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Neuroprotective potential of polysaccharides from the mycelial extract of *Schizophyllum commune*

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Abstract The fruiting body of split gill macrofungus was collected and isolated. Its ribosomal RNA (rRNA) gene was sequenced to identify the species. Based on ITS1 and ITS4 regions, the nucleotide sequence was matched with *S. commune* showing 99.89% identity. Moreover, to prepare the crude polysaccharide (CP) from mycelium, the pressurized hot water extraction method and ethanol precipitation were utilized. The neuroprotective activity was evaluated by applying 100 µM H_2O_2-induced oxidative stress on the SH-SY5Y cell line. Cell morphology was observed by fluorescent microscopy using Hoechst 33342 staining. A 250 µg/ml crude polysaccharide concentration reduced intracellular nuclear deformation compared to H_2O_2 alone with apoptosis protection of cells stained with Annexin-V and propidium iodide (PI). The cell cycle was estimated by PI staining and flow cytometry. The results showed that 125-250 µg/ml of crude polysaccharide reduced neuronal apoptosis. Furthermore, the crude polysaccharide mechanism of action reduced apoptosis in the sub-G1 phase compared to the H_2O_2 group. This is the first report on the neuroprotective effect of crude polysaccharides extracted from *S. commune* performed *in vitro on* the SH-SY5Y cell line.

Keywords: *Schizophyllum commune* mycelium, Polysaccharide, Neuroprotective

Introduction

Neurodegenerative disease occurs when nerve cells in the brain and neurons deteriorate and cannot recover, causing them to lose function. As a result, the brain gradually becomes smaller by the progressive loss of the nervous system causing many diseases such as Alzheimer's and Parkinson's (Kovacs *et al.*, 2014; Kovacs, 2016; Przedborski *et al*., 2003). The primary agents of neuronal degeneration and death in this group of diseases have not yet been clearly identified, but 90-95% of patients are elderly. Therefore, age is a

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critical factor in disease occurrence, but the disorder can also occur in younger populations (Lutz *et al*., 2014; Hung *et al*., 2010; Hou *et al*., 2019; Lin and Beal, 2006).

Both external environmental and internal genetic interactions are responsible for most neurodegenerative diseases, and studies are ongoing to determine optimal patient treatment. Recently, medicinal herbs or natural products such as mushrooms and macrofungi have been applied to prevent and restore degenerative disorders of the brain and nerve cells. *Schizophyllum commune* is a fast-growing macrofungus found in every region of Thailand. It is highly nutritious and contains protein, iron, phosphorus, calcium, vitamins and minerals (Adejoye *et al*., 2007; Hobbs, 2005; Han *et al*., 2005), with the main bioactive compound a non-ionic polysaccharide called schizophyllan (Ooi and Liu, 1999; Yoshiba *et al*., 2015). The pharmacological activity of schizophyllan has been reported in many fields such as antifungal, antibacterial, cancer therapy, tumor treatment, and as a tonic or elixir (Zhou *et al*., 2015; Du *et al*., 2017; Liu *et al*., 2018).

Polysaccharide compounds of mushrooms and macrofungi are found in the fruiting body, the mycelia and the liquid medium (Friedman, 2016). The several steps in cultivating mushrooms take at least 4-5 weeks. Therefore, the study investigated whether the polysaccharide extract from mycelia of *S. commune* for neuroprotective effects against H_2O_2-induced neuronal cell loss in neuroblastoma cells (SH-SY5Y) and examined the polysaccharide extract induced an anti-apoptotic effect.

Materials and methods

Morphological and molecular identification

The mycelia of *S. commune* was isolated from the fruiting body on dead rubber trees under natural conditions in Than-To District, Yala Province**.** The pure culture was isolated and transferred to a spawn bag to produce the fruiting body following the method described by Garcia *et al.* **(**2018**).** The harvested fruiting body was morphologically and molecularly identified which was performed by amplification and rRNA sequencing using ITS1 and ITS4 primers**.** Nucleotide sequences were compared with those deposited in the GenBank database using the BLAST **(**Basic Local Alignment Search Tool**)** program **(**https**://**www**.**ncbi**.**nlm**.**nih**.**gov/blast**.**cgi**).**

Cell culture and chemicals

The human neuroblastoma cell line (SH-SY5Y) was obtained from the American Type Culture Collection (ATCC, USA). Cells were cultured in Dulbecco's modified Eagle's medium and Ham's F12 (1:1 mixture) (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 units/ml of penicillin, and 100 mg/ml of streptomycin (Gibco, USA) in a 5% CO_2 incubator at 37 ℃.

Extraction of polysaccharide from mycelia of S. commune

For mycelial cultivation of *S. commune*, a sterile cellophane membrane (9 cm diameter) was placed on the surface of autoclaved potato dextrose agar (PDA, Merck, Germany). Then, 0.5 cm^3 of pure culture of *S. commune* was placed in the center of the cellophane membrane. After incubation at room temperature for 10 days, the mycelia were harvested by lifting the mycelial mass from the culture medium. To extract the polysaccharides, the mycelial mass was mixed with distilled water at a ratio of 1:10 (w/v) and then autoclaved under a pressure of 15 pounds per square inch at 121°C for 30 min. After cooling, the supernatant was collected and precipitated by mixing with 95% ethanol at a ratio of 1:1 (v/v) and incubated overnight at 4 ℃. To precipitate the polysaccharides, the overnight solution was centrifuged at 7,000 rpm for 10 mins, and the supernatant was discarded. The precipitated crude polysaccharide was washed with ethanol and lyophilized (Nik Ubaidillah *et al*., 2015).

Analysis of crude polysaccharide composition

Schizophyllan analysis

Crude polysaccharide analysis to detect schizophyllan was performed on an HPLC system. (ThermoSpectra Series Complete HPLC AS3000, P4000, UV1000, SN4000, USA) The separation phase was an Agilent Eclipse Plus C18 column (100 mm × 4.6 mm, 3.5 μm) with a 0.8 ml/min flow rate at 25 ℃. The mobile phase consisted of eluent A (water) and eluent B (methanol). Gradient elution run time was 0–5 min, 90% A; 5–10 min, 85% A. Absorbance of the analytes was detected at 205 nm (Su *et al.*, 2016).

Carbohydrate and protein contents

The crude polysaccharide was analyzed to quantify carbohydrate content using the phenol sulfuric acid colorimetric method outlined in Dubois *et al*. (1956). Soluble carbohydrate percentage was calculated based on absorption at

490 nm using a spectrophotometer and compared to glucose as the standard. Protein content was measured by the Bradford assay with a percentage calculated based on absorption at 595 nm using a spectrophotometer and compared to BSA as the standard.

Cytotoxicity test of crude polysaccharide

Non-toxic doses of crude polysaccharides were determined by the MTT colorimetric assay following the method described by Siripong *et al*. (2012). SH-SY5Y cells ($1x10^4$ cells per well) were seeded into each well of 96-well culture plates and incubated overnight to allow for attachment. Cells were then treated with 25-500 µg/ml crude polysaccharide for 24 h, followed by adding MTT solution and incubating at 37 ℃ for four h. The amount of MTT formazan was determined by measuring the absorbance at 550 nm using a microplate reader. All MTT assays were performed in triplicate.

The optimal concentration of hydrogen peroxide (H_2O_2)

Cytotoxicity of H_2O_2 on SH-SY5Y cells was determined using the MTT method. All concentrations of H_2O_2 (3-1,000 µM) were freshly prepared with the medium. The half-maximal inhibitory concentration (IC_{50}) of H_2O_2 was calculated to determine H_2O_2-induced oxidative stress dosage.

Cytotoxicity test of crude polysaccharides against H_2O_2-induced cells death

The overnight seeded SH-SY5Y cells (3×10^4 cells per well) in 96-well culture plates were pretreated with various doses of non-toxic crude polysaccharides for 24 h and then challenged with H_2O_2 at the optimal concentration (IC_{50}) to induce oxidative stress of SH-SY5Y cells for another 24 h. MTT solution was then added to all the wells and incubated in the dark at 37 ℃ for four h. The amount of MTT formazan was determined by measuring the absorbance at 550 nm using a microplate reader. All MTT assays were performed in triplicate.

Morphological changes using fluorescence microscopy

Hoechst 33342 staining was used to visualize nuclear changes and apoptotic body formation characteristics. SH-SY5Y cells (1×10^5 cells/well) were seeded into a petri dish 33x15 mm with a cover slip. The cells were pretreated with crude polysaccharides for 24 h and then damaged with 100 µM

H_2O_2 for another 24 h. Finally, cells were fixed and incubated with Hoechst 33342 (Sigma, USA). Morphological changes in the cells were observed under an inverted fluorescence microscope.

Cell cycle analysis

SH-SY5Y cells were seeded in a petri dish 90x15 mm at a density of 1×10^6 cells/dish. The cells were pretreated with crude polysaccharides for 24 h and then challenged with 100 µM H_2O_2 for another 24 h. Cells were harvested using 0.1% trypsin-EDTA, fixed in 70% ethanol, and kept at -20 ℃ overnight. After fixation, the pellets were washed with PBS to remove ethanol and mixed with 25 µl of 1 mg/ml RNase A, 50 µl of 100 µg/ml propidium iodide (PI) and 425 µl of PBS to make up the volume of 500 µl. After incubation for 30 min in the dark, the DNA contents of the cells were analyzed using a flow cytometer (BD, USA).

Analysis of apoptosis by Annexin V-FITC/PI assay

SH-SY5Y cells were seeded in a petri dish 90x15 mm at a density of 1×10^6 cells/dish. The cells were pretreated with crude polysaccharides for 24 h and then challenged with 100 µM H_2O_2 for another 24 h. Cells were harvested using 0.1% trypsin-EDTA. SH-SY5Y cells were double-stained using Annexin V-FITC/PI apoptosis detection kit (BD, USA) according to the manufacturer's instructions. Samples stained with Annexin V and PI was quantitatively analyzed using a flow cytometer.

Statistical analysis

All the experiments in this study were performed in triplicate except as noted otherwise**.** Statistical analyses included t-tests and one-way analysis of variance **(**ANOVA**)** using GraphPad Prism software version 5 using one-way ANOVA using Dunnett's *post hoc* test to compare the values between control and treatment groups**.** The test results were considered statistically significant at $p< 0.05$, $p<0.01$ *and* $p<0.001$**.**

Results

Morphological and molecular identification

Pure isolated mycelium collected from Than-To District Yala Province was cultivated at Entrepreneur Mushroom Farms in Chanthaburi Province. The

obtained fruiting body was 1-5 centimeters in size and had a fan-shaped or flabellate form. The cap was 1-4 centimeters wide. The upper surface showed tiny hairs with split gills on the lower side. This is because the gills, which produce basidiospores, are on their surface. These superficial external characteristics confirmed the identification of the mushroom species corresponding to *S. commune*, as shown in Figure 1.

Amplifying the rRNA gene with universal primers ITS1 and ITS4 obtained a PCR product size of about 600 bp. The nucleotide sequences had the highest level of similarity at 99.68% homology with *S. commune* in GenBank (accession number MH857808.1).

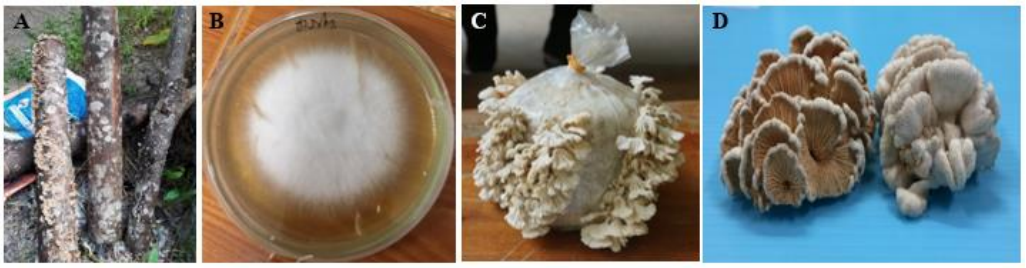

Figure 1. (A) The fruiting bodies of *S. commune* growing on rubber wood from Than-To District, Yala Province; (B) Pure mycelial culture isolated from the fruiting body; (C) The fruiting body of *S. commune* was produced on the cultivation bag using seeded isolated pure mycelium; (D) Morphology of cultivated mushrooms

Extraction of crude polysaccharides from mycelia of S. commune

Crude polysaccharides were extracted from the mycelia of *S. commune* with water using a steam autoclave, then precipitated crude polysaccharides with ethanol. The resulting crude polysaccharides were freeze-dried. The mycelia of *S. commune* weighed 21.43 g and yielded 0.807 g of crude polysaccharide extract, yielding 3.77%. The crude polysaccharide extract at 1 mg/ml was analyzed for carbohydrate and protein content. The result showed that total carbohydrate and protein content were 0.54±0.006 mg/ml and 0.06±0.003 mg/ml, respectively. HPLC analysis showed that schizophyllan was an active compound in the crude polysaccharide extract from *S. commune*. A chromatogram of crude polysaccharides using the HPLC method was presented in Figure 2, which showed that 1 g of crude polysaccharide contained 21.0g of schizophyllan.

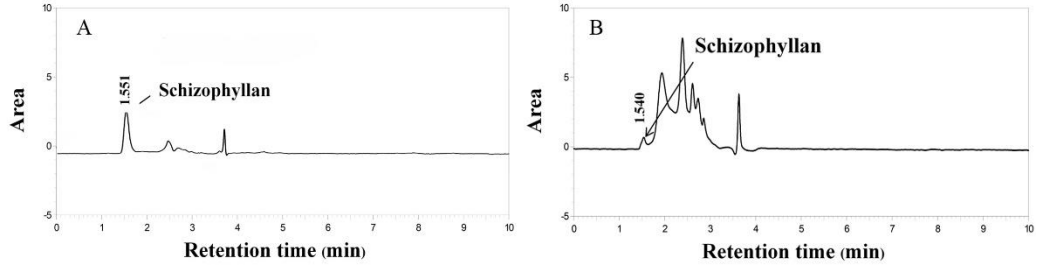

Figure 2. (A) HPLC chromatograms of Schizophyllan standards; (B) HPLC chromatograms of crude polysaccharide extract, the first peak is Schizophyllan

Crude polysaccharide cytotoxicity test

The results showed that the crude polysaccharides had no significant effect on SH-SY5Y cell viability. In detail, treatments with 25, 50, 125, 250 and 500 μg/ml of crude polysaccharide showed survival of SH-SY5Y cells were 99.94%, 95.35%, 102.82%, 92.38% and 88.06%, respectively. However, high concentrations of crude polysaccharides (500 μg/ml) showed slight cytotoxicity to SH-SY5Y cells and reduced cell viability to less than 90%. (Figure 3A). Therefore, crude polysaccharide concentrations of 25-250 μg/ml were chosen for further study.

Optimal concentration of hydrogen peroxide (H_2O_2)

The optimal concentration of H_2O_2 was required for cell viability of 50% within 24 h. After H_2O_2 treated with 0, 3, 10, 30, 100, 300 and 1000 μM, the percentage of cells survival were presented 100%, 100.30%, 109.97%, 71.24%, 56.43%, 31.82% and 18.38%, respectively. However, the concentration of H_2O_2 at 30 -1000 μM showed significantly decreased in percentage of cell viability compared with untreated cells ($p<0.001$) (Figure 3B). The optimal concentration of H_2O_2 at 100 μM (56.43% cell survival) was chosen for subsequent experiments.

Effects of crude polysaccharides against H_2O_2-induced cytotoxicity

The SH-SY5Y cells were pretreated with various concentrations of crude polysaccharides against H_2O_2-induced cytotoxicity. The result showed that cell viability pretreated with 25, 50, 125 and 250 μg/ml of crude polysaccharides were presented at 52.48%, 53.63%, 66.63% and 72.83%, respectively (Figure 3C). However, it appeared at only 250 μg/ml of crude polysaccharide extract

pretreatment, conferring significant protection against H_2O_2-induced cell damage ($p<0.05$).

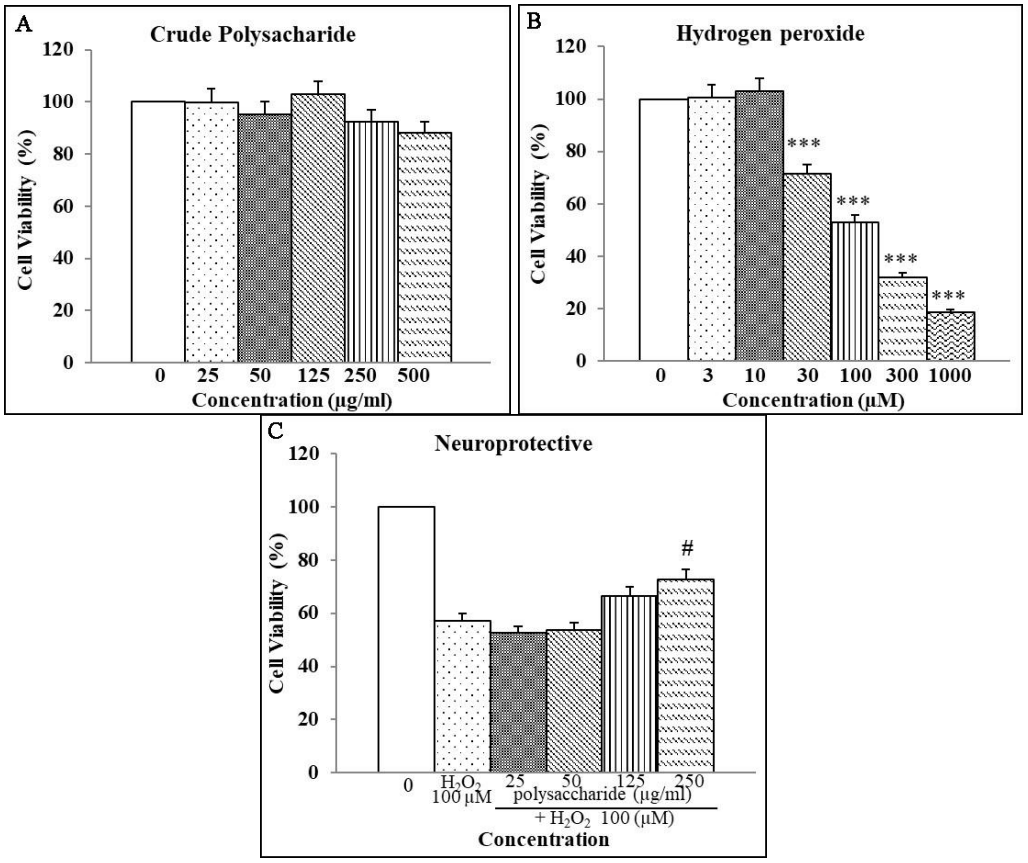

Figure 3. Crude polysaccharides exerted neuroprotective effects against H_2O_2-induced damage in SH-SY5Y cells: (A) Percentage cytotoxicity of SH-SY5Y cells after 24 h incubation with various concentrations of crude polysaccharide. Data are shown as means ±SE (n=3); (B) Percentage cytotoxicity of SH-SY5Y cells after 24 h incubation with various concentrations of H_2O_2. Data are shown as means ± SE (n=3). $^{***}p<0.001$, compared to untreated cells; (C) The effect of crude polysaccharides against H_2O_2-induced cytotoxicity in SH-SY5Y cells. Cells were pretreated with crude polysaccharide for 24 h and then co-treated with 100 µM H_2O_2 for 24 h. Data are shown as means ± SE (n=3). $^{\#}p<0.05$, compared to treatment with H_2O_2

Morphological changes using fluorescence microscopy

Morphological changes were assayed cytologically using Hoechst 33342 staining and determined by fluorescence microscopy. The blue fluorescent Hoechst 33342 is a cell-permeable nucleic acid dye commonly used to explore fragmentation and chromatin condensation by staining the nucleus of cells.

Morphological observations in the nucleus of SH-SY5Y cells pretreated with 250 µg/ml crude polysaccharide and then co-treated with H_2O_2 showed significant differentiation compared to the untreated control. The untreated cells (Figure 4A) were an intact oval shape and the nucleus was a less bright blue that also denoted regular intact cells. Cells treated with 100 µM H_2O_2 (Figure 4B) exhibited typical features of apoptosis, such as chromatin condensation, apoptotic body formation, cell shrinkage and cell decrement. The nucleus obviously showed the condensed chromatin and apoptotic body that was identically fluorescent. Pretreatment with 250 µg/ml of crude polysaccharides (Figure 4C) prevented morphological nuclei changes damaged by H_2O_2 in cells compared to 100 µM H_2O_2 alone.

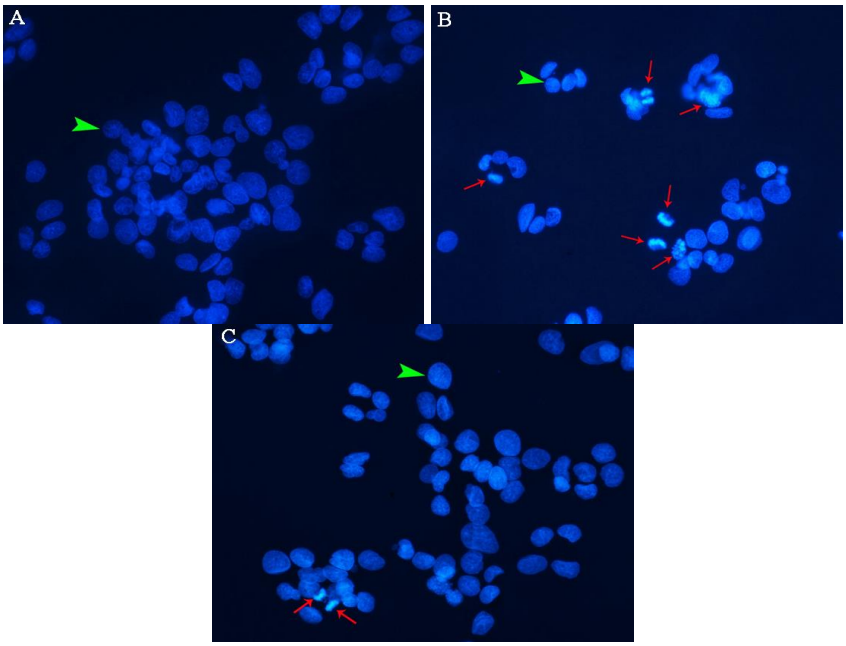

Figure 4. Nucleus alteration of SH-SY5Y cells by Hoechst 33342 technique: (A) Untreated cells; (B) Cells treated with 100 µM H_2O_2. (C) Cells were pretreated with 250 µg/ml of crude polysaccharides and co-treated with 100 µM H_2O_2

(►) Viable cell (⟶) Apoptosis cell

Effects on cell cycle progression

Characterization of apoptotic are morphologic changes, nuclear condensation and reduced DNA content to be confirmed with propidium iodide staining and analysis by flow cytometry. The sub-G1 phase examined the DNA damage in cell cycle progression. Results showed that DNA damaged content of the untreated cell and induced with H_2O_2 were 55.8 ±05.0% and 55.21 ±35.0% in the sub-G1 phase (Table 1, Figure 5). Conversely, cells pretreated with 25, 50, 125 and 250 µg/ml crude polysaccharide and co-treatment with H_2O_2 showed a reduction of DNA damage in the sub-G1 phase were 20.40 ±0.50%, 14.00 ±1.00%, 13.90 ±0.20% and 12.95 ±0.05%, respectively compared to treated with H_2O_2. However, cells pretreated with 50, 125 and 250 µg/ml of crude polysaccharide showed significantly decreased DNA damage in the sub-G1 phase ($p<0.01$ compared to H_2O_2). In the progression of the cell cycle, it can be seen that there was no significant difference in DNA content in G1, S and G2/M phases among pretreatments with crude polysaccharides against H_2O_2.

Table 1. DNA content (%) in various phases of the cell cycle after pretreatment with crude polysaccharides (CP) and co-treatment with 100 µM H_2O_2

Treatment	Percentage of DNA content in various phases of the cell cycle			
	Sub-G1	G1	S	G/2M
Untreated cells	55.8 ±05.0	70.52 ±90.0	10.21 ±60.0	65.17 ±55.1
H_2O_2	55.21 ±35.0	95.46 ±05.0	85.15 ±45.0	65.15 ±05.0
CP 25 µg/ml+ H_2O_2	40.20 ±50.0	40.47 ±40.0	05.22 ±25.1	15.10 ±15.2
CP 50 µg/ml+ H_2O_2	00.14 ±00.1$^{\#\#}$	65.47 ±75.1	10.24 ±90.3	25.14 ±15.1
CP 125 µg/ml+ H_2O_2	90.13 ±20.0$^{\#\#}$	90.52 ±70.0	70.18 ±40.2	50.14 ±50.1
CP 250 µg/ml + H_2O_2	95.12 ±05.0$^{\#\#}$	55.53 ±35.0	05.20 ±05.0	45.13 ±35.0

Data were means ± SE (n=2). $^{\#\#}p < 0.01$ compared to 100 µM H_2O_2 as the inductive oxidative stress treatment, Sub-G1: DNA damage content (%), G1: Preparation for DNA synthesis (%), S: DNA synthesis (%) and G2/M: DNA replication (%).

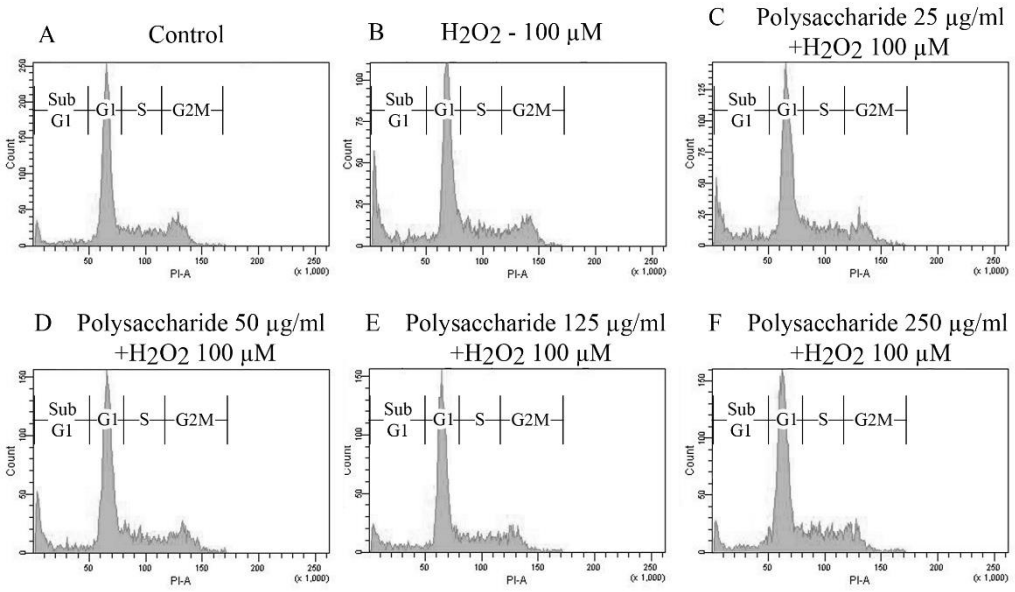

Figure 5. DNA histograms of cell cycles stained by propidium iodide and analyzed using flow cytometry: (A) Untreated cells; (B) Cells treated with H_2O_2 100 µM; (C)-(F) Cells pretreated with crude polysaccharides at 25, 50, 125 and 250 µg/ml concentrations

Effects on apoptosis in SH-SY5Y cells

The crude polysaccharides protective against H_2O_2-induced cell death were examined. The cells were co-stained with Annexin-V and PI for flow cytometry analysis. The results were shown in four quadrants dot plot (Figure 6) the lower left quadrant represents viable cells (Annexin-V^-/PI^-), the lower right quadrant represents early apoptosis (Annexin-V^+/PI^-), the upper right quadrant represents late apoptosis (Annexin-V^+/PI^+) and the upper left quadrant represents death cell (Annexin-V^-/PI^+). The H_2O_2-induced cells presented the percentage of late apoptotic at 54.20%. Crude polysaccharides at 25, 50, 125 and 250 µg/ml showed neuroprotective effects against H_2O_2-induced cells in late apoptosis were 47.35%, 45.20%, 38.75% and 31.30%, respectively. Therefore, crude polysaccharides showed anti-apoptotic properties against H_2O_2-induced SH-SY5Y cells.

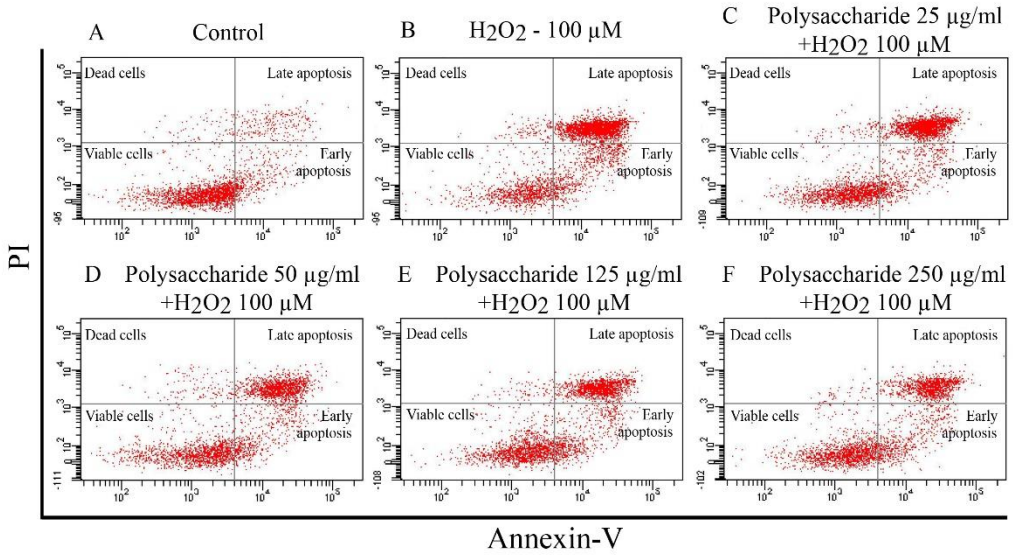

Figure 6. Effects of crude polysaccharides on H_2O_2-induced cell death in SH-SY5Y cells. Cells treated with H_2O_2, or H_2O_2 + crude polysaccharide (25-250 µg/ml) for 24 h were subjected to flow cytometry analysis after PI and Annexin-V staining. H_2O_2 treatment alone increased late apoptotic cells and necrotic cells, while pretreatment of crude polysaccharides protected against H_2O_2-induced cells

Discussion

Previous studies mainly concentrated on the production of schizophyllan, a constituent of exopolysaccharides directly extracted from the liquid-culture medium (Mansoldo *et al*., 2020; Mohammadi *et al*., 2018; Singh *et al*., 2017). In the current study, the solid culture-derived polysaccharides belong to endopolysaccharide, which was extracted from the mycelium, and exopolysaccharide, a jelly-like layer of extracellular secretion attached to the mycelium. Moreover, our extracted polysaccharide was confirmed to evaluate the carbohydrate and protein contents, including HPLC analysis showed schizophyllan as a component. Crude polysaccharide extract is white and fluffy similar to the schizophyllan standard.

Polysaccharide activity from *S. commune* mycelium was investigated as an antioxidant (Deng *et al*., 2021), immune-boosting and anti-inflammatory effects (Du *et al*., 2017). The result of polysaccharides from *S. commune* mycelium on injury or death of cultured neurons was also examined. Neuroblastoma, SH-SY5Y cells were tested using a cellular model of neuronal degeneration or cell death caused by H_2O_2, an oxidative stress agent.

Polysaccharides, when tested concurrently with H_2O_2, can potentially reduce H_2O_2-induced injury or death of SH-SY5Y cells through the apoptosis inhibition mechanism. Cell cycle mortality analyses showed that SH-SY5Y cells tested for polysaccharide co-incubated with H_2O_2 had a lower mortality rate than cells in the treatment of the H_2O_2-only. Results demonstrated the neuroprotective effect of the polysaccharide extract, concurring with previous reports that many mushrooms contained neuroprotective agents such as *Daldinia concentrica* (Lee *et al*., 2002), *Paxillus panuoides* (Lee *et al*., 2003), *Ganoderma lucidum* (Aguirre-Moreno *et al*., 2013), *Ganoderma leucocontextum* (Chen *et al*., 2018), *Cantharellus cibarius* (Lemieszek *et al*., 2018), *Lignosus rhinocerus* (Kittimongkolsuk *et al*., 2021), *Hericium Mushrooms* (Chen *et al*., 2022) and *Sanguinoderma rugosum* (Sam *et al*., 2022). Polysaccharide extract was also reported to have immunostimulating and inhibitory effects on cancer cells (Zhong *et al*., 2015). This is the first report on the neuroprotective effect of crude polysaccharides extracted from *S. commune* mycelium, possibly related to schizophyllan as the main active ingredient of crude polysaccharides or other synergistic compounds in *S. commune* mycelium.

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