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Hepatoprotective and antioxidant properties of *Schizophyllum commune* fruiting body

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Abstract

The split gill fungus, *Schizophyllum commune*, is an edible mushroom acknowledged for its medical importance and hepatoprotective activity. This study assessed the hepatoprotective and antioxidant activities of extraction from *S. commune*. The dried fruiting body of *S. commune* was extracted with different solvents (hexane, chloroform, ethyl acetate, and methanol) and by the hot water extraction method, followed by ethanol precipitation to provide crude polysaccharide. The extracts were evaluated for their scavenging activity, total phenolic content, and flavonoid content, with cytotoxicity determined in human hepatoma cells (HepG2). The hepatoprotective effect of *S. commune* extracts was evaluated using hydrogen peroxide (H_2O_2)-induced toxicity on HepG2 cells, while protective effects and morphological changes were assessed using Hoechst 33342 staining assay. Lipid peroxidation and reduced glutathione levels induced by H_2O_2 were studied to assess possible mechanisms of hepatoprotection by *S. commune* extracts. The five extracts showed DPPH free radical scavenging activities with IC_{50} values of 0.57±0.02 mg/mL, 0.40±0.01 mg/mL, 0.36±0.00 mg/mL, 0.32±0.00 mg/mL and 1.00 ±0.05 mg/mL. Antioxidant activity IC_{50} values of DPPH scavenging correlated with total phenolic and total flavonoid contents. Treatment of HepG2 cells with polysaccharide extract before H_2O_2 exposure increased cell viability, while polysaccharide extract inhibited lipid peroxidation and increased glutathione levels against H_2O_2-induced damage. Results revealed that the polysaccharide extract from the fruiting body of *S. commune* had hepatoprotective potential and showed promise as a potential therapeutic agent for liver diseases.

Keywords: *antioxidant; hydrogen peroxide; hepatoprotective; polysaccharide extract; Schizophyllum commune.*

1. Introduction

Oxidative stress is an important contributor to the pathophysiology of various pathological conditions including cardiovascular dysfunctions, atherosclerosis, inflammation, carcinogenesis, reperfusion injury and neurodegenerative diseases (Aruoma, Grootveld, & Bahorun, 2006). The liver is the most significant and important organ in the vertebrate body, involving the metabolism of food molecules, detoxification and biotransformation. (Kumar, Kumar, & Sinha, 2019). It is also the most important site of intermediary metabolism by processing both endogenous (e.g., cholesterol, fatty acids and proteins) and exogenous substances (e.g., drugs) and converting them into less-harmful chemicals by detoxification or elimination. Prolonged

exposure to exogenous compounds such as drugs, alcohol or chemicals causes liver dysfunction due to increased reactive oxygen species (ROS) in hepatic cells, leading to oxidative stress (Lister et al., 2020; Yoon, Baber, Chondhary, Kuter, & Pysopoulos, 2016). Many bioactive compounds have protective effects against oxidative stress-induced cell death (Ali et al., 2004; Jaydeokar, Bandawane, Bibave, & Patil, 2014). Mushrooms possess naturally occurring antioxidants as phenolic and polysaccharide compounds that can prevent and cure liver damage (Mau, Tsai, Tseng, & Huang, 2005; Selima, Aminul, Ugur, & Narayan, 2012). Several mushroom species including *Ganoderma lucidium* (Kao, Jesuthasan, Bishop, Glucina, & Ferguson, 2013), *Grifola frondora* (Masuda, Ito, Konishi, & Nonbu, 2010), *Lentinula edodes* (Wu, & Hansen, 2008) and *Schizophyllum commune* contain antioxidants.

Schizophyllum commune or split gill mushroom is commonly found in many countries including Laos, Myanmar, India and Thailand. *S. commune* has a protein content of 16-27%, (Aletor, 1995; Longvah, & Deosthale, 1998) low-fat content, fatty acids and amino acids essential to the human diet (Longvah, & Deosthale, 1998), together with a polysaccharide known as Schizophyllan, which has a chemical structure consisting of a β (1-3) glucan chain linked via β (1-6) glucan (Zhang, Kong, Fang, Nishinari, & Phillips, 2013). Multifunctional biological properties include anti-tumor, antimicrobial, immunomodulatory and anti-inflammatory functions (Lindequist, Niedermeyer, & Julich, 2005; Joel, & Bhimba, 2013). *S. commune* methanol and ethyl acetate extracts inhibited superoxide radicals (Mirfat, Noorlidah, & Vikineswary, 2010), while the ethyl acetate fraction exhibited high inhibition against lipid peroxidation in egg yolk (Mayakrishnan et al., 2013). However, no reports are available detailing the antioxidant and associated hepatoprotective activities of *S. commune* extracted by different organics-solvents and polysaccharides.

2. Objectives

This study investigated the hepatoprotective activity of different organic solvents and polysaccharide extract from the fruiting body of *S. commune* against liver damage mediated by hydrogen peroxide (H_2O_2) in human hepatocellular liver carcinoma cells (HepG2 cells) to determine its antioxidant activity.

3. Materials and Methods

3.1 Materials and chemicals

Schizophyllum commune (Fr.) were cultured from a certified organic farm in Chantaburi Province, Thailand. The fruiting body was dried in a hot air oven at 50 °C for 4 days, then ground into powder and stored for further extraction. Hexane, chloroform, ethyl acetate, methanol, ethanol and DMSO were purchased from RCl Labscan Co., Ltd., Thailand. Folin-Ciocalteu phenol reagent was purchased from Sigma-Aldrich, Switzerland. Standard gallic acid was purchased from Sigma-Aldrich, Germany. Standard quercetin was purchased from Sigma-Aldrich, India. Standard BSA and Coomassie reagent were purchased from Thermo Scientific, USA. Standard D-glucose was purchased from VWR Life Science, USA. HepG2 (human hepatocellular liver carcinoma cell line, ATCC) was purchased from USA. Minimum Essential Medium (MEM) was purchased from Gibco, USA. Fetal bovine serum (FBS) was purchased from Gibco, UK. Hydrogen peroxide (H_2O_2) was purchased from Polkie Odczynniki Chemiczne S. A. MTT (thiazolyl blue tetrazolium bromide) was purchased from Sigma-Aldrich, China. Hoechst 33342 was purchased from Invitrogen, USA. 2-Thiobarbituric acid (TBA) was purchased from Sigma-Aldrich, UK. Trichloroacetic acid (TCA) was purchased from Merck, Germany. Standard glutathione was purchased from Sigma-Aldrich, USA. DTNB (5, 5'-dithiobis (2-nitrobenzoic acid) was purchased from Fluka Analytical, USA. All reagents and chemicals were of analytical grade.

3.2 Preparation of extracts

Solvent extraction

The dried powdered fruiting body of *S. commune* (100 g) was extracted by maceration using hexane, ethyl acetate, chloroform and methanol. Crude extracts were obtained by evaporating the solvent in a rotary evaporator and then stored at -20 °C until use. Percentage yields of the crude extracts were calculated as [weight of crude mushroom extract (g) / weight of dry mushroom (g) x 100%] (Bugrahan et al., 2017).

3.3 Polysaccharide extraction

The dried powdered fruiting body (100 g) was extracted with distilled water at 85 °C and centrifuged. The supernatant was concentrated under reduced pressure at 40 °C and precipitated with six volumes of 95% ethanol (Zhong et al., 2015). The

solution was freeze-dried to provide crude polysaccharides and then stored at -20 ºC until use. The percentage yield of the crude extract was calculated as above.

3.4 Schizophyllan analyses

The polysaccharide extract was filtered through a 0.2 µm filter and 20 µL of the filtrate was loaded into the HPLC system. (Thermo, model: Spectra Series Complete HPLC AS3000, P4000, UV1000, SN4000, USA) Separation was performed on an Agilent Eclipse Plus C18 column (100 mm × 4.6 mm, 3.5 µm) using a 0.8 mL/min flow rate at 25 °C. The mobile phase consisted of eluent A (Water) and eluent B (Methanol). A gradient program was used for elution comprising 0–5 min, 90% A; 5–10 min, 85% A. Absorbance of the sample solution was measured at 205 nm (Su, Lai, Lin, & Ng, 2016).

3.5 Measurement of carbohydrate and protein contents

Total carbohydrate content of the polysaccharide was determined by the phenol–sulfuric acid colorimetric method using D-glucose as the standard (Masuko et al., 2005). Protein in the polysaccharide was also estimated by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as the standard.

3.6 Total phenolic determination

Phenolic content was determined by the Folin- Ciocalteu method following Thomas, Priyadarshini, & Michael (2012). Briefly, 10 µL of each extract was mixed with 20% Folin-Ciocalteu phenol reagent (150 µL*)* in 96-well plates, followed by adding 50 µL of sodium carbonate (Na_2CO_3) after 3 min. The mixture was incubated for 2 h in the dark and absorbance was measured at 765 nm using a microplate reader (TECAN, Infinite M200 Pro, Switzerland). Total phenolic content was expressed as gallic acid equivalent (GAE) in mg per g of extract.

3.7 Total flavonoid determination

Flavonoid content of each extract was determined using an aluminum chloride colorimetric assay. (Ghasemi, Ghasemi, & Ebrahimza, 2009). Briefly, 50 µL of each extract was mixed with 150 µL of methanol, 10 µL of 10% aluminum chloride ($AlCl_3$) and 10 µL of 1M potassium acetate, followed by 30 µL of distilled water. Samples were mixed and kept for 30 min in the dark. Absorbance was measured at 415 nm using a microplate reader. Total flavonoid content was expressed as quercetin equivalent (QE) in mg per g of extract from a calibration curve of quercetin standard solution.

3.8 DPPH free radical scavenging activity

Scavenging ability was determined by the stable radical DPPH (2, 2-diphenyl picrylhydrazyl) as described by Gerhauser et al. (2013). Various 5 µL concentrations of different extracts (hexane, chloroform, ethyl acetate, methanol and polysaccharide) were mixed with 195 µL ethanolic solution containing DPPH (0.05 mg/mL) and left for 30 min in the dark. Absorbance was measured at 515 nm using a microplate reader (TECAN, Infinite M200 Pro, Switzerland). Ethanol was used as a blank. Sample concentration that scavenged 50% of DPPH free radical (IC_{50} value) was calculated.

3.9 Cell cultures

HepG2 cells (human hepatocellular liver carcinoma cell line) were obtained from ATCC (ATCC®. HB- 8065™). Cells were cultured in Minimum Essential Medium (MEM), supplemented with 1 % penicillin-streptomycin and 10% FBS. Cells were grown in 5% CO_2 at 37 °C. HepG2 showing more than 80% cell confluence was used.

3.10 Cell viability

The MTT assay was performed to assess cytotoxicity following Siripong, Rassamee, Piyaviriyakul, Yahuafai, & Kanokmedhakul (2012). In brief, HepG2 cells were seeded in 96-well plates at a density of 1×10^4 cells per well and allowed to grow overnight. Then, the cells were exposed to various concentrations of each extract of *S. commune* (100-1000 µg/mL) or H_2O_2 (100-1000 µM) for 24 h. After exposure, 5 mg/mL MTT was added to each well and the plates were further incubated for 3 h. Then, the reaction mixture was carefully taken out, and 100 µL of DMSO was added to each well and mixed gently by pipetting up and down. Absorbance of the plates was measured at 550 nm.

3.11 Hepatoprotective activity assay

The cytoprotective activity of test samples on H_2O_2-induced cell injury was investigated. Cell densities of 1×10^4 cells per well were plated in 96-

well plates with culture medium for 24 h, then exposed to test samples for 1 h and added with H_2O_2 100 µM for 24 h. Following treatment, the cells were added to a 5 mg/mL MTT solution in MEM, and further incubated for 3 h. The resulting formazan was dissolved in 100 µL of DMSO. The developed color was read at 550 nm using a microplate reader. Cell viability was determined as described above and expressed as a percentage with the vehicle-treated control cells as 100% (Siripong et al., 2012).

3.12 Morphological changes induced by Hoechst 33342 staining

Hoechst 33342 staining was performed to observe changes in nuclear morphology. Briefly, HepG2 cells were seeded at a density of $1x10^4$ cells/mL in 6-well plates and incubated for 24 h at 37 °C. Then, the cells were treated with polysaccharide extract at final concentrations of (400, 800 and 1000 µg/mL). After 1 h, H_2O_2 (100 µM) was added and the cells were incubated for 24h. After treatment, the HepG2 cells were stained with 5 µg/mL Hoechst 33342 for 20 min at room temperature (Phonghanpot, & Jarintanan, 2021). Finally, the cells were imaged under a fluorescence microscope (Nikon, & Yokohama, Japan).

3.13 Lipid peroxidation (LPO)

HepG2 cells were plated at 1×10^5 cells per well in 6-well plates and allowed to stabilize for 24 h before treatment with 1 mL of polysaccharide extract (400-1000 µg/mL). After 1 h, H_2O_2 (100 µM) was added and the cells were incubated for 24 h. Cells were then gently washed twice with PBS and harvested in 1.5 mL of PBS by scraping. Following centrifugation, the pellet was re-suspended in 100 µL of 25 mM Tris-HCl buffer (pH 7.4) and subjected to ultrasonication for 5 min. The protein content of the cell suspension was measured using BSA as the standard. The extent of lipid peroxidation was estimated by measuring levels of malondialdehyde (MDA) using the thiobarbituric acid reactive substances (TBARS) assay. One milliliter of the reaction mixture was mixed with 2 mL of thiobarbituric acid (15%), trichloroacetic acid (10%) and hydrochloric acid (0.25 N) at a ratio of 1:1:1. The mixture was heated in a 95 °C water bath for 40 min and cooled at room temperature for 10 min. Following centrifugation, the absorbance of the supernatant was measured at 532 nm. Positive and negative controls consisted of cells treated with H_2O_2 and medium alone, respectively. A standard calibration curve was prepared from 1,1,3,3-tetramethoxypropan (0.3-10 µg/mL), a commercial form of malondialdehyde (MDA). The lipid peroxidation was calculated from the standard curve. The result was expressed as LPO level (%) Chourasiya and Sharma, S. (2021).

3.14 Glutathione level

The glutathione level in HepG2 cells was measured following the methods of Chandra et al. (2002) and Al-Sheddi et al. (2015). In brief, HepG2 cells were plated at 1×10^5 cells per well in 6-well plates for 24h and then exposed to polysaccharide extract (400-1000 µg/ mL) and H_2O_2 (100 µM) for 24 h. After respective exposures, cells were centrifuged and cellular protein was precipitated with 1 mL TCA (10%) on ice for 30 min. The supernatant was taken by centrifugation at 13000 rpm for 10 min. Then, 2 mL of 0.4 M Tris buffer (pH 8.9) containing 0.02 M EDTA and 0.01 M 5, 5'pol-dithionitrobenzoic acid (DTNB) were added to the supernatant. The absorbance of the developed yellow color was read at 412 nm after incubating for 10 min at 37 °C. The total glutathione was determined from the standard curve of reduced glutathione(GSH).The result was expressed as GSH level (%)

3.15 Statistical analysis

Data, presented as the mean ± SE, were representative of at least three independent experiments. Statistical analysis was performed using SPSS version 18. Differences among means were analyzed by one-way analysis followed by a Dunnett's post hoc test to compare values between the control and treatment groups. Graphs were drawn using GraphPad Prism software version 5.

4. Results and Discussion

4.1 Quantification of total bioactive compounds

Several *in vitro* and *in vivo* studies revealed that the presence of phenolic compounds, flavonoids and polysaccharide compounds in mushroom extracts was related to important biological properties such as antioxidant, immunomodulatory, antimicrobial and anticancer activity (Ooi, & Liu, 2000). Preventive effects on chronic diseases, in particular antiallergic, antibacterial, antifungal, anti- inflammatory, antioxidative, antiviral, cytotoxic,

immunomodulating, antidepressive, antihyperlipidemic, antidiabetic, digestive, hepatoprotective, neuroprotective, nephroprotective, osteoprotective and hypotensive activities were confirmed by many studies and clinical trials (Venturella, Ferraro, Cirlincione, & Gargano, 2021). Flavonoids, phenolic acids and polysaccharides are among the most important phytochemical constituents of *S. commune*. Here, we evaluated their contents in several *S. commune* extracts. Results of the quantitative determination of total phenolics and flavonoids from different extracts and yields of crude extracts (mg extract/g) are presented in Table 1. Extracted yields of each solvent in descending order were methanol (6.99%), ethyl acetate (1.04%), chloroform (0.78%) and hexane (0.65%) demonstrating higher yields with increasing solvent polarity. The polysaccharide extract with hot water appeared as a white powder with a yield of 8.91%.

Table 1 Total phenolic and flavonoid contents (mg/g) in *S. commune* extracts.

Extract	Yield %)w/w(	Total phenolic content) mg GAE/g of extract(a	Total flavonoid content) mg QE/g of extract(b
Hexane	0.65	16.09±0.43	3.37±0.04
Chloroform	0.78	24.29±0.24	4.53±0.26
Ethyl acetate	1.04	25.93±0.80	3.98±0.10
Methanol	6.99	16.93±0.88	3.20±0.11
Polysaccharide	8.91	11.98±0.12	2.34±0.09

Data are mean±SE of three independent experiments

[a] mg GAE/g gallic acid equivalent per g of extract, [b] mg QE/g quercetin equivalent per g of extract

Total phenolic contents of *S. commune* extracts were determined. The highest values were observed in the ethyl acetate extract (25.93±0.80 mg GAE/g) followed by chloroform (24.29±0.24 mg GAE/g) methanol (16.93±0.88 mg GAE/g), and hexane (16.09±0.43mg GAE/g). Conversely, total flavonoid contents of *S. commune* extracts exhibited a slightly different descending order as chloroform (4.53±0.26 mg QE/g of extract), ethyl acetate (3.98±0.10 mg QE/g of extract), hexane (3.37±0.04 mg QE/g of extract) and methanol (3.20±0.11 mg QE/g of extract).

Chemical compositions of *S. commune* polysaccharide extracts were analyzed by HPLC. The phenol–sulfuric acid and Bradford methods were used to evaluate total carbohydrate content and total protein content, respectively. Results showed that the total carbohydrate content and total protein content of the polysaccharide extract were 1.57±0.07 µg/mL and 0.29 ± 0.01 µg/mL, respectively. Schizophyllan (0.38 ± 0.04 mg/g extract) was the major active compound in the polysaccharide extract of *S. commune* fruiting body but absent in the organic solvent extracts. A chromatogram of polysaccharides using the HPLC method is presented in Figure1. A and B. Our results concurred with Mirfat et al. (2010) who determined dried fruiting body phenolic contents in methanol, ethyl acetate and dichloromethane from *S. commune*.

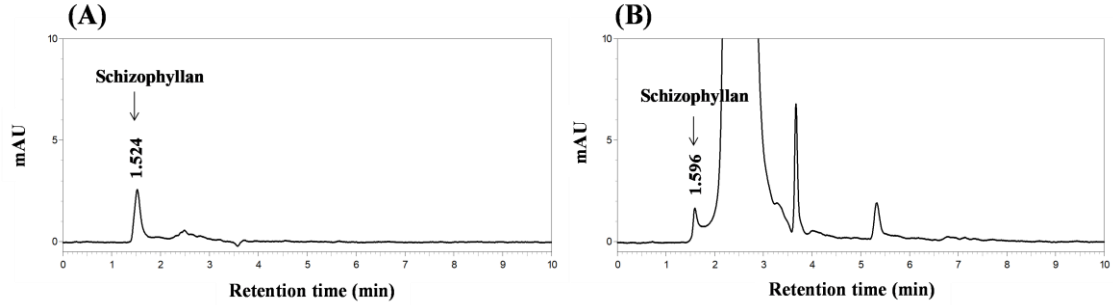

Figure 1 (A) HPLC chromatograms of Schizophyllan standards, (B) HPLC chromatograms of polysaccharide extract: the first peak is Schizophyllan.

4.2 DPPH radical scavenging activity of *S. commune* extracts

Free radical scavenging activities of *S. commune* extracts were tested using a methanol solution of the stable free radical DPPH. This decreases significantly when exposed to radical scavengers by providing a hydrogen atom or electron as a stable diamagnetic molecule. *S. commune* extract was evaluated using gallic acid as the standard control. Table 2 shows the DPPH radical scavenging activities of all extracts of *S. commune*. Results revealed that the methanolic extract exhibited the highest radical scavenging activity with an IC_{50} value of 0.32±0.00 mg/mL, similar to the ethyl acetate extract at 0.36±0.00 mg/mL and followed by the chloroform extract at 0.40±0.01 mg/mL, hexane at 0.57±0.02 mg/mL and polysaccharide extract at 1.00±0.05mg/mL. This data agreed with previous studies (Mirfat et al., 2010), confirming the methanol extract of *S. commune* as the most potent antioxidant agent.

Table 2 DPPH free radical scavenging activities of *S. commune* extracts.

Extract	**IC_{50} (mg/mL)**
Hexane	0.57±0.02
Chloroform	0.40±0.01
Ethyl acetate	0.36±0.00
Methanol	0.32±0.00
Polysaccharide	1.00±0.05
Gallic acid	0.002±0.01

Data are mean ± SE of three independent experiments

4.3 Effects of *S. commune* extracts against H_2O_2-induced cytotoxicity

Herbal medicines are globally claimed to provide relief against many liver diseases but scientific evidence is lacking. Our results suggested valid hepatoprotective activity of *S. commune* extract in HepG2 cells. Cytotoxic effects of the extracts were assessed by the MTT assay. Results showed that the hexane and methanol extracts did not have any effect ($p > 0.01$ and 0.001) on HepG2 cell viability (Figure 2A and 2D) at concentrations lower than 200 µg/mL, while the chloroform and ethyl acetate extracts (Figure 2B and 2C) had no effect ($p > 0.001$) on HepG2 cell viability at concentrations lower than 400 µg/mL.

As shown in Figures 2E and 2F, the polysaccharide extract at 100-1000 µg/mL and schizophyllan, as the main active compound in the polysaccharide extract, had no effect on cell viability. The non-cytotoxic extract of more than 80% was selected to study the hepatoprotective effect against H_2O_2-induced cytotoxicity in HepG2 cells.

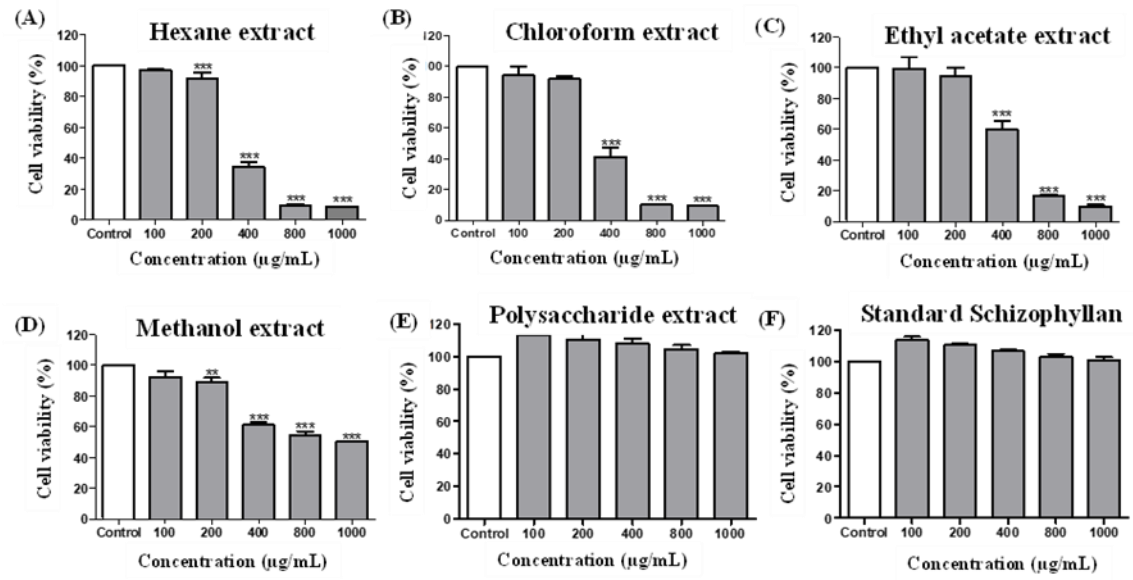

Figure 2 Cytotoxicity assessment by MTT assay in HepG2 cells (1×10^4cells/well) following exposure of various concentrations of hexane extract (A), chloroform extract (B), ethyl acetate extract (C), methanol extract (D), polysaccharide extract (E) and standard Schizophyllan (F) for 24 h. Values are mean ± SE of three independent experiments. **p<0.01, ***p<0.001 versus the control.

As shown in Figure 3, H_2O_2 ranging from 100 to 1000 µM induced cell death in a concentration- dependent manner and also demonstrated that H_2O_2 at 100 µM could be used to induce oxidative stress-mediated cytotoxicity in a cell system. To illustrate the cytoprotective effects of non- cytotoxic extracts (hexane, chloroform, ethyl acetate, methanol, polysaccharide and schizophyllan) on the viability of HepG2 cells against cytotoxicity induced by the H_2O_2, the HepG2 cells were incubated with various concentrations of test extracts for 24 h and then with a cytotoxic concentration (100 µM) of H_2O_2. The H_2O_2- induced cell death and hepatoprotective effect were determined by MTT and morphological changes. The protective potential of each extract, as observed in HepG2 cells, is presented in Figure 5. A reduction in cell viability was observed in HepG2 cells following exposure to H_2O_2 (100 µM) for 24 h. Cell viability was observed in HepG2 cells pre-exposed to hexane, chloroform, ethyl acetate and methanol extracts at 12. 5, 25, 50, 100 and 200 µg/ mL. Results showed that the hexane, ethyl acetate and methanol extracts did not have protective potential for viability of HepG2 cells exposed to 100 µM H_2O_2 (Figure 4A, 4B and 4C), while methanol extracts at concentrations of 100 and 200 µg/mL showed cell viability of 56.25±0.41 and 59.55±0.67%, respectively ($p > 0.01$ and 0.001) (Figure 4D). Among all the organic solvents, only these two concentrations showed slightly effective protection on HepG2 cells treated with 100 µM H_2O_2. Interestingly, the polysaccharide extract showed highly significant ($p < 0.001$) protection against H_2O_2- induced cytotoxicity, expressed as percentage of cell viability at concentrations of 200, 400, 800 and 1000 µg/ mL at 63. 29±0. 97, 77. 75±3. 56, 86. 65±4. 42 and 98. 86±2. 97% , respectively (Figure 4E) . A similar effect of standard schizophyllan at concentrations of 400, 800 and 1000 µg/mL exhibited viability of HepG2 cells at 69.29±1.80, 85.48±2.03 and 98.23±3.22%, respectively ($p < 0.001$) (Figure 4F). However, the polysaccharide extract was effective in protecting the viability of HepG2 cells damaged by H_2O_2 at a dose of 200 µg/ mL, lower than standard schizophyllan, possibly as a result of synergistic protective effects. Alterations in the morphology of HepG2 on exposure to polysaccharide extract and H_2O_2 are shown in Figure 5(A–E). Cells exposed to 100 µM of H_2O_2 showed reduced normal morphology of HepG2 cell adhesion capacity compared with the control. Most of the cells exposed to H_2O_2 lost their typical morphology, showed higher amounts of cell debris and appeared smaller in size (Figure 5B). HepG2 cells exposed to

increasing concentrations of polysaccharide extract for 24 h before H_2O_2 exposure were restored to their original morphology in a concentration-dependent manner (Figure 5C– E). Results suggested that exposure of HepG2 cells to the polysaccharide extract significantly increased viability against H_2O_2- induced cell death but extracts from the organic solvents did not show cytoprotective properties. Our results support previous studies that polysaccharides from mushrooms exhibit significant hepatoprotective effects against H_2O_2. Several studies also demonstrated that various compounds isolated from mushrooms, such as schizophyllan, showed prominent cytoprotective potential against cell damage (Kukan et al., 2004; Soares et al., 2013). Our study affirmed for the first time the hepatoprotective activity of the polysaccharide extract of *S. commune*, with the potential to protect against cell damage induced by H_2O_2. Therefore, we selected the polysaccharide extract to examine morphological changes, lipid peroxidation and glutathione levels in the following experiment.

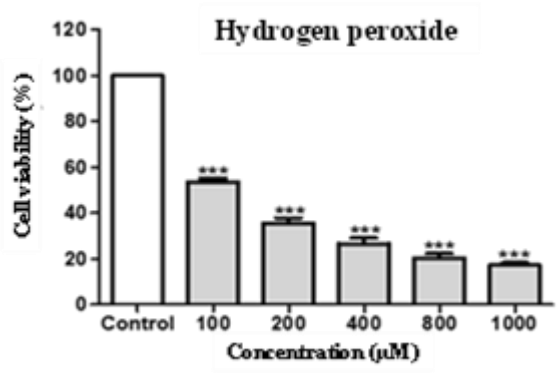

Figure 3 Cytotoxicity assessment by MTT assay in HepG2 cells (1×10^4cells/well) following exposure of various concentrations of hydrogen peroxide (H_2O_2) for 24 h Values are mean ± SE of three independent experiments. ***p<0.001 versus the control.

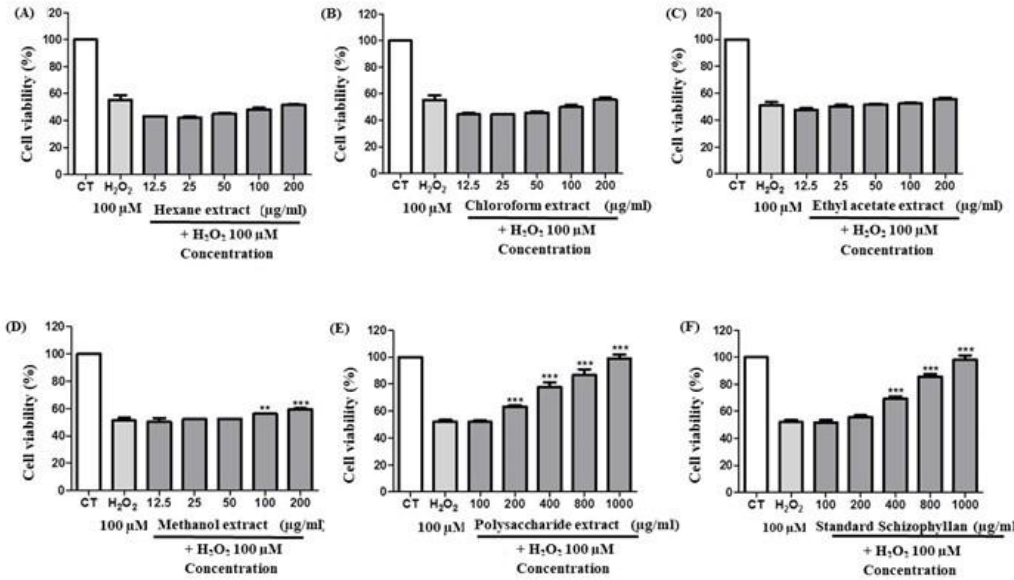

Figure 4 Assessment of protective potential of compounds on cell viability of HepG2 cells by MTT assay. Cells (1×10^4cells/well) were exposed to various concentrations of non-cytotoxic extracts for 24 h, and then exposed to H_2O_2 at 100 µM for 24 h. Values are mean ± SE of three independent experiments.
**p<0.01, ***p<0.001 versus H_2O_2 exposure.

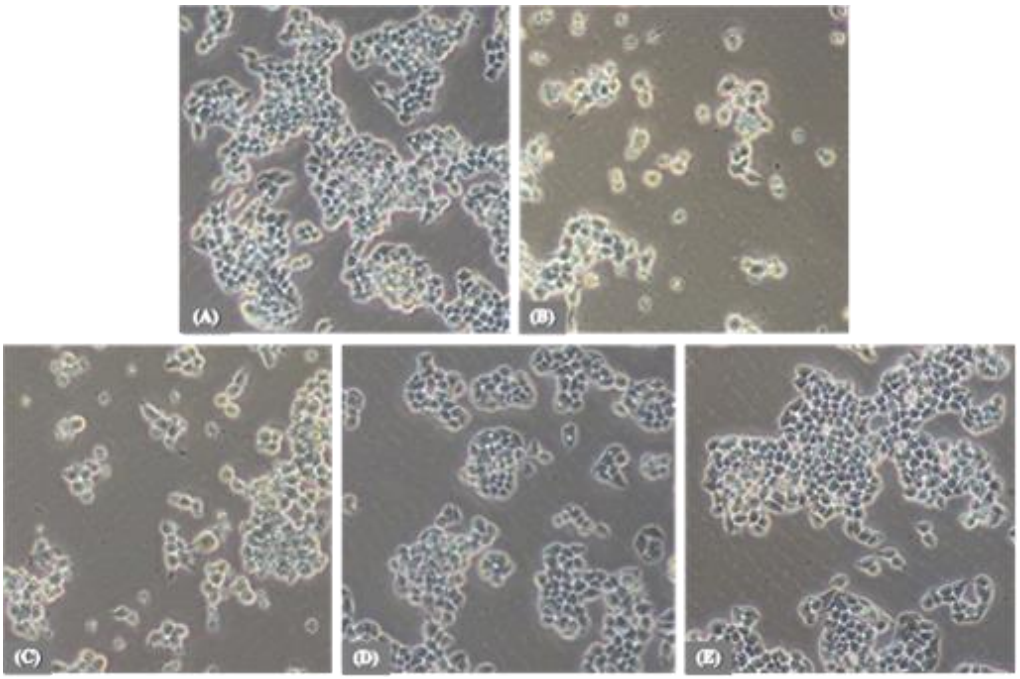

Figure 5 Morphological changes in HepG2 cells observed by contrast inverted microscopy at 20x magnification. Control group (A), H_2O_2 100 µM (B), polysaccharide 400 µg/ mL + H_2O_2 100 µM (C), polysaccharide 800 µg/mL + H_2O_2 100 µM (D) and polysaccharide 1000 µg/mL + H_2O_2 100 µM (E).

4.4 Morphological changes

Cells with homogeneous chromatin distribution were considered viable, whereas the presence of chromatin condensation and/or nuclear fragmentation was indicative of hallmarks of apoptosis. The morphological changes observed in HepG2 cells pre-exposed to polysaccharide extract and then H_2O_2 for 24 h are summarized in Figure 6. As shown in Figure 6B, H_2O_2 at 100 µM concentration induced characteristic morphological changes, such as cell shrinkage, appearance of a rounded fruiting body and loss of adhesion capacity compared to the control (Figure 6A). HepG2 cells exposed to 400, 800 and 1000 µg/mL concentrations of polysaccharide extract were restored to their original morphology similar to the control in a concentration-dependent manner (Figure 6C, 6D and 6E, respectively). Preservation of the normal morphology of HepG2 cells observed by fluorescence microscopy indicated the preventive role of the compounds against H_2O_2-induced morphological damage.

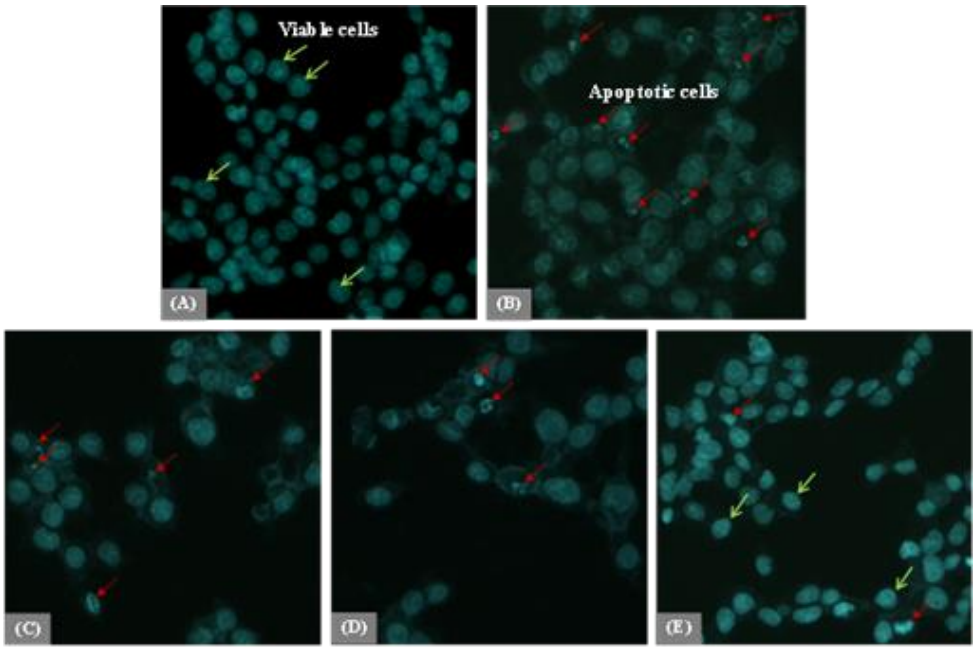

Figure 6 Detection of nuclear morphological changes in HepG2 by Hoechst 33342 staining, observed by fluorescence microscopy at 20x magnification. Control group (A), H_2O_2 100 µM (B), polysaccharide 400 µg mL + H_2O_2 100 µM (C), polysaccharide 800 µg/mL + H_2O_2 100 µM (D) and polysaccharide 1000 µg/mL + H_2O_2 100 µM (E).

4.5 Protective effect of polysaccharide extract on lipid peroxidation

To evaluate the protective effect of the polysaccharide extract on oxidative cell damage caused by H_2O_2, lipid peroxidation was measured as the formation of MDA in the homogenates of HepG2 cells (Figure 7). The increase in lipid peroxidation in HepG2 cells by H_2O_2 may be due to enhancement in hepatic MDA from the peroxidation of polyunsaturated fatty acids. In this assay, percentage inhibition of lipid peroxidation of the polysaccharide extract significantly reduced lipid peroxidation induced by H_2O_2 as 375.38±35.16% at 400 µg/mL, 231.54±0.94% at 800 µg/mL and 157.69±51.14% at 1000 µg/mL, respectively compared with cells treated with H_2O_2. This result indicated that the polysaccharide extract displayed a significant protective capability against H_2O_2-induced cell damage.

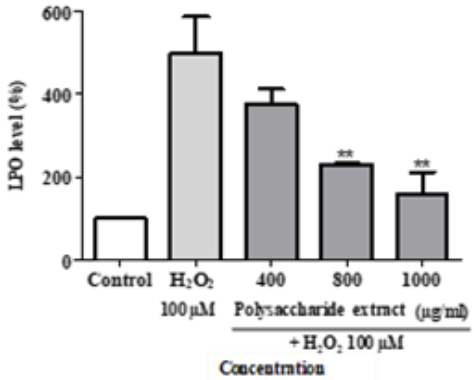

Figure 7 Effects of polysaccharide extract on lipid peroxidation (LPO) level of HepG2 cells following H_2O_2 - induced oxidative damage. Cells (1×10^5 cells in 6-well plates) were treated with polysaccharide extract for 24 h before H_2O_2-induced oxidation. Values represent mean ± SE. **$p<0.01$ versus H_2O_2 exposure.

4.6 Protective effect of polysaccharide extract on glutathione level

To evaluate the effect of the polysaccharide extract on antioxidant defense systems, the glutathione level was measured in H_2O_2 exposed HepG2 cells. Glutathione is a main non-enzymatic antioxidant and plays an important role in protective effects against oxidative stress. (Scharf et al., 2003). Our results showed that the glutathione level in H_2O_2 exposed HepG2 cells significantly decreased by 67.21% ($p < 0.001$) compared to the control (Figure 8). However, exposure of cells to polysaccharide extract significantly increased the glutathione level in H_2O_2 exposed HepG2 cells in a dose-dependent manner. Increases in GSH level were 77.76±4.36% at 400 µg/ mL, 85. 96±6. 44% at 800 µg/ mL and 95.39±2.15% at 1000 µg/mL, respectively (Figure 8). Our results showed that pre-exposure of cells to the polysaccharide extract effectively restored the glutathione level. It is well known that exogenous exposure to H_2O_2 can increase intracellular ROS generation and induce cellular oxidative damage in hepatic cells, while hepatocyte antioxidant defense mechanisms can eliminate this. Glutathione is a main non-enzymatic antioxidant, which plays an important role in the cellular defense system against oxidative stress (Scharf et al., 2003). Exposure of HepG2 cells to the polysaccharide extract quenched the intracellular destructive peroxide and increased the concentration of reduced glutathione

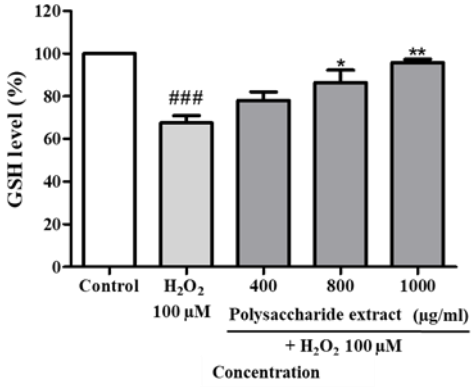

Figure 8 Effects of polysaccharide extract on glutathione(GSH) level of HepG2 cells following H_2O_2- induced oxidative damage. Cells (1×10^5 cells in 6-well plates) were pre-treated with polysaccharide extract for 24 h before H_2O_2-induced oxidation. Values represent mean ± SE
*p<0.05, **p<0.01 versus H_2O_2 exposure; ### p<0.001 H_2O_2 versus the control.

5. Conclusions

Our results demonstrated that the scavenging activity and cytoprotective potential of the polysaccharide extract attenuated H_2O_2-induced cytotoxicity in HepG2 cells. The protective effect was mediated by both inhibiting lipid peroxidation and restoring glutathione levels. Findings suggested that constituents in the fruiting body of *S. commune* can protect HepG2 cells against H_2O_2-induced oxidative damage through antioxidant activity. Further work is necessary to find specific active compounds and elucidate the extracting mechanism involved in the hepatoprotective activity. Moreover, the animal model should be good feasibility to prove the ability of the polysaccharide extract of *S. commune* for hepatoprotective.

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