Original Article

ANTIOXIDANT ACTIVITY OF POLYSACCHARIDES EXTRACTED FROM *Lentinus edodes* MYCELIA AND THEIR PROTECTIVE EFFECTS ON INS-1 CELLS

ATIVIDADE ANTIOXIDANTE DE POLISSACARÍDEOS EXTRAÍDOS DE *Lentinus edodes* MICÉLIO E SEUS EFEITOS PROTECTORES EM CÉLULAS INS-1

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ABSTRACT: The disruption of the delicate balance between the generation of reactive oxygen species (ROS) and antioxidant scavenging systems can lead to many health problems. Polysaccharides extracted from fungi fruit body and myceliums have several potential health benefits. In this study, the antioxidant capacity and cell protective effects of a polysaccharide isolated from *Lentinus edodes* mycelia (LMP) were investigated. The antioxidant properties of LMP were screened using radical scavenging (1, 1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, and superoxide anion), reducing power and inhibition of lipid peroxidation assays. Intracellular reactive oxygen species (ROS), in response to H_2O_2, was determined by the dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay. Rat insulinoma (INS-1) cells apoptosis was detected using flow cytometry analysis. The results showed that LMP exhibited significantly strong radical scavenging activity. At the concentration of 2.0 mg mL^{-1}, DPPH radical, hydroxyl radical, and superoxide anion radical scavenging rates are $80.32 \pm 2.58\%$, $92.56 \pm 3.11\%$ and $93.73 \pm 2.82\%$, respectively. The reducing power and inhibition of lipid peroxidation activity of LMP were as strong as the positive control. LMP alleviated the intracellular reactive oxygen species level and inhibited islet cell apoptosis significantly. The results open perspectives for studies of LMP in the treatment of diabetes.

KEYWORDS: *Lentinus edodes* mycelia. Polysaccharide. Antioxidant. Oxidative stress.

INTRODUCTION

The disruption of the delicate balance between the generation of reactive oxygen species (ROS) and antioxidant scavenging systems can lead to health problems (XIA et al., 2011). A large amount of evidence has suggested that the production of oxygen derived free radicals is associated with many diseases, such as diabetes, Alzheimer's and Parkinson's disease, cancer as well as being involved in the degenerative process of aging (DURACKOVÁ, 2010). To alleviate damage to the human body, many antioxidants have been synthesized industrially. However, these synthetic antioxidants are suspected of being responsible for liver damage and certain types of cancer (LI et al., 2011). Therefore, it is important to develop and use effective and non-poisonous antioxidants that are able to scavenge free radicals in human bodies and maintain health.

In recent years, there has been a growing trend of finding natural antioxidants from edible mushrooms. *Lentinus edodes* is a mushroom widely consumed as a nutritious dietary and medicinal food in China. Many bioactive materials have been found in this mushroom, for example, polyphenolic compounds, polysaccharides, and dietary fiber (CHEN et al., 2012). Polysaccharides are biopolymers comprised of monosaccharides linked together through glycosidic bonds (ZHANG et al., 2007). Several recent studies have indicated that *Lentinus edodes* polysaccharides exhibit significant antioxidant activity by scavenging free radicals (CHEN et al., 2012; TSRIMUANG et al., 2011; CHEN et al., 2008). *Lentinus edodes* polysaccharides can be obtained from both the fruiting body and the mycelia. Generally, mature *Lentinus edodes* are harvested from plastic bags about 2 months after maturity, whereas the mycelia can be cultivated through submerged cultures. Thus, the mycelia of *Lentinus edodes* are substitute products for the mature fruiting bodies, which can be used for the production of polysaccharides. The polysaccharides extracted from *Lentinus edodes* mycelia are expected to become a new biologically active substance.

Type 2 diabetes is a serious metabolic disorder characterized by defects in the control of blood glucose (RAINS et al., 2011). Pancreatic beta cell dysfunction is closely related to the development and progression of type 2 diabetes, which is related to the loss of beta cells leading to a

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reduced beta cell mass (LEE et al., 2011). There is growing evidence that beta cell loss is largely because of apoptosis induced by oxidative stress (LEE et al., 2013; LIN et al., 2012; MEHMETI, et al., 2011). Although there has been a study on the antioxidant capacity of polysaccharides from the *Lentinus edodes* fruiting body carried out by Tsrimuang *et al* (TSRIMUANG, et al., 2011), there is still relatively little information concerning the antioxidant activities of *Lentinus edodes* mycelia polysaccharides and their function in reducing oxidative stress in islet cells. To fully develop this polysaccharide resource and extend the potential application of *Lentinus edodes* mycelia, the antioxidant activities of polysaccharides extracted from *Lentinus edodes* mycelia harvested from submerged cultures were analyzed to screen for natural antioxidants which could be used to protect islet cells.

MATERIALS AND METHODS

Preparation of Lentinus edodes mycelia polysaccharide (LMP)

The origin of the *Lentinus edodes* samples is *Lentinus edodes 973* stored in the School of Life Science, Liaoning University, China. The procedures for the preparation and purification of the *Lentinus edodes* mycelia polysaccharide have been described previously（CAO et al., 2013）. Briefly, the dried fungal biomass was boiled for 4 h. After centrifugation at $8000 \times g$ for 30 min, the crude polysaccharide was extracted from the supernatant using four volumes of absolute ethanol (4:1 v/v) at 4 °C for 10 h. The supernatant was discarded after centrifugation at $8000 \times g$ for 20 min. The pellet was washed, redissolved in distilled water, applied to a Sephadex G-200 column (2.0 × 60 cm) and eluted with H_2O at a flow rate of 1.0 mL min^{-1}. Fractions were further purified on a diethylaminoethyl (DEAE)-32 column (2.5 × 40 cm), with phosphate buffer solution-water used as the mobile phase and a gradient mode elution. A polysaccharide fraction identified as LMP was obtained.

DPPH radical scavenging assay

The DPPH radical scavenging assay was conducted according to the procedure of Song *et al* with minor modifications (SONG et al., 2010). DPPH solution (0.1 mM, in 95% ethanol solution) was prepared and used fresh on the day of each test. Two mL of DPPH solution was incubated with 2.0 mL of the LMP solution at varying concentrations (0.25, 0.5, 1.0, 1.5, and 2.0 mg mL^{-1}). The reaction mixture was mixed well and incubated for 30 min at room temperature. The absorbance was then measured at 517 nm against a blank. The DPPH radical scavenging activity was measured and calculated using the following equation:

Scavenging effect (%)=(1− A $_{Sample}$ / A $_{Control}$)×100%

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging effect of LMP was measured according to the procedure of He *et al* (2012) with slight modifications (He et al., 2012). 1.0 mL of sample solution at different concentrations (0.25, 0.5, 1.0, 1.5, and 2.0 mg mL^{-1}) was mixed with 1.0 mL of 6 mM $FeSO_4$. Then 1.0 mL of 6 mM H_2O_2 was added to start the reaction. 1.0 mL of 6 mM salicylic acid was added after 10 min. Finally, the reaction mixture was incubated for 30 min at room temperature and the absorbance was recorded at 510 nm with a visible spectrometer (details of machine manufacturer, location). Ascorbic acid was used as the positive control. The scavenging activity of the polysaccharides against the hydroxyl radicals was calculated according to the following equation:

HO radical scavenging activity (%) = (1 − (A_{sample} − A_{blank}) / $A_{control}$) × 100%

Superoxide anion radical scavenging assay

The measurement of superoxide anion radical scavenging activity was investigated according to the method reported by Zha *et al* (ZHA et al., 2009). 1.0 mL of 86 µM nitroblue tetrazolium (NBT), 1.0 mL of 469 µM nicotinamide adenine dinucleotide (NADH), 1.0 mL of LMP at different concentrations (0.25, 0.5, 1.0, 1.5, and 2.0 mg mL^{-1}) and 0.4 mL phenazine methosulfate (PMS) were mixed in test tubes to initiate the reaction. The reaction mixture was then incubated at ambient temperature for 5 min and the absorbance of the mixture solution was determined at 560 nm with a visible spectrometer (details of machine manufacturer, location). Ascorbic acid was used as the positive control. The superoxide anion radical scavenging capability of LMP was calculated as follows:

Scavenging activity (%) = [1 − (A_{sample} − A_{blank}) / $A_{control}$] × 100%

Reducing power assay

The measurement of reducing power was conducted according to the method reported by Soares *et al* (SOARES et al., 2009). 1.0 mL of LMP solution at Different concentrations (0.25, 0.5, 1.0, 1.5, and 2.0 mg mL^{-1}) were mixed with 2.5 mL of

phosphate buffer (50 mM, pH 7.0) and 2.5 mL of 1% potassium ferricyanide. The mixture was then incubated at 50 °C for 20 min. Then, 2.5 mL of 10% trichloroacetic acid was added to the mixture to terminate the reaction, followed by centrifuging at $3000 \times g$ for 10 min. Finally, 1.25 mL of the supernatant was mixed with 1.25 mL of distilled water and 0.25 mL of a 0.1% $FeCl_3$ solution. The absorbance was immediately measured at 700 nm by using a spectrometer. Ascorbic acid was used as the positive control. A higher absorbance of the reaction mixture indicated a high reducing power.

Assay for inhibition of liver lipid peroxidation

The assay for inhibition of liver lipid peroxidation was performed using the method described by Ma et al (MA et al., 2012) with some modifications. Healthy Rat livers were washed with 0.15 M NaCl and homogenized in 10 volumes (v/w) of normal saline at 4 °C. Reaction mixtures contained 2.5 mL of liver homogenate, 1.4 mL of 0.2 mM PBS (pH 7.4), 1.0 mL of 10 µM $FeSO_4 \cdot 7H_2O$, 2.5 mL of 100 mM ascorbic acid and 1.0 mL of various concentrations of LMP (0.25, 0.5, 1.0, 1.5, and 2.0 mg mL^{-1}). The mixtures were incubated at 37 °C for 30 min, followed by centrifugation ($3000 \times g$, 10 min). After the addition of 1.0 mL of the TBA reagent to the supernatant, the tubes were placed in a boiling water bath for 15 min. Absorbance was then measured at 530 nm by a spectrophotometer. The percentage of lipid peroxidation inhibition by the samples was then calculated. Ascorbic acid was used as the positive control.

Measurement of intracellular ROS

Intracellular reactive oxygen species (ROS) were measured by a fluorescent dye technique as described by Lu *et al* (LU et al., 2008). 1×10^5 INS-1 cells were seeded on plate overnight, then treated with 0.5 mM H_2O_2, and then incubated with or without 2.0 mg mL^{-1} LMP 3 h. INS-1 cells were incubated for 30 min with 10 µM DCFH-DA at 37 °C in the dark in phosphate buffered saline (PBS). Images were obtained with a fluorescence microscope with excitation at 488 nm and emission at >500 nm. The fluorescent intensities of individual cells were analyzed using the InterVision 2D program (Noran Instruments Inc, WI, USA).

Flow cytometry analysis

Flow cytometry analysis was performed to determine this event using the Annexin V-FITC Apoptosis Detection Kit (KeyGen, Nanjing, China).

Cells were seeded and treated with 0.5 mM H_2O_2 followed by incubating with or without 2.0 mg mL^{-1} LMP 3 h. Cells were harvested by trypsinization, then washed twice with cold PBS and centrifuged at $1000 \times g$. About 1-5×10^5 cells were resuspended in 500 µL of binding buffer and transferred to a flow cytometry glass tube. Five microliters of Annexin V-FITC and 5 µL of propidium iodide were added and the tube was incubated in the dark for 15 min at room temperature. Apoptotic cells were measured using a FACScan flow cytometer.

Data analysis

Statistical analysis was performed using SPSS 18.0 (IBM Corp., Armonk, NY, USA). All data were expressed as mean ± standard deviation (SD). Statistical comparisons were carried out using one-way analysis of variance (ANOVA) and randomized block analysis of variance. The Students-Newman-Keuls (SNK) method was employed for intergroup comparison. Associations between categorical variables were verified by the Chi-squared test. Statistical significance was determined as $p < 0.05$.

RESULTS AND DISCUSSION

DPPH radical scavenging activity

DPPH (1,1-diphenyl-2-picrylhydrazyl) is a compound that has been widely used to determine the free-radical scavenging ability of different materials (CHEN et al., 2011). Alcoholic solutions of DPPH have a characteristic maximum absorption at 517 nm. When an antioxidant that can provide electrons or hydrogen atoms is added to DPPH, the absorbance at 517 nm disappears because the DPPH radical is scavenged (LI et al., 2011). The scavenging ability of LMP on the DPPH radical is shown in Figure 1A. The results indicated that LMP showed evident scavenging activities on the DPPH radical in a concentration-dependent manner. High levels of scavenging abilities were found for all samples used in the test. The scavenging ability of LMP on DPPH radical was 36.69 ± 1.98% at 0.25 mg mL^{-1} and increased to 80.32 ± 2.58% at 2.0 mg mL^{-1} which is close to that of ascorbic acid. The scavenging ability of ascorbic acid on DPPH radical was 92.3± 3.18%. The EC$_{50}$ value of LMP on DPPH is 0.39 mg mL^{-1}, which is higher than the result of Tsrimuang *et al* (TSRIMUANG et al., 2011). These results indicated that LMP is a strong free radical-scavenging polysaccharide that could help prevent oxidative stress.
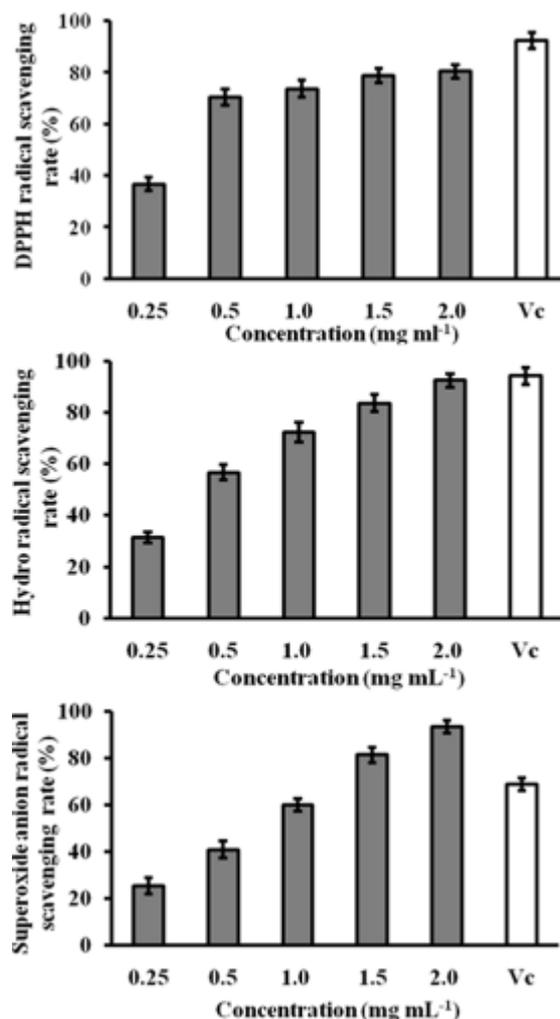
Figure 1. Free radical scavenging capacity of LMP. (A) DPPH radical scavenging activity of LMP. (B)Hydroxyl radical scavenging activity of LMP. (C) Superoxide anion scavenging activity of LMP. Vitamin C (Vc) was used as positive control. Data were mean ± SD of three parallel measurements.

Hydroxyl radical scavenging activity

The hydroxyl radical has a very short *in vivo* half-life of approximately 10^{-9} s and a high reactivity. This makes it a very dangerous compound in adjacent bimolecular reactions (CHEN et al., 2012). These radicals can attack biological molecules such as DNA, proteins and lipids. Thus, the removal of hydroxyl radicals is important for the protection of living systems (LIU et al., 2012). As shown in Fig. 1B, the results indicated effective hydroxyl radical scavenging activities at all tested concentrations of LMP. The scavenging ratios were improved with the increase of polysaccharide concentration. The highest scavenging rate was 92.56 ± 3.11% at the concentration of 2.0 mg mL^{-1}, which was similar to that of ascorbic acid (The scavenging rate was 95.21 ± 4.56%). These results show that LMP is a good scavenger for the hydroxyl radical. The scavenging activity of LMP on the hydroxyl radical is also higher than that of

polysaccharides from the fruiting body of *Lentinus edodes* (CHEN et al., 2012). This indicates that *Lentinus edodes* mycelium could be a new resource of bioactive polysaccharides.

Superoxide radical scavenging activity

The superoxide radical is normally formed first in cellular oxidation reactions, and then produces other types of cell damaging free radicals and oxidizing agents (LIU et al., 2012). Although the superoxide radical is a weak oxidant in most organisms, it could produce hydrogen peroxide and hydroxyl radicals through dismutation and other types of reaction *in vivo* (ZHA et al., 2009). Superoxide radical is also known to indirectly initiate lipid peroxidation as a result of the formation of hydrogen peroxide. Therefore, scavenging the superoxide anion is extremely important in antioxidant work. In the present study, the scavenging ability of LMP on the superoxide

radical was exhibited in a concentration-dependent manner as shown in Figure 1C. The highest scavenging ability of 2.0 mg mL^{-1} LMP was 93.73 ± 2.82%, which was better than that of ascorbic acid (The scavenging rate was 69.58 ± 2.36%). Chen *et al* (2012) reported that the polysaccharides from the fruiting body of *Lentinus edodes* could scavenge the superoxide radical, with a scavenging rate of about 90% (CHEN et al., 2012). Our results showed that LMP had a higher scavenging rate. However the mechanism and the basis of the LMP structure should be investigated in future studies.

Reducing power

The reducing power of a compound may serve as an indicator of its potential antioxidant activity. Higher absorbance values indicate stronger reducing power (LIU et al., 2012). The reducing power of LMP was investigated and showed in Figure 2A. As shown in the figure, the reducing power of the samples correlated well with increasing concentrations. At the lower concentration, the reducing power was far lower than that of ascorbic acid. However, at the higher concentration, the reducing power of LMP was close to that of ascorbic acid. The reducing power of

LMP in our data suggested that it was likely to contribute to the observed antioxidant effect.

Activity of inhibition on liver lipid peroxidation

The liver peroxidation inhibition activity of LMP is shown in Figure 2B, The data revealed that LMP demonstrated great capacity for the inhibition of lipid peroxidation. The inhibition rate of LMP increased from 12.53 ± 1.22% to 61.76 ± 3.38% as the concentration of LMP increased from 0.25 to 2.0 mg mL^{-1}, suggesting that LMP possesses relatively a strong liver peroxidation capacity and the inhibitory effects were concentration dependent. In the concentration range tested, the inhibiting effect of LMP was lower than that of ascorbic acid. It is generally admitted that the initiation of a peroxidation sequence in a cell or polyunsaturated fatty acid is because of the abstraction of a hydrogen atom from the double bond in the fatty acid (MA et al., 2012). Many studies showed that lipid peroxidation is associated with various pathological events, such as insulin resistance, inflammation, postischemic reperfusion injury, atherosclerosis, ethanol toxicity, and even cancer. (MIRMIRAN P et al., 2014; WANG et al., 2012). Therefore, it is very important for health to prevent the lipid peroxidation.
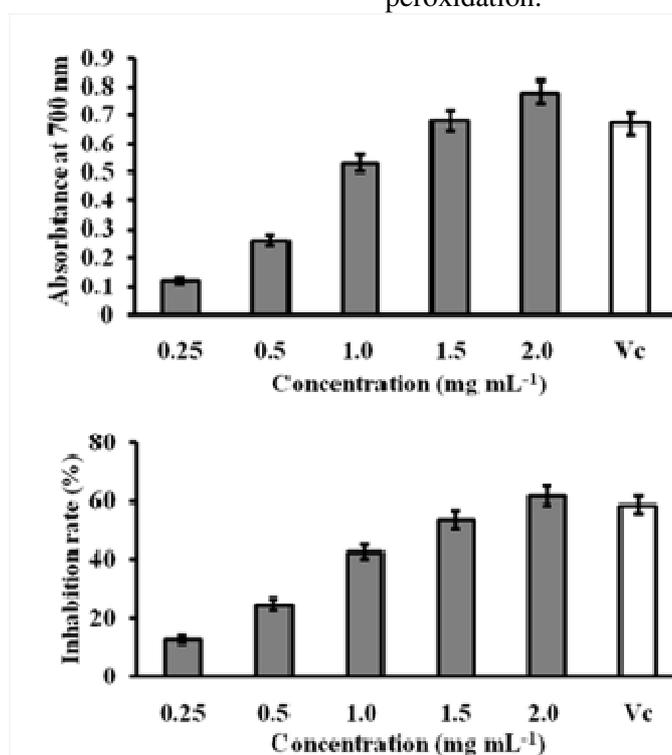

Figure 2. Reducing powers and Inhibitory activity on liver lipid peroxidation of LMP. (A) Reducing power of LMP. (B) Inhibitory activity on liver lipid peroxidation of LMP. Data were mean ± SD of three parallel measurements.

LMP attenuate intracellular ROS

The antioxidant capacity in β cells is very low because of the weak expression of antioxidant enzymes such as glutathione peroxidase and superoxide dismutase in pancreatic islets compared with that in various other tissues (FUJIMOTO et al., 2011). It is believed that the generation of ROS ultimately culminates in β cell dysfunction and death (MCCABE et al., 2006). As shown in Figure 3A, many INS-1 cells were detached within 3 h of H_2O_2 loading. Most of the LMP loaded cells treated with H_2O_2 remained adherent. The data demonstrated that LMP prevented cell death induced by H_2O_2. The number of coherent cells after LMP loading for 3 h was significantly higher than

that after 3 h loading of H_2O_2. Statistical analysis demonstrated that the decrease in the adherent cell number induced by H_2O_2 loading were significant (Figure 3B). To determine whether the cell death of INS-1 cells was because of oxidative stress, the DCFH-DA assay was applied to evaluate intracellular ROS, as shown in Figure 4A. The intracellular ROS decreased significantly after LMP loading in INS-1 cells in the presence of H_2O_2 for 3h. Dichlorofluorescein fluorescence was assayed using confocal microscopy. The ROS content of H_2O_2-treated INS-1 cells was more than nine fold compared with the control cells (Figure 4B). The loading of LMP significantly decreased the level of ROS.
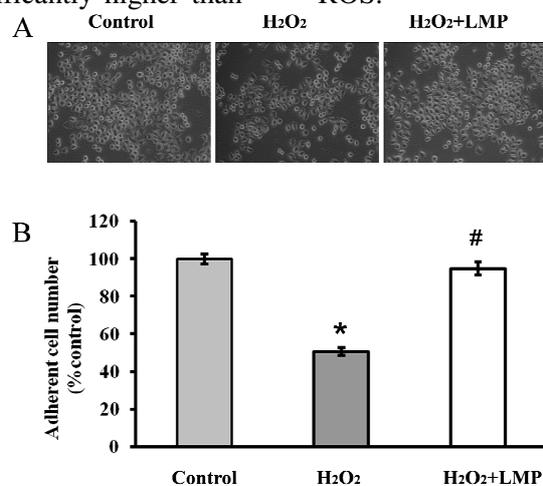

Figure 3. LMP protected cell viability after H_2O_2 exposure. (A) The cell images were obtained with a phase-contrast microscope at 3 h after treated with H_2O_2 in the absence or presence of 2.0 mg mL^{-1} LMP. (B) The numbers of adherent cells were expressed as percentage of the number of control cells, Data were mean ± SD of three parallel measurements. *$p<0.05$ vs. the levels of control. #$p<0.05$ vs. the levels of cells untreated with H_2O_2
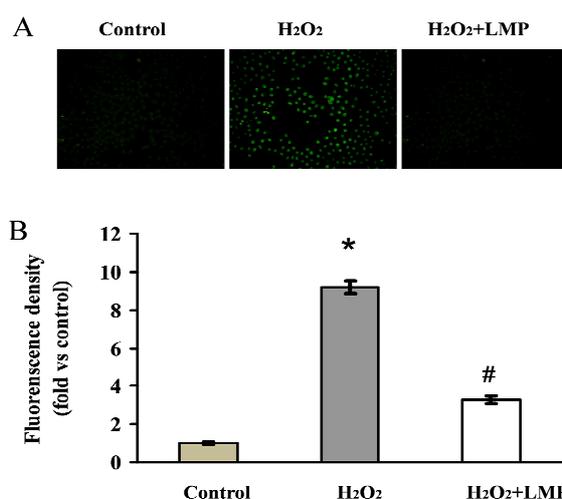

Figure 4. Effect of LMP on intracellular ROS.(A) The cell images were obtained with fluorescence microscope at 3 h after treated with H_2O_2 in the absence or presence of 2.0 mg mL^{-1} LMP. (B) Fluorescent intensities of DCFH-DA are expressed as fold increases of the of control level, Data were mean ± SD of three parallel measurements *$p<0.05$ vs. the levels of control. #$p<0.05$ vs. the levels of cells untreated with LMP.

LMP inhibit cell apoptosis induced by H₂O₂

Many studies have shown that reduced β cell mass is one of important determining risk factor for diabetes. (XU et al., 2009) Pancreatic β cells are sensitive to ROS damage which can induce cell apoptosis in many kinds of cell lines. It has been proposed that treatment using antioxidants can prevent pancreatic β cells apoptosis. Our results indicated that 0.25 mM H_2O_2 can induce the

apoptosis of pancreatic β cell with a 10.23 ± 0.87% apoptosis rate, whereas LMP can reduce the pancreatic β cell apoptosis to a 3.26 ± 0.28% apoptosis rate (Figure 5). The results indicated that LMP can inhibit pancreatic β cell apoptosis by reducing oxidative stress, which suggests that LMP has the potential to be used as an antioxidant for diabetes prevention.
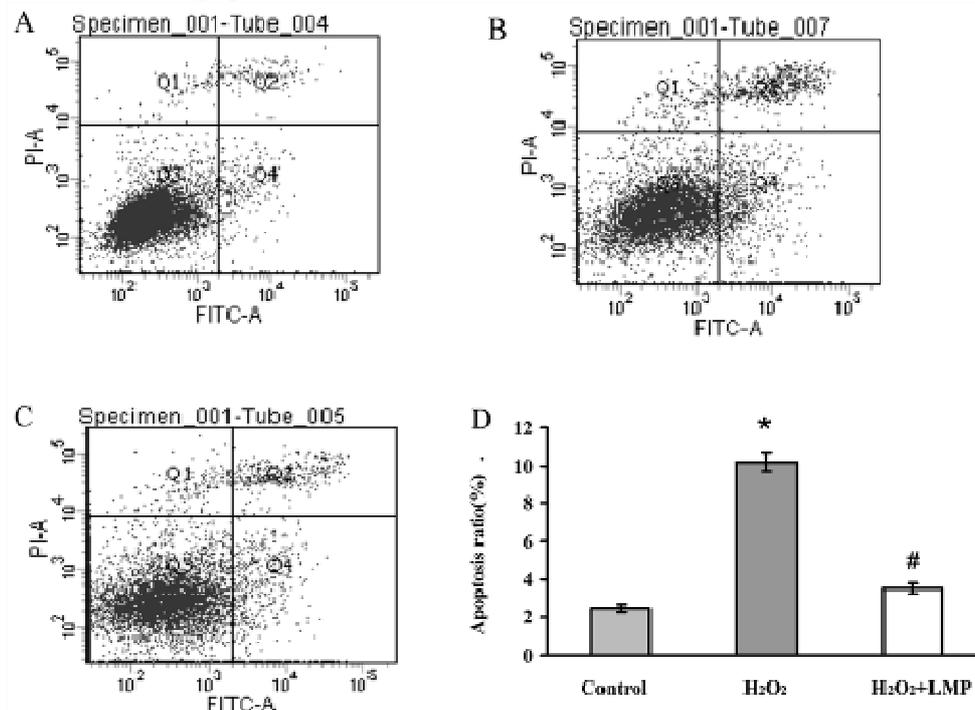

Figure 5. LMP inhibited INS-1 cell apoptosis induced by H_2O_2. (A) Control group. (B) Measurement of INS-1 cell apoptosis with H_2O_2 treatment. (C) Measurement of INS-1 cell apoptosis induced by H_2O_2 in the presence of 2.0 mg mL^{-1} LMP. (D) Statistical analysis of apoptosis rate. Data were mean ± SD of three parallel measurements *$p<0.05$ vs. the levels of control. #$p<0.05$ vs. the levels of cells untreated with LMP.

CONCLUSIONS

LMP exhibited significantly strong radical scavenging activity.

The radical scavenging rate of the DPPH radical, hydroxyl radical and superoxide anion radical were 80.32 ± 2.58%, 92.56 ± 3.11% and 93.73 ± 2.82%, respectively. The reducing power and inhibition of lipid peroxidation activity were as strong as the positive control.

LMP reduced the level of intracellular reactive oxygen species and inhibited cell apoptosis significantly, which appeared to be because of its antioxidant characteristics. The results open perspectives for studies of LMP in the treatment of diabetes

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RESUMO: O estresse oxidativo tem uma função biológica importante em muitos organismos. A ruptura do equilíbrio delicado entre a geração de espécies reativas de oxigênio (ROS) e sistemas de eliminação de antioxidantes pode levar a muitos problemas de saúde. Polissacarídeos extraídos do corpo fungos frutas e micélio têm vários benefícios potenciais para a saúde. Neste estudo, foram investigados os efeitos celulares e capacidade de proteção antioxidante de um polissacarídeo isolado a partir de Lentinus edodes micélios (LMP). As propriedades antioxidantes da LMP foram rastreadas utilizando eliminação de radicais (1, 1-difenil-2-picrilhidrazil (DPPH), hidroxilo e anião superóxido), e a redução da potência de inibição de ensaios de peroxidação lipídica. Espécies reativas de oxigénio intracelulares (ROS), em resposta ao H_2O_2, foi determinada pelo ensaio de diacetato de dicloro-di-hidro-f luoresceina (DCFH-DA). INS-1 células a apoptose foi detectada utilizando análise de citometria de fluxo. Os resultados mostraram que LMP exibiu significativamente forte atividade de eliminação de radicais. Na concentração de 2 mg mL^{-1}, DPPH radical, radical hidroxilo e anião superóxido velocidades de eliminação de radicais livres são 80,32 ± 2,58%, 92,56 ± 3,11% 93,73 ± 2,82%, respectivamente. O poder redutor e inibição da peroxidação lipídica de LMP foram tão forte quanto o controlo positivo. LMP aliviou a nível de espécies reativas de oxigênio intracelular e apoptose de células da ilhota inibiu significativamente. Sugere-se que LMP pode ser utilizado como um reagente complementar para a diabetes a prevenção e terapia.

PALAVRAS-CHAVE: Lentinus edodes mycelia. Polissacarideo. Antioxidante. Estresse oxidativo.

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