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Full Length Research Paper

Inhibition of *Lentinus edodes* polysaccharides against liver tumour growth

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The objective of this study was to examine the *in vitro* antitumour activities of *Lentinus edodes* polysaccharides. The present results demonstrated that *L. edodes* polysaccharides at 100 and 200 mg/kg dose-dependently suppressed liver tumour growth. The polysaccharides were still found to be dose-dependently and enhance spleen and thymus index and inhibit expression of Caspase-3 in mice with liver cancer. It may be concluded that *L. edodes* polysaccharides is beneficial to therapy of liver tumour.

Key words: Liver cancer, *Lentinus edodes* polysaccharides, caspase-3, spleen index.

INTRODUCTION

The most active constituents in mushrooms are polysaccharides, which have been found to boost the human immune system, showing anticancer and anti-viral activities. Medicinal mushroom extracts have been considered as important remedies for the prevention and treatment of many diseases for thousands of years especially in the Orient (Israilides and Philippoussis, 2003; Kidd, 2000; Wasser and Weis, 1999). A plethora of medicinal effects has been demonstrated for many traditionally used mushrooms including antibacterial, antiviral, antifungal, antitumour and immuno-potentiating activities (Hobbs, 2003; Ooio and Liu, 1999). Among the various bioactive components which have been demonstrated to be most effective as antitumor and immunomodulatory agents are polysaccharides and polysaccharopeptides. One of the most widely edible mushrooms, *Lentinus edodes*, is largely cultivated in China, Japan and other Asian countries because of its taste and nutritional values, its named is Xianggu in Chinese and Shiitake in Japanese. A b-(1 / 3)-D-glucan isolated from *L. edodes* is well known for its medicinal effect (Cheung, 2008). Chihara et al. (1969) have demonstrated that lentinan exhibited anti-cancer bioactivities (Chihara et al., 1969), so lentinan has been attracting much attention.

Liver cancer or hepatic cancer is properly considered to be a cancer which starts in the liver, as opposed to a cancer which originates in another organ and migrates to the liver, known as a liver metastasis. For a thorough understanding of liver cancer it is important to have an understanding of how the liver functions. In the present study, we extracted *L. edodes* polysaccharides and analysed its chemical structure. Then, anti-tumour activity of the polysaccharides was evaluated.

MATERIALS AND METHODS

MATERIALS

L. edodes mushroom was purchased from a local market. Polysaccharides was extracted from *L. edodes* by traditional water-boiling method.

Capillary zone electrophoresis operations

Before the experiments, the three-electrode system was fixed in corresponding positions of the electrochemical cell and the disk-shaped copper working electrode was carefully adjusted to make an effective injection to the off-side of the capillary by the three-dimension positioner.

Before each run in CE experiments, the capillary was sequentially

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rinsed with 1.0 mol L^{-1} hydrochloric acid, doubly distilled water, 1.0 mol L^{-1} sodium hydroxide 3 min for each and running buffer till the current inside of the capillary reached stable state. This was important to get a reproducible EOF. The optimal conditions of this experiment were 12 kV as separation voltage, 0.045 mol L^{-1} NaOH as buffer solution, 8 s as sampling time and 0.60 V as detection potential.

Chemical hepatocarcinogenesis scheme

Hepatocarcinogenesis was evaluated using the mice liver preneoplastic foci assay (Semple-Roberts et al., 1987, Ito et al., 1989 and Williams, 1982). Thirty male mice (15–20 g weight, 2 months of age) were randomly allocated into three groups of 10 animals each and were housed in a well-ventilated environment. They were fed with rodent chow and given tap water. For the induction of preneoplastic foci, mice were treated according to the chemical hepatocarcinogenesis scheme modified by Semple et al. (1987). Mice in the positive control group (Group 2) were injected intraperitoneally with a single dose of diethylnitrosamine (DEN) dissolved in water (100 and 200 mg/kg body weight) for 20 days. Group 3, with the same chemical hepatocarcinogenesis scheme as in the positive group (Group 2), received polysaccharides (0.5%, w/v) instead of drinking water for 20 days. Mice in the negative control group (Group 1) were given an intraperitoneal injection of the same volume of water for 20 days. At the end of the experiment, mice were killed and tumours were removed and weighed. Spleens and thymuses were removed and weighed.

Spleen index is calculated as (spleen weight/body weight) ×100
Thymus index is calculated as (thymus weight/body weight) ×100

MTT colorimetric assay

Cancer cell line (5×10^3 cells/0.33 cm^2) was plated in 96-well plates 'Nunclon TM MicrowellTM' (Nunc) and was incubated at 37°C. After 24 h, cells were treated with the extracts (final concentration 0.01 to 100 µM). Untreated cells were used as controls. Microplates were incubated at 37°C in humidified atmosphere of 5% CO_2, 95% air for 3 days and then cytotoxicity was measured with colorimetric assay based on the use of tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). The results were read on a multiwell scanning spectrophotometer (Multiscan reader), using a wavelength of 570 nm. Each value was the average of 8 wells (standard deviations were less than 20%).

Immunohistochemical methods

The sections were cut from a tissue microarray containing cores of all 10 tumors, prepared from the original paraffin-embedded blocks. Immunohistochemical staining was performed on new sections from all cases using monoclonal antibodies for cyclin D1 (1:750 dilution, Dako), and Caspase-3 (1:40 dilution). All antibodies required antigen retrieval at high temperature, but required no other pretreatment.

Biotinylated alpha mouse IgG obtained from horse serum (ABC Kit, Vector Laboratories, Burlingame, CA) was applied as a secondary antibody at 1:400 in PBS for 30 min at room temperature. Immunostaining was performed using the avidin–biotin peroxidase complex technique (ABC Kit, Vector Laboratories) applied for 30 min. Finally, 3,3-diaminobenzidine tetrahydrochloride (DAKO, Glostrup, Denmark) in distilled water was used as the chromogen for 10 min, and sections were counterstained using Mayer's hematoxylin.

The grade of $p27^{Kip1}$ protein expression in each specimen was evaluated according to the percentage of positively stained cells among the total number of counted cancer cells; all positive cells were counted regardless of intensity of staining and used as labeling index (LI).

Statistical analyses

The differences between the groups were analyzed by Mann–Whitney U test. Spearman correlation was used for correlation analysis (StatView, version 5.0.1, SAS Institute, Cary, NC). Significant difference was considered as $P < 0.05$.

RESULTS AND DISCUSSION

Chemical composition of polysaccharides

In the present work, we used a similar approach for the separation of the monosaccharides obtained after the hydrolysis of the polysaccharides. According to capillary zone electrophoresis results, these monosaccharides are Man, Ara, Glu, Gal, Xyl. The resulting electropherogram (recorded with indirect UV detection, with 2,6-pyridinedicarboxylic acid as chromophore) obtained with reversed EOF upon addition of cetyltrimethylammonium bromide (CTAB) is shown. It can be seen that the nine analytes migrate in eight peaks. The Rha and Xyl have the highest total mobility, followed by Glu and Man. Ara and Gal elute then. It will be shown below that the resulting electrophoretic peak pattern allows for identification of the plant polysaccharides.

Antitumour and immunity activities of *L. edodes* polysaccharides

The water soluble extract of the mycelial culture of *L. edodes* (designated as LEM) was isolated from solid medium of sugar-cane bagasse and defatted rice bran (Sugano et al., 1982). Several studies have shown that *L. edodes* extract and its purified fractions have many physiological activities including antitumor (Azuma et al., 2000; Sugano et al., 1985), antiviral (Suzuki et al., 1990; Suzuki et al., 1989), and immunomodulating activities (Tochikura et al., 1988). The antitumor activity of *L. edodes* extract has been demonstrated by activation of macrophages *in vitro* (Sorimachi et al., 1990; Suzuki et al., 1988).

Table 1 shows the antitumour activities for the *L. edodes* polysaccharides at 100 and 200 mg/kg body weight. The inhibitory activities increased proportional to polysaccharides extract concentration. The results showed that *L. edodes* polysaccharides had significantly higher inhibitory activity against tumour growth.

Hong Fu and Wen-yuan Guo contribute equally to this work

Table 1. Inhibitory effect of *Lentinus edodes* polysaccharides on tumour.

Group	Tumour weight (g)	Inhibition rate (%)
Normal control	0	-
Model control	0.874±0.016	-
Polysaccharides-treat 1	0.428±0.012	51
Polysaccharides-treat 2	0.251±0.009	71

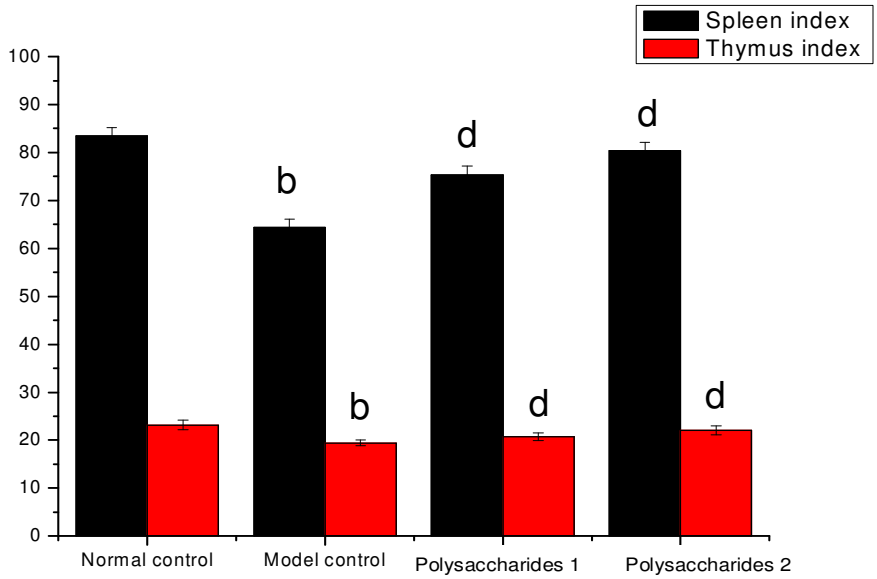

Figure 1. Effect of *Lentinus edodes* polysaccharides on spleen and thymus indexs. Values are mean ± SD. $^{b}P < 0.01$, compared with the control group using unpaired t test; $^{d}P < 0.01$, compared with the model control group using unpaired t test.

The majority of the spleen's functions are related to the immune system or the blood supply. The spleen removes old red blood cells, called erythrocytes, from the blood supply and removes, stores and produces white blood cell lymphocytes. These stored lymphocytes produce antibodies and assist in removing microbes and other debris from the blood supply (Brown et al., 2003; Corsi et al., 2007).

The main function of the thymus is to develop immature T-cells into immunocompetent T-cells. This process begins with the production of pre-T cells in the bone marrow and their subsequent transport to the thymus via the blood. The pre-T cells are then taken into the cortex of the thymus. Here, a series of molecular events take place allowing the cells to recognize certain antigens. Some of the cells recognize self-components, and these are eliminated by a process of negative selection. Those that fail the selection die and those that live proceed to the medulla and eventually into the blood stream where they act upon foreign agents in the body (van Baarlen et al., 1988; Louise Markert et al., 2010; Globerson, 2002).

The effect of treatment of rats with doses of *L. edodes* polysaccharides (100 and 200 mg/kg/body weight) on Spleen and Thymus indexes are shown in Figure 1. The Spleen and Thymus indexes were significantly reduced ($p< 0.05$) in model mice compared to normal control mice. Significant increase in Spleen and Thymus indexes was observed after 20 days of administration of *L. edodes* polysaccharides. Spleen and Thymus indexs were 80.36±0.77 and 22.03±0.91, respectively, at the doses of 200 mg/kg/body weight.

Cyclin D1 is encoded by an oncogene on Chromosome 11 and regulates progress through the cell cycle by activating p34 protein kinase (Motokura et al., 1991). It is clonally rearranged in numerous neoplasms, most characteristically mantle-cell lymphoma and parathyroid tumors. A critical target of pro-proliferative cell signaling pathways is regulation of the cyclin D1 kinase, a holoenzyme complex formed by cyclin D1 and cyclin-dependent kinases-4 (cdk4) and cdk6. Cyclin D1 regulates catalytic activity of this complex; its up-regulation is sufficient to promote progression of hepatocytes from Go through the G1 restriction point and into S-phase of the cell cycle (Albrecht et al., 1995; Loyer

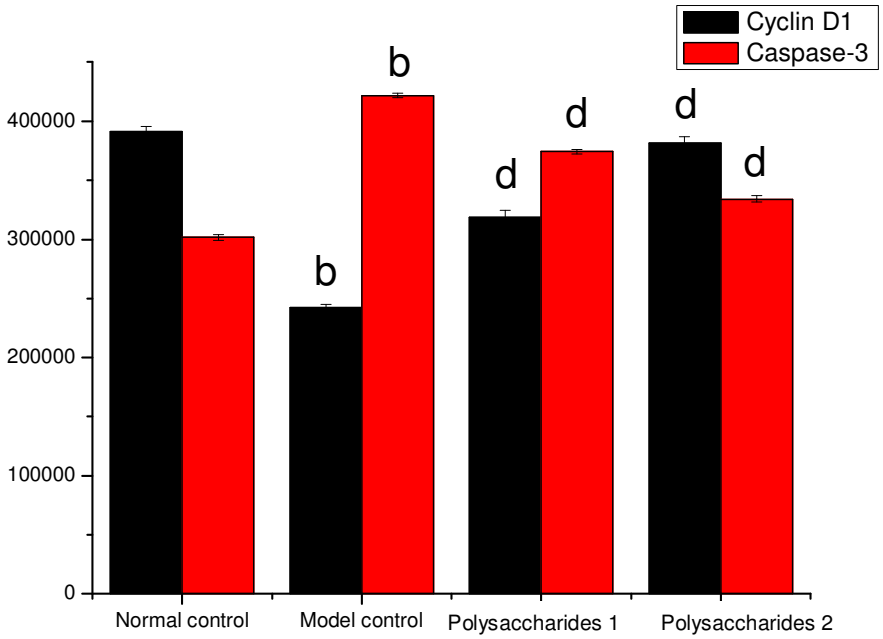

Figure 2. Effect of *Lentinus edodes* polysaccharides on expression of Cyclin D1 and Caspase-3. Values are mean ± SD. $^{b}P < 0.01$, compared with the control group using unpaired t test; $^{d}P < 0.01$, compared with the model control group using unpaired t test.

et al., 1996; Albrecht and Hansen, 1999; Nelsen et al., 2001).

As shown in Figure 2, expression of Cyclin D1 in model control mice was significantly reduced (241954.37 ± 3128.49 versus 391382.23 ± 4003.32), whereas expression of Caspase-3 was markedly enhanced compared to normal control (421742.41 ± 1839.42 versus 301614.05 ± 2531.82). The expression of Cyclin D1 in polysaccharides-treated mice substantially increased as compared with that in the model control group, whereas expression of Caspase-3 was markedly decreased. Expression of Cyclin D1 and Caspase-3 were 381539.32 ± 5263.26 and 334106.53 ± 2719.36, respectively, at the dose of 200 mg/kg body weight.

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