


Purification, Structural Characterization, and Biological Activity of Polysaccharides from *Lentinus velutinus*

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ABSTRACT

A polysaccharide (LVP) was purified from fruiting body of *Lentinus velutinus* by ethanol precipitation fractionation and DEAE and Sephadex G-100 column chromatography. The yield of purified polysaccharide was 0.025%. Molecular characteristics of LVP were determined by gel permeation chromatography, FT-IR spectroscopy, and thin-layer chromatography. Our results revealed that LVP is a polysaccharide composed of only glucose units, and has a molecular weight of 336 kDa. Biological activity assays indicated that LVP exhibits both cytotoxic and antioxidant activity. LVP showed specific cytotoxicity against cancer cells (HeLa and HepG2 cells), and alterations in cancer cell morphology were found after LVP treatment.

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1. Introduction

For thousands of years, mushrooms have served as both food and medicine to mankind, including use as traditional medicines in East Asia [1–3]. In recent decades, modern science has discovered specific bioactive compounds in mushrooms, revealing their pharmaceutical properties and associated mechanisms. The medicinal properties of mushrooms involve immunomodulatory, anticancer, anti-inflammatory, antidiabetes, antihepatopathy, and antioxidant functions [2,4,5]. Polysaccharides isolated from mushrooms are among the bioactive compounds with medicinal benefits. For instance, lentinan from *Lentinus edodes*, the D-fraction from *Grifola frondosa*, schizophyllan from *Schizophyllum commune*, polysaccharides peptide from *Coriolus versicolor*, and polysaccharide-K from *Trametes versicolor* are mushroom-derived polysaccharides with powerful anticancer and immunostimulating activities [6].

Lentinan is a high molecular weight β -glucan isolated from the fruiting body of *L. edodes*. Bioactivity of the purified polysaccharide was first demonstrated by growth inhibition of Sarcoma 180 in implanted mice [7,8]. Since then, its functions and related mechanisms have been extensively studied with regard to their anticancer and immunostimulating activities. A number of studies have revealed that lentinan acts on cancer cells both directly and indirectly. Its effects have also been assessed in

clinical trials [9]. Since cancer is a leading cause of death worldwide, searching for new anticancer treatments is a top priority in biological research. To date, a number of polysaccharides with anticancer activity were purified from mushroom species [3].

Since *L. edodes* is one of the medicinal mushrooms with numerous pharmacological properties, it is possible that other species of *Lentinus* can show the pharmacological effects. Previously, we evaluated the cytotoxic effect of crude extracted from *L. sajor-caju*, *L. squarrosulus* and *L. velutinus* and found that crude polysaccharide extracted from the fruiting body of *L. velutinus* exhibits cytotoxic activity on human cancer cell lines [10]. *L. velutinus* is a wild mushroom found in tropical regions of South America, Southeast Asia, and southern Africa. It can be cultured but is not considered edible due to its tough texture. In this study, *L. velutinus* polysaccharide (LVP) was purified by ethanol precipitation fractionation and DEAE and Sephadex G-100 column chromatography. The structural characteristics of the LVP were determined. Further, its cytotoxic and antioxidant activities were investigated.

2. Materials and methods

2.1. Mushroom strain

Lentinus velutinus strain WCR1104 was collected from the Ratchaburi Province, Thailand. A pure culture was maintained on potato dextrose agar

(PDA; 200 g/L potato infusion, 20 g/L dextrose, and 15 g/L agar) at 4 °C and sub-cultured monthly.

2.2. Mushroom cultivation

Pure culture of *L. velutinus* was prepared on PDA at 30 °C ± 2 °C for 7 days. For the spawn production, pieces of the peripheral colony were cut and transferred to sorghum grain and incubated at 30 °C ± 2 °C for 10 days. Mushroom cultivation was performed in a plastic bag of sterilized sawdust (91.8% sawdust, 5% rice bran, 2% gypsum (CaSO₄·2H₂O), 1% lime (Ca(OH)₂), and 0.2% Epsom salt (KH₂PO₄) with 70% Rh). The bags were inoculated with 5 g of spawn and incubated at 30 °C ± 2 °C. After 45 days, the mycelium had run throughout the bag, the plastic bags were removed and the mushroom substrate was covered with casing soil. The humidity was maintained at 80%–90% by watering.

2.3. Extraction and purification of polysaccharides

The fruiting bodies of *L. velutinus* were dried at 60 °C in hot air oven and blended into fine particles. The particles were de-fatted with 95% ethanol at room temperature overnight while shaking at 180 rpm. The supernatant was then discarded. The mushroom biomass was boiled three times with 20 volumes of water for 3 h each. After centrifugation at 8,000 rpm for 30 min, the supernatant was concentrated by boiling and deproteinized with one volume of Sevag's reagent (5:1 CHCl₃:*n*-BuOH) three times. Next, the aqueous solution was extracted for crude polysaccharide by adding cold ethanol (final concentration of 80%) and kept at 4 °C overnight. The supernatants were discarded after centrifugation at 8,000 rpm for 30 min. The precipitate was washed with cold pure ethanol, centrifuged, and washed again. The pellet was collected and air-dried to obtain crude polysaccharide of *L. velutinus*.

The ethanol-precipitated fraction was used for subsequent separation. The crude polysaccharides were dissolved in sterile water and serially precipitated with equal volume and three volumes of 95% cold ethanol, respectively. The precipitates were separated by centrifugation at 8,000 rpm for 30 min at 4 °C, washed with cold pure ethanol, and air-dried. Next, the sample obtained from ethanol precipitation was separated by DEAE-Sepharose column chromatography. The sample was re-dissolved in sterile water, and the solution (30 mL at 5 mg/mL) was loaded onto a DEAE-FF column (5 mL) equilibrated with water using a P-50 pump. The extract was step eluted with water, 0.05 M, 0.1 M, 0.2 M,

and 0.5 M NaCl solutions at a flow rate of 1 mL/min. Each 2 mL fraction of eluate was collected and monitored spectrophotometrically at 490 nm using the phenol-sulfuric method [11]. The same peak fractions were collected, combined, and dialyzed with water for 5 days. Each fraction was precipitated with four volumes of cold 95% ethanol, centrifuged, and air-dried. The fraction obtained from DEAE-FF chromatography was further purified using gel filtration. The sample was re-dissolved in sterile water and loaded onto a Sephadex G-100 column (50 mL at 50 mg/mL). The sample was eluted with 50 mM Na-phosphate buffer, pH 7.0 (with 0.15 M NaCl) at a flow rate of 0.1 mL/min. The fractions (5 mL each) forming the same peak were collected, dialyzed, and lyophilized to obtain three purified LVP samples. The polysaccharides were stored at –20 °C for further use.

2.4. Determination of molecular weight

The homogeneity and molecular weight of the samples were determined by gel permeation chromatography (GPC) with a Shimadzu LC-10ADvp HPLC pump (Shimadzu, Kyoto, Japan), equipped with an OHPak SB-804 HQ column (8.0 mm × 300 mm: Shodex, Tyjyo, Japan) and eluted with water at a flow rate of 0.5 mL/min. Elution was monitored with a refractive index detector. As standards, a series of dextran solutions covering a molecular weight range of 342–393 kDa were run under the same conditions. The elution volumes of the standard solutions were plotted against the logarithms of their respective molecular weights, and the average molecular weights of the polysaccharides samples were determined on the same graph.

2.5. Determination of total carbohydrate content

The carbohydrate content of the samples was determined with the phenol-sulfuric method. Briefly, 0.1 mL of sample was mixed with 0.1 mL of 5% phenol, followed immediately with 0.5 mL of concentrated sulfuric acid, and shaken well. After 10 min of shaking, the mixture was placed at 25 °C–30 °C for 20 min. The absorbance of the mixture was measured at 490 nm. The total carbohydrate content was calculated with D-glucose as a standard (0–100 µg/mL).

2.6. Determination of monosaccharide composition

The monosaccharide content of the samples was determined using thin-layer chromatography (TLC) according to [12], with some modifications. Briefly,

the polysaccharide (4 mg) was hydrolyzed with 4 mL of 2 M trifluoroacetic acid in a sealed glass tube at 100 °C for 18 h. The excess acid was removed by co-distillation with methanol at 60 °C, and the hydrolyzed products were then dried. The hydrolyzed polysaccharide and standard were re-dissolved in water and spotted on TLC plates. The TLC plates were irrigated with a two-solvent system, such as *n*-butanol-acetone-water (4:3:1, v/v/v) or *n*-butanol-acetic acid-isopropanol-water (8:4:7:3) at 25 °C. The solvent was allowed to ascend to the top of the plate. The plate was then removed and placed to dry. The different monosaccharides were developed by dipping the plates into a methanolic solution of 0.3% (w/v) N-(1-naphthyl) ethylenediamine and 5% (v/v) sulfuric acid, followed by incubation at 120 °C for 10 min. The spots, colors, and retention factor values were recorded.

2.7. FT-IR spectrophotometer

The polysaccharide sample was grounded into a fine powder and analyzed with a Spectrum 100 FT-IR Spectrometer (PerkinElmer, Waltham, MA, USA) to detect functional groups.

2.8. Determination of cytotoxicity using MTT assay

HeLa (human epithelial cervix carcinoma), HepG2 (human hepatocellular liver carcinoma), and LLC-MK2 (monkey rhesus kidney) cell lines were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum and 50 µg/mL gentamycin. The cultures were maintained at 37 °C in a humidified incubator with 5% CO₂. Cytotoxicity was evaluated by MTT assay. Briefly, 1.2×10^4 cells were seeded in each well of 96-well plates and incubated at 37 °C in a humidified atmosphere with 5% CO₂. After 24 h, the media was removed, and fresh media (100 µL) containing polysaccharides (0–2000 µg/mL) was added to each well, followed by further incubation for 24 or 48 h. The media was then removed and the cells were incubated with fresh culture media containing MTT (0.5 mg/mL) for 4 h at 37 °C. Finally, the media was removed, DMSO was added (100 µL/well), and the absorbance of each well was measured at 540 nm in a microtiter plate reader. The percentage of viable cells was determined according to the formula; $\text{viable cells (\%)} = (A_a - A_0) / (A_b - A_0) \times 100$, where A_a is the absorbance of the system with the addition of polysaccharides, A_b is the absorbance of the system without the addition of polysaccharides, and A_0 is the absorbance of the system without the addition of cells. Assays were performed in triplicate.

2.9. Determination of antioxidant activity by DPPH free radical scavenging assay

DPPH free radical scavenging assays were performed according to [13], with some modifications. A volume of 30 µL of each sample (0–2000 µg/mL) and 0.2 mM DPPH radicals (in methanol) were added to each well in 96-well plates and mixed. Ascorbic acid (1–200 µg/mL) was used as a standard. The plates were then kept in the dark for 30 min at RT. The absorbance was measured at a wavelength of 515 nm. The radical scavenging activity was calculated as a percentage of DPPH discoloration based on the formula $SA (\%) = 100 - (100 \times (A_a - A_c) / A_b)$, where A_a is the absorbance of the system with polysaccharide and DPPH, A_b is the absorbance of the system with only the DPPH, and A_c is the absorbance of the system with only the polysaccharide. Assays were performed in triplicate.

3. Results and discussion

3.1. Extraction and purification of polysaccharides

The crude polysaccharide was extracted from the dried fruiting bodies of *L. velutinus* with a yield of 3.99% on dry weight basis. The crude extract was then fractionated by re-precipitation with ethanol by adding one and four volumes, and three fractions were yielded 1.23%, 1.82% and 1.37% on dry weight basis, respectively. These fractions were tested for cytotoxicity, and only the fraction prepared with four volumes of ethanol showed effects against the cell lines. Ethanol has anti-solvent properties, which can precipitate various sizes of polysaccharides in a concentration-dependent manner. High molecular weight polysaccharides are precipitated at low concentrations of ethanol, whereas high concentrations of ethanol favor precipitation of small polysaccharides [14,15]. The fraction was carried out on a DEAE-FF column and step eluted with water and 0.05 M, 0.1 M, 0.2 M, and 0.5 M NaCl. Using the phenol-sulfuric method, each eluted step showed a single peak (Figure 1). The fraction eluted by 0.5 M NaCl yielded 0.093% on dry weight basis and revealed cytotoxicity against the HeLa and HepG2 cell lines. Then, the fraction was further purified by Sephadex G-100 column chromatography, and three symmetrical peaks were obtained (Figure 2). Interestingly, only the second peak, with a yield of 0.025% on dry weight basis, showed cytotoxic activity against the HeLa and HepG2 cell lines. This polysaccharide fraction was named LVP.

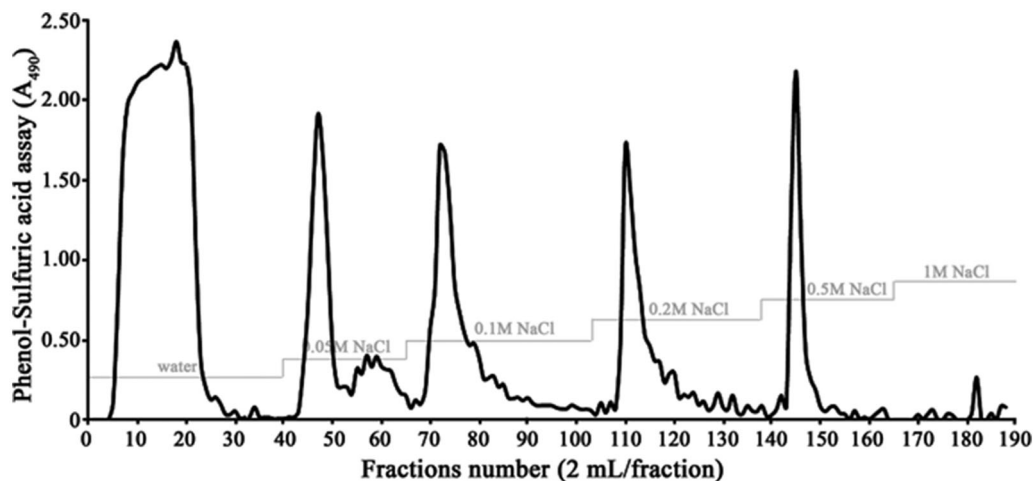


Figure 1. Elution profile of polysaccharides on DEAE column.

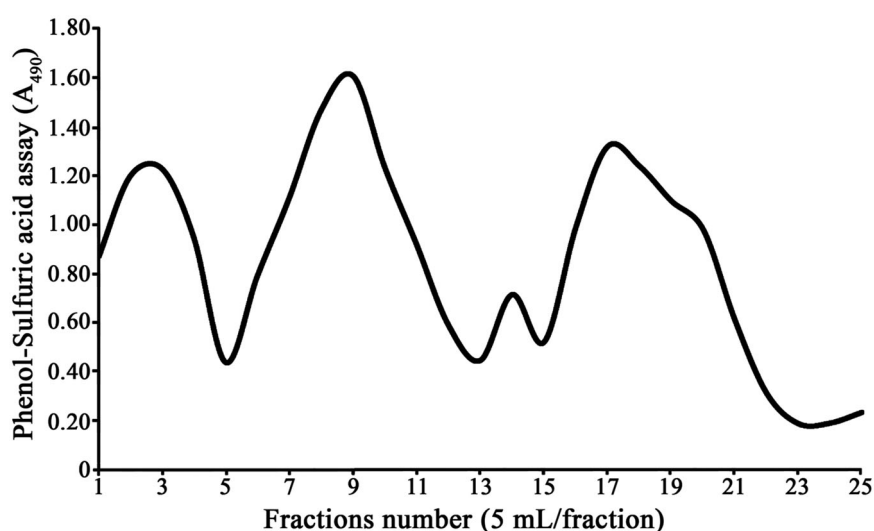


Figure 2. Elution profile of polysaccharides on Sephadex G-100 column.

3.2. Characterization of LVP

The molecular weight of LVP was determined by GPC. The results showed a symmetric peak with a retention time of 14.051 min and an average molecular weight of 335,970 Da. The polydispersity index (DPI, M_w/M_n) was about 1.56. The DPI indicated that LVP is a natural polymer containing monomer units arranged in chains of different lengths.

Total polysaccharide content was measured by the phenol-sulfuric method. This revealed that LVP contains 99.01% polysaccharide. No absorbance was detected at 260 or 280 nm, indicating that LVP contains neither protein nor nucleic acid.

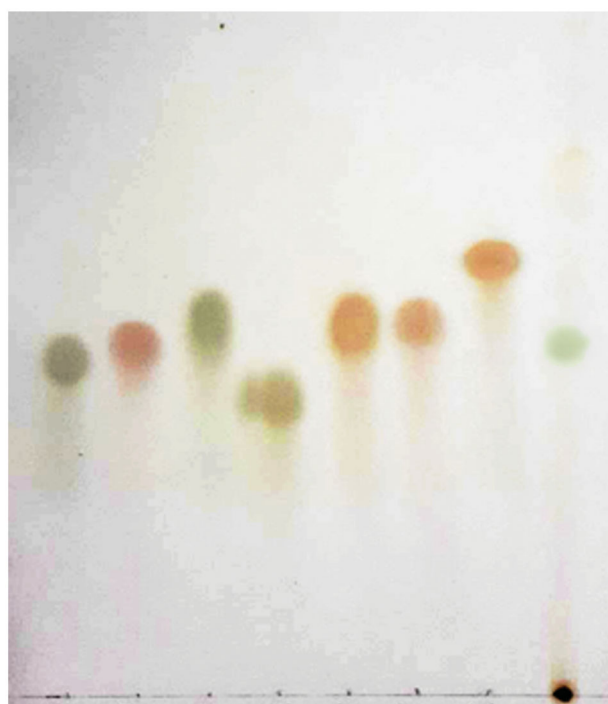
The monosaccharide composition of LVP was preliminarily determined by TLC. The result revealed that the hydrolysate of LVP showed only one green spot (Figure 3). Its appearance was the same in color and retention as the standard glucose spot. This indicated that LVP was composed of only glucose subunits.

The FT-IR spectrum of LVP displayed the typical signal pattern expected for a carbohydrate moiety, with several bands in the anomeric region (Figure 4). O-H stretching is seen as a broad peak at $3200\text{--}3400\text{ cm}^{-1}$, while bands in the $2800\text{--}3000\text{ cm}^{-1}$ region are due to C-H stretching. The band of associated water is around 1600 cm^{-1} . The peak at 1460 cm^{-1} is from the CH_2 stretching vibration. The absorptions at about 1200 cm^{-1} and between $1000\text{--}1200\text{ cm}^{-1}$ are assigned to be the stretching vibrations of C-O in the sugar ring and C-O-C, respectively. The peaks in the range of $750\text{--}950\text{ cm}^{-1}$ are anomeric regions. These results indicate that LVP is a polysaccharide with sugar ring structures. Furthermore, there were no absorption peaks between $1700\text{--}1750\text{ cm}^{-1}$, indicating the absence of uronic acid.

3.3. Determination of cytotoxicity by MTT assay

The cytotoxic effects of LVP against human cancer cell lines, HeLa and HepG2, and the normal cell

line, LLC-MK2, were evaluated using MTT assays at various concentrations (10, 50, 100, 250, 500, 1000, 1500, and 2000 $\mu\text{g}/\text{mL}$). LVP showed significant cytotoxicity against the cancer cell lines in a concentration- and time-dependent manner. The inhibition of LVP (2000 $\mu\text{g}/\text{mL}$) on HeLa and HepG2 cells were 22.44% and 21.92%, respectively, at 24 h, and 49.97% and 51.83%, respectively, at 48 h (Figure 5). In addition, the morphologies of the HeLa and HepG2 cells were observed after treatment with



Glu Fru Man Gal Ara Sor Xyl LVP

Figure 3. Chromatographic spots from thin-layer chromatography (TLC) for monosaccharides of the LVP, which was hydrolyzed with 2 M TFA for 18 h. The standards used were glucose (Glu), fructose (Fru), mannose (Man), galactose (Gal), arabinose (Ara), sorbitol (Sor), and xylose (Xyl).

LVP. At low concentrations, the cancer cells displayed no detectable morphological changes, and they could adhere to the plate surface and elongate. At concentrations higher than 1000 $\mu\text{g}/\text{mL}$, however, apoptosis was detected in both cancer cell lines. Cells and their nuclei were swollen and round, and the cell density and adherent capacity decreased (Figure 6). These results clearly indicate that LVP exhibited cytotoxic activity against cancer cell lines. Importantly, LVP exhibited no effect on the normal cell line, LLC-MK2, at the tested concentrations (Figure 5), indicating that the cytotoxic effects are specific to cancer cells.

A number of studies have indicated that polysaccharide extracts from mushrooms inhibit the proliferation of cancer cells. For example, LEP, an ethanol precipitate of a dried powder extracted from *L. edodes* mycelium, showed cytotoxicity against HepG2 cells. The treated cells were shrunken, rounded, and detached from the surface. The viability of HepG2 cells treated with LEP at 200 $\mu\text{g}/\text{mL}$ for 24 h was 59.9% [16]. Li et al. [17] evaluated the effects of acid, water, and alkaline extract of crude polysaccharide from eight Chinese mushrooms on HeLa and HepG2 cell proliferation. The viability of these cells treated with extracts at 600 $\mu\text{g}/\text{mL}$ for 48 h was 0%–67.9%. The most common polysaccharides derived from mushrooms are those that are non-digestible by humans, such as glycan, as these molecules have a beta bond that human enzymes cannot break. These molecules can be absorbed in the gastrointestinal tract and activate the immune system through various mechanisms [18]. In innate immunity, polysaccharides play a role in antigen-presenting cells. After oral administration, they enter the small intestine and are captured by macrophages. Moreover, these macrophages also create

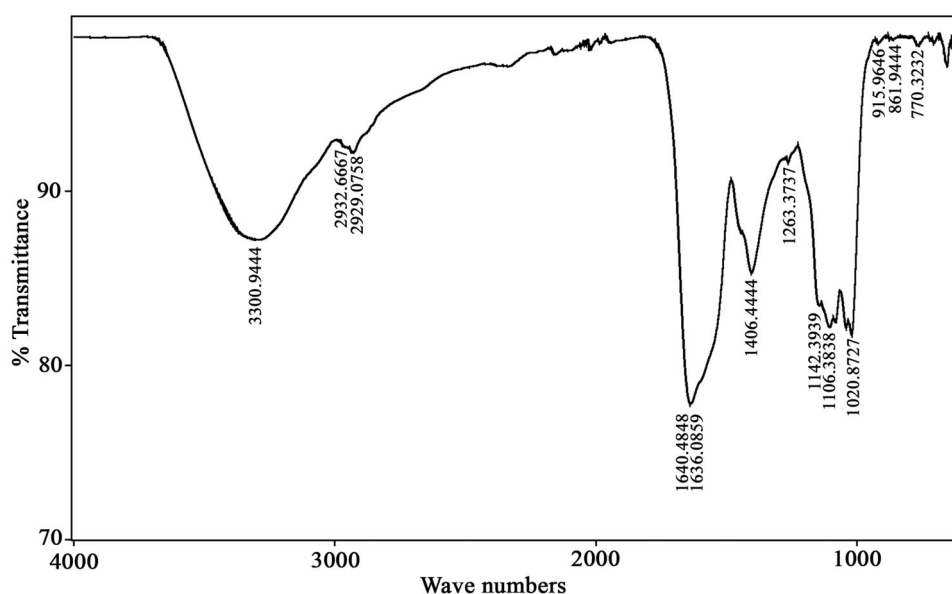


Figure 4. IR spectrum of the LVP ($600\text{--}4000\text{ cm}^{-1}$). LVP fractions were analyzed using ATR-FTIR.

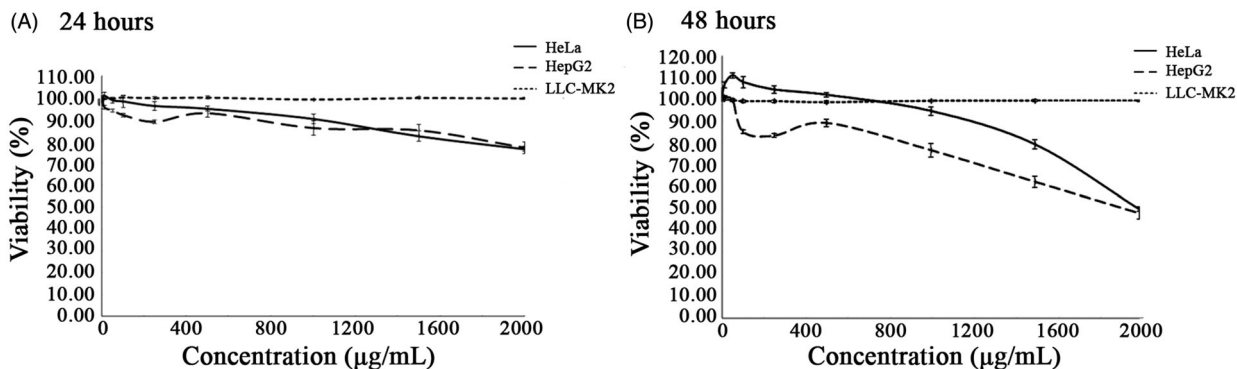


Figure 5. Cell viability of the human cancer cell lines, HeLa and HepG2, and the normal cell line, LLC-MK2, after treatment with LVP at concentrations of 0, 10, 50, 100, 250, 500, 1000, 1500, and 2000 $\mu\text{g/mL}$ for 24 h (A) and 48 h (B). Error bars indicated means \pm standard deviation ($n = 3$).

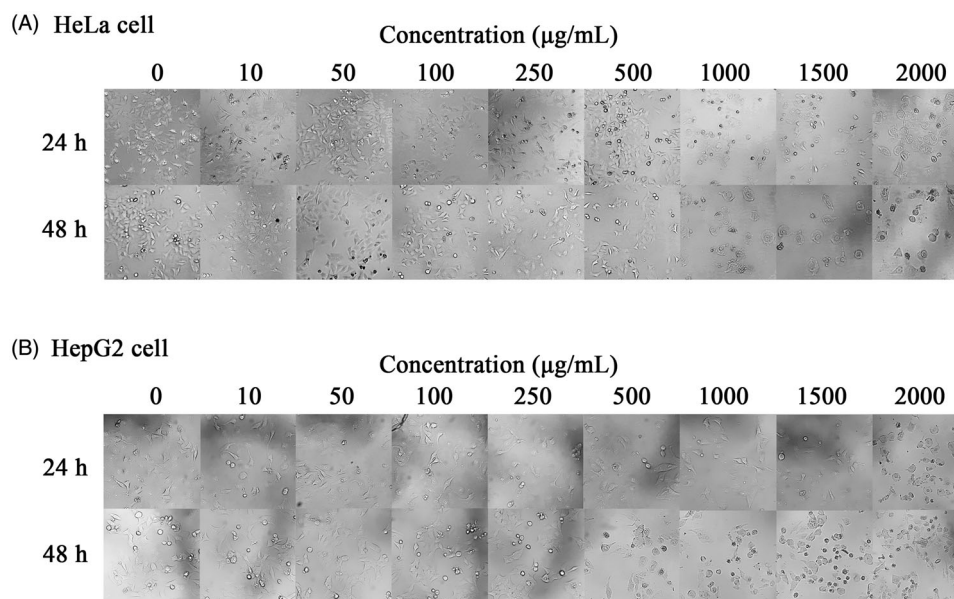


Figure 6. Morphology of the human cancer cell lines, HeLa (A) and HepG2 (B), after treatment with the LVP at various concentrations (0, 10, 50, 100, 250, 500, 1000, 1500, and 2000 $\mu\text{g/mL}$) for 24 and 48 h.

cytokines, which are toxic to cancer cells [19,20]. In addition, mushroom polysaccharides act as pathogen-derived molecules in adaptive immunity and can associate with several cell surface receptors such as dectin-1, complement receptor-3, scavenger receptor, lactosylceramide, and toll-like receptors. They can activate signaling pathways that promote innate immune responses and induce the production of reactive oxygen species and inflammatory cytokines [3,19,20]. In this study, the LVP inhibited growth of HeLa and HepG2 cells. It would be interested to determine inhibition of proliferation of different types of cancer cells both *in vitro* and *in vivo*.

3.4. Determination of antioxidant activity by DPPH free radical scavenging

The polysaccharides were evaluated for antioxidant activities by scavenging the DPPH radical. LVP caused a concentration-dependent response in the

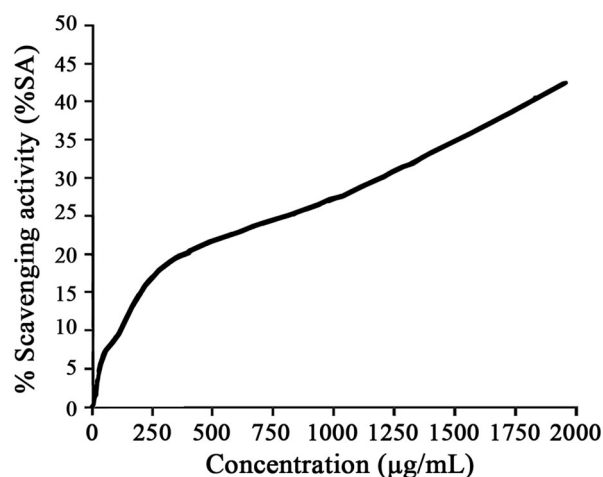


Figure 7. Free radical scavenging activity of LVP on DPPH at various concentrations (0, 10, 50, 100, 250, 500, 1000, 1500, and 2000 $\mu\text{g/mL}$). Error bars indicate means \pm standard deviation ($n = 3$).

assay. At a concentration of 2000 $\mu\text{g/mL}$, antioxidant activity reached approximately 40% SA (Figure 7).

Several studies have identified purified polysaccharide with strong antioxidant and anticancer activities [21,22], and these two effects may be related. Cancer cells have higher oxidative stress levels compared with normal cells, and ROS may alter the signaling pathways that contribute the cell malignancy. Therefore, antioxidant polysaccharides could scavenge free radicals and prevent the oxidative damage associated with cancer development, potentially slowing cancer development and leading to apoptosis [23].

Disclosure statement

We declared that there is no conflict of interest.

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