharides on tumor cells is not only the first step to screen edible fungi with significant anti-cancer activity, but

also an important way to analyze the antitumor mechanism of edible fungi polysaccharides.

Since the first report of Brander on the antitumor effect of yeast cell-wall polysaccharides Zymosan [4], scientists have been taking more and more interests in edible fungi polysaccharides, and its antitumor activity was drawn especially extensive attention [5-7]. At least 50 species of edible fungi had certain anti-cancer effects, and most of the functional ingredients were found to be polysaccharides [8]. The polysaccharides in edible fungus mycelia and fruit body could achieve its treatment of tumor by either

enhancing the host immune function to suppress tumor growth, or directly killing tumor cells [9, 10]. For any kind of edible fungi polysaccharides, clarification of its antitumor mechanism is indispensable before its clinical treatment of tumor disease.

Recently, the characteristics of *Pleurotus* polysaccharides were acquired more and more attention, especially its antitumor activity [11-14]. During our previous researches, five strains with excellent mushroom cultivation values selected from 256 protoplast regeneration isolates of *Pleurotus ostreatus* P89 were found to be able to reduce the death rate, improve the blood leukocytes quantity, and enhance the peroxidase and catalase activity of S180-bearing tumor mice by feeding the mycelium polysaccharides immediately after the S180 cell injection (data not shown). However, the antitumor activity of the fungal polysaccharides remains unclear. In this paper, we detected the tumor cell activity after the tumor cells were mixed with mycelium polysaccharide by MTT [3-(4,5)-dimethylthiazol-2-yl]-3,5-diphenyltetrazolium bromide] method, in order to elucidate the *in vitro* effects of mushroom polysaccharide on the activity of mouse sarcoma S180 cells.

## 2. Materials and Methods

### 2.1. Materials

*Pleurotus ostreatus* P89, a traditional variety generally used in the mushroom cultivation, was applied to prepare the protoplast using the method previously reported. The protoplasts were regenerated and 256 isolates were purified and conducted to mushroom cultivation. Five strains, p11, p23, p44, p105, p176, were proved to exhibit good cultivation characteristics and production values.

Clean type mice infected by S180 sarcoma cells were fed 12 days before the S180 sarcoma cells were harvested from

the mice fresh ascites. The S180 cells were used to do the following experiments immediately after they were harvested.

All chemicals were of the highest quality available and were used as received. 5 mg/ml MTT (American Sigma) were prepared with physiological saline.

### 2.2. Experiment Methods

#### 2.2.1. Mycelium Polysaccharides Extraction and Content Determination

18-day fungal mycelium in PD (Potato Dextrose) broth were harvested by 4 layers gauze filtration and rinsed 3 times with pure water. The mycelium polysaccharides were extracted by hot water extraction method [15], purified by Sevag solution [16], and measured by anthracene ketone colorimetric method.

#### 2.2.2. S180 Cell Activity Determination by MTT Method

The *in vitro* effects of edible fungus polysaccharides on the cell activity of sarcoma S180 were detected by MTT method [17] with some adjustments. The S180 cells in ascites of 12-day infected mouse were counted under microscope (Olympus, Japan) and immediately diluted by mycelium polysaccharides with a final cell density of  $4 \times 10^6$ /ml and a final polysaccharide concentration from 250 µg/ml to 1250 µg/ml, following by taking 100 µl mixture into 96-hole panel, reacting with 20 µl MTT solution for 4-8 hours, discarding the suspension, adding 150 µl DMSO, shaking at room temperature and with the speed of 70-80 r/min until the solution was in the uniform color, then taking 100 µl suspension into another 96-hole panel to measure A570 values by Biotek multi-function microplate instrument (Gene company, USA). Replacement of fungal polysaccharides with saline water in the detection system was served as blank group. Each treatment was repeated five times. The inhibition rate of cell activity was calculated with the formula

$$\text{cell activity inhibition rate \%} = [1 - \text{average A570 (polysaccharides group)} / \text{average A570 (blank group)}] \times 100.$$

#### 2.2.3. S180 Cell Mortality Rate Assay

The cell mortality rate of fresh S180 cells treated by fungal polysaccharides was measured by Trypan blue

dyeing method. Replacement of fungal polysaccharides with saline water was served as blank group. The dead cell percentage was calculated according to the formula:

$$\text{dead cell percentage (\%)} = (\text{blue cell number} / \text{total cell number}) \times 100.$$

#### 2.2.4. Statistical Analysis

The statistical analysis of original data was processed with DPS (data processing system) version 7.05.

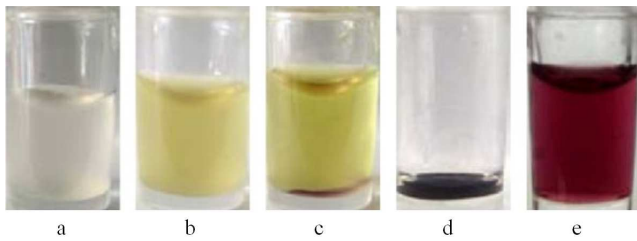
## 3. Results

### 3.1. Content Determination of Fungal Polysaccharides

Using glucose standard curve:  $y = 0.0044x + 0.0034$  ( $R^2 = 0.9992$ ), the polysaccharide concentration of strains p11, p23, p44, p105, and p176 was 2069 µg/ml, 2348 µg/ml, 1957 µg/ml, 2396 µg/ml, and 3484 µg/ml respectively.

### 3.2. The Adjusted MTT Detection Method

The color of fresh S180 cells (Figure 1a) changed from grey (Figure 1a) to light yellow (Figure 1b) after adding MTT solution, and purple precipitation (Figure 1c) appeared at the bottom. After 4 hours reaction, the suspension was discarded (Figure 1d). The purple precipitation was dissolved into bright red solution after the immediate addition of DMSO and gentle shake at room temperature (Figure 1e). The color changes had shown the adjusted MTT method was proper for S180 cell activity determination.



**Figure 1.** Scheme of MTT method: S180 cell suspension (a) changes into bright yellow after adding MTT (b) and produces purple precipitation (c); Discarding the suspension after 4 hours (d) and adding DMSO, the purple precipitation is dissolved into red solution (e).

### 3.3. In Vitro Detection of S180 Cell Activity Affected by Fungal Polysaccharide

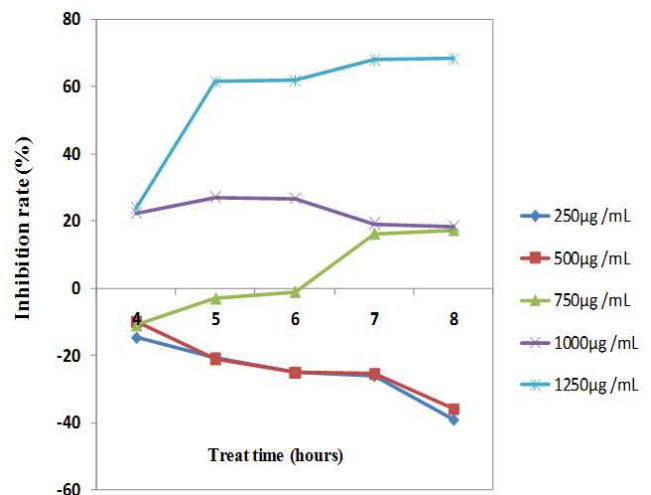
S180 sarcoma cells were treated by strain P44 mycelium polysaccharides of 250-1250 $\mu$ g/ml for 4-8 hours with the physiological saline as blank control. The color changes had shown that the effects of fungal polysaccharides on the S180 cell activities were dose-dependent and time-dependent (Figure 2).



**Figure 2.** The color changes in MTT detection of S180 cell activity treated with physiological saline (a) and *Pleurotus ostreatus* P44 polysaccharides 1250 $\mu$ g/ml (b), 750 $\mu$ g/ml (c), and 250 $\mu$ g/ml (d) after treated with 4-8 hours.

As the treat time prolonged from 4 hours to 8 hours, the solution color became lighter in the blank control group, P44 polysaccharides 1250  $\mu$ g/ml group and 750  $\mu$ g/ml group, showing the S180 cell activity dropped significantly, but remained unchangeable in the P44 polysaccharides 250 $\mu$ g/ml group, showing the S180 cell activity had not been affected. But contrasting to color changes in the blank group, the

solution color changed more lighter and more rapidly in the groups of P44 polysaccharide 1250  $\mu$ g/ml and 750 $\mu$ g/ml meaning the inhibition of S180 cell activity, and the unchangeable solution color in the group of P44 polysaccharides 500 $\mu$ g/ml meaning the enhancement of S180 cell activity. Because the color change happened on the 5 hours in the group of P44 polysaccharides 1250  $\mu$ g/ml and 7 hours in the group of P44 polysaccharides 750 $\mu$ g/ml, the increase of polysaccharides concentration from 750 $\mu$ g/ml to 1250  $\mu$ g/ml increased the inhibition ability to S180 cell activity.



**Figure 3.** Inhibition rate of *Pleurotus ostreatus* P44 polysaccharides on activity of S180 cells.

**Table 1.** Inhibition rate (%) of *Pleurotus ostreatus* polysaccharides on S180 cell's activity.

Strain	Concentration ( $\mu$ g/mL)	Treat time (hours)				
		4	5	6	7	8
p11	250	-7.47	-6.31	-12.78	-24.69	-33.33
	500	-3.4	-0.16	-0.14	-8.36	-15.27
	750	2.46	2.9	7.29	14.22	14.4
	1000	6.71	8.73	14.7	18.62	23.09
	1250	17.13	28.33	46.67	53.56	55.6
p23	250	-49.33	-59.17	-67.09	-97.78	-119.5
	500	-22.14	-22.11	-26.97	-45.5	-56.84
	750	-6.82	-10.54	-10.67	-23.28	-21.76
	1000	-4.94	-1.56	9.33	16.05	8.89
	1250	9.85	12.79	21.45	21.58	26.5
P44	250	-14.71	-20.68	-25.06	-26.14	-39.25
	500	-9.99	-21.02	-25.05	-25.61	-35.98
	750	-10.88	-2.98	-0.99	16.23	17.29
	1000	22.28	27.00	26.60	19.24	18.43
	1250	23.90	61.65	61.85	68.10	68.40
p105	250	-15.26	-22.5	-22.45	-43.06	-45.82
	500	-10.04	-18.73	-18.36	-39.11	-30.66
	750	-3.43	-8.97	-14.27	-19.11	-33.55
	1000	-0.19	-0.63	0.75	0.82	3.02
	1250	1.38	5.64	8.46	8.49	24.37
p176	250	-23.64	-30.15	-35.3	-54.69	-57.38
	500	-14.87	-20.98	-27.15	-33.21	-37.39
	750	-6.31	-7.47	-12.78	-24.69	-33.33
	1000	6.24	42.51	44.92	45.45	49.57
	1250	10.71	57.49	62.56	63.24	63.72

Note: the minus before numbers indicates enhancement of the S180 cell's activity.

The inhibition of P44 mycelium polysaccharides under high concentration and enhancement under low concentration on S180 cell activity could be confirmed by the A570 value measurement and inhibition rate calculation (Figure 3).

This phenomenon was also found in the treatment of mycelium polysaccharides of other strains. Based on the inhibition rate of all the strains in Table 1, results had shown that:

(1) In general cases, the polysaccharides of five strains inhibited the S180 cell activity under high concentration (1000-1250µg/ml), had no significant effect under moderate concentration (about 750 µg/ml), and enhanced the S180 cell activity under low concentration (250-500µg/ml). Strain P44 exhibited the highest inhibition rate (68.4%) under high concentration of 1250µg/ml and treat time of 7-8 hours. Strain P23 had the highest enhancement (119.5%) under low concentration of 250µg/ml and treat time of 8 hours.

(2) Generally speaking, the effects of polysaccharides increased during 4-8 hours, but remained stable more than 7 hours. However, the strains P23, P44 and P105 enhanced the S180 cell activity for short time processing (4-5h), but inhibited the cell activity for long time processing (6-8h), as the bold numbers shown in table 1.

3.4. Inhibition of S180 Cell Activity Could Cause Cell Death Under High Concentration of Fungal Polysaccharides

Whether the inhibition of S180 cell activity had any effects on the cell’s survivance, we conducted a Trypan blue dyeing test for the treatment showing the highest inhibition rate: P44 polysaccharides with concentration of 1250µg/ml (Figure 3). Results had shown in Figure 4.

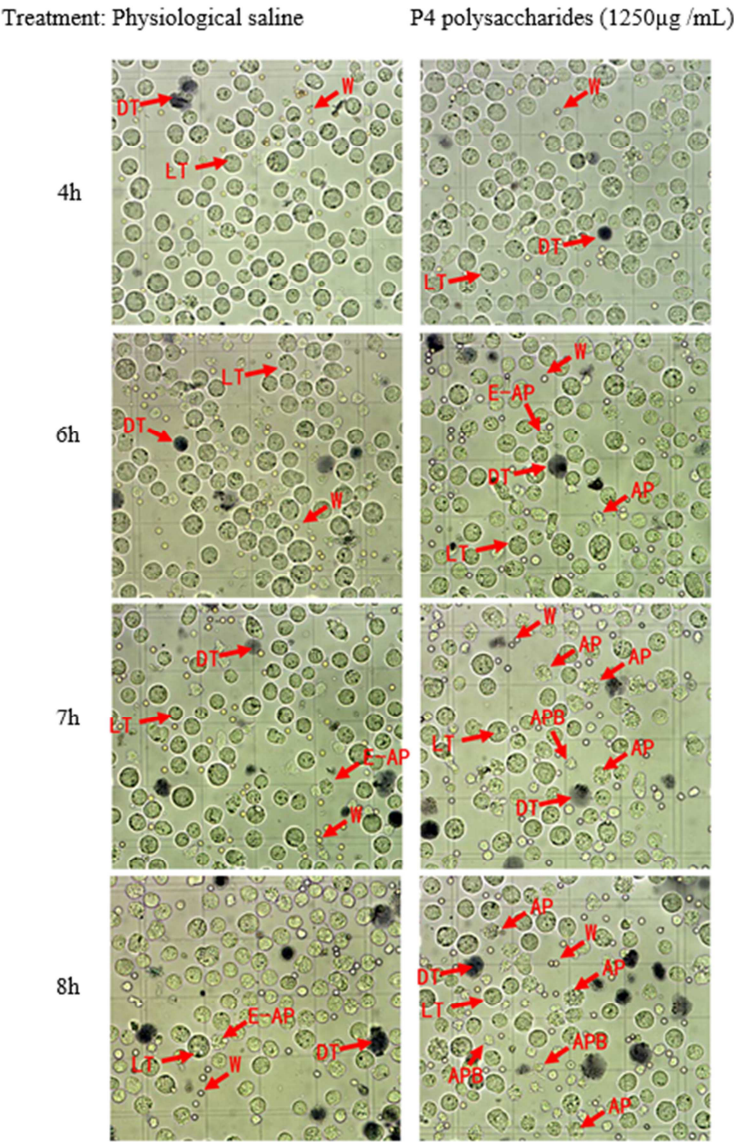


Figure 4. Direct visualization of S180 cells under 1250 µg/ml *Pleurotus ostreatus* P44 polysaccharides in 4, 6, 7 and 8 hours.

LT: live S180 tumor cells; DT: dead S180 tumor cells; E-AP: early apoptosis cells alike; AP: apoptosis cells alike; APB: apoptosis body alike; W: white blood cells (leukocyte).

In the group of physiological saline treatment, as the treat time prolonged from 4 to 8 hours, the dead cells took the percentage of 2.8%, 4.3%, 4.8% and 5.7% in 4, 6, 7, 8 hours respectively. Except the dead cells, there were little abnormal cells found in the cells before 7-hour treatment. Some cells similar as the early stage of apoptotic cells (named as E-AP cells in this paper) was scarcely found after 7-hour treatment (< 1%). The E-AP cell's membrane edge didn't furl neatly, its cytoplasm density increased, but it could not be stained by Trypan blue dye.

In the group of P44 polysaccharides treatment, the dead cells took the percentage of 3.0% in 4 hours, 6.3% in 6 hours, 7.1% in 7 hours and 11.9% in 8 hours. The E-AP cells appeared early than the physiological saline treatment, and about 1% cells were found in 6-hour treatment. Another kind of abnormal cell similar as apoptotic cells (named as AP cells in this paper) was also found in 6-hour treatment. There were some small vesicular bulbs on the AP cell's membrane, the AP cell's content was condensed, and it could not be strained by Trypan blue dye either. The third kind of abnormal cell similar as apoptotic body (named as APB cells in this paper) was found in the 7-hour treatment. The APB cell was condensed into a very small cell with irregular cell membrane, and multiple granular structures were wrapped into the vesicular bulb on the APB's outer membrane. The number of AP and APB cells increased significantly in the 8-hour treatment.

In two kinds of processing conditions, there were some mononuclear cells or lymphocytes leukocytes leaking into ascites. In selected concentration and time, P44 polysaccharides had no significant effect on these immune cells.

## 4. Discussion

Tumor cell activity could be determined by Typhan blue, neutral red dyes, LDH leakage method, and MTT method. Among them, the MTT method was quick, easy and did not require radioactive isotope or special testing instrument. Therefore, the MTT method suited to mass detection characteristics, and acquired widely application on *in vitro* antitumor activity assay [18-21]. The improved MTT method in this paper could shorten the time within 8 hours and visualize results by eyes, making it possible to rapidly detect the reaction between fungal polysaccharide and tumor cells.

The results of this study indicated that the effects of fungal polysaccharides on S180 cell activity were strictly dose-dependent and time-dependent. The main reason possibly relied on the fact that the mushroom polysaccharides needed to change the tumor cell's signaling pathways in order to influence the syntheses or degradation of specific proteins within tumor cells [22, 23]. The on-off of related signaling pathways and the activation or degradation of specific proteins were all sensitive and strictly regulated in the cell. One of the possible mechanisms was that inhibition-related signal pathways were "on" and the inhibition-related proteins were synthesized in the present of high concentration of fungal polysaccharides, but they were "off" or degraded under low concentration of fungal polysaccharides. Although we have

just acquired some preliminary results in this paper, we have not only built an improved MTT method suitable for *in vitro* determination of tumor cell activity, but also found an interesting phenomenon that low concentration of fungal polysaccharides could enhance the tumor cell activity, suggesting that we should carefully utilize the fungal polysaccharides especially in the clinical application to tumor therapy to avoid unpredictable results.

## 5. Conclusion

We proposed an improved MTT method to simply and rapidly test the antitumor activity of *P. ostreatus* polysaccharides, and we found its antitumor activity was dramatically changed from inhibition to enhancement of S180 cell activity when the polysaccharide concentration decreased from 1250 µg/ml to 250 µg/ml. Moreover, the treat time affects the antitumor activity significantly. By detecting the S180 cells after treated by 1250 µg/ml *P. ostreatus* polysaccharides, apoptosis cells alike were visualized after 7 hours. The above results suggested that the antitumor mechanism should be further clarified before the clinical application of *P. ostreatus* polysaccharides.

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