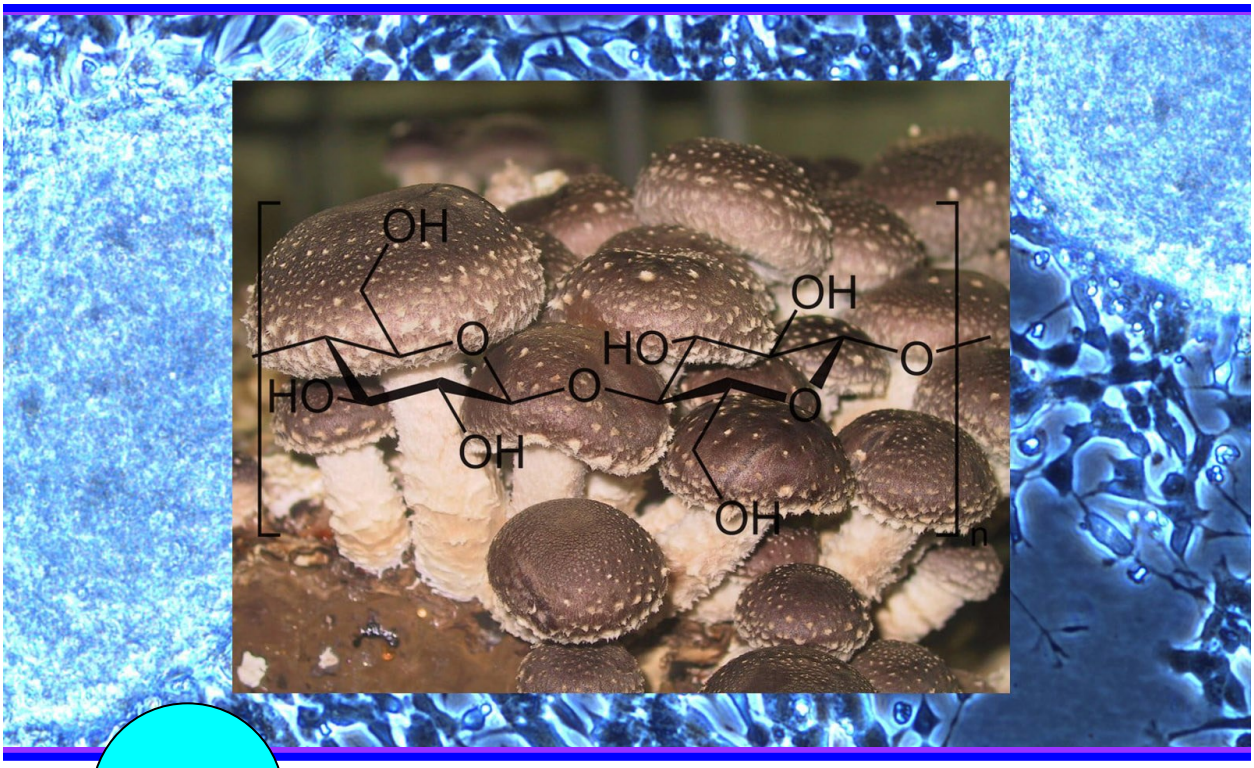

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Mechanism of anti-cancer effect of β-glucan on SH-SY5Y cell line

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Abstract

Anti-cancer property of fungi derived β-glucan (*Lentinula edodes*) on several cancer cell lines have been reported. In this work, the SH-SY5Y cell lines were treated with various concentrations of β-glucan (62.5, 125, 250 and 500 µg/mL) and the viability of the cells was tested using the XTT assay after 24 hours. Cleaved PARP, BCL-2, 8-hydroxy-desoxyguanosine (8-oxo-dG), cleaved caspase 3, Bax, total oxidant, and total antioxidant levels in the cells were measured by commercial kits. β-Glucan significantly decreased the cell viability in SH-SY5Y cells. ELISA tests demonstrated that β-glucan therapy dramatically increased 8-oxo-dG, cleaved caspase 3, Bax, cleaved PARP, total oxidant. However, β-glucan treatment did not change the BCL-2 protein level. Altogether, β-glucan caused significant cytotoxicity in SH-SY5Y cells by inducing oxidative stress, increasing DNA damage, and ultimately increasing apoptosis.

Introduction

Neuroblastoma, the most prevalent extra-brain solid tumor of infancy, is the primary reason of death in children. More than 40 percent of patients are noted to be at excessive risk, including children suffering metastatic disease (Moreno et al., 2017). Despite the existence of multiple therapeutic approaches, including immunotherapy, surgery, chemotherapy, and radiotherapy, the resistance and recurrence of neuroblastoma often occur, necessitating an alternative effective therapy of the cancer (Suebsoonthron et al., 2017). About a third of patients are resistant to first line treatment and have very bad consequences (Ladenstein et al., 2008). In addition, because of the intensity of the multimodal therapy, survivors are exposed to considerable stress from long-term effects (Laverdière et al., 2005). Late treatment toxicities, such as the emergence of secondary cancers, put neuroblastoma patients at risk of serious or life-threatening diseases, emphasizing the need for extensive and effective preclinical drug screening (Nolan et al., 2020). More research into the discovery of new therapeutic agents that reduce mortality due to neuroblastoma while also having less side effects is needed in this context. SH-SY5Y cells are human neuroblastoma cells obtained from a 4-year-old female's bone marrow biopsy with neuroblastoma. SH-SY5Y cells are frequently used as a model for neuronal activity and differentiation *in vitro* (Suebsoonthron et al., 2017). Food items and natural ingredients have long been used as health treatments thanks to the widespread belief that they have mild side effects. As a result, insights into the mechanisms by which foods and natural ingredients can have potential beneficial effects is necessary for human societies.

β-Glucans are a type of polysaccharide that is distinguished by its position in the cell wall. It is abundant in barley and oats (Mantovani et al., 2008). Many illnesses, including atherosclerosis, infectious disease, hyperlipidemia, diabetes, cancer, and obesity, are now known to benefit from β-glucans in the prevention and treatment. Different properties of β-glucans, such as immune activation and bile acid trapping, mediate the effects (Chen, 2013; Chen and Huang, 2009). β-Glucan administration has been confirmed to cause substantial

stimulation of reticuloendo-thelial system phagocytic activity, with subsequent enhancement of host defense mechanisms, since the 1970s (Sima et al., 2019). Granulocytes, monocytes, dendritic cells, and macrophages were reported to have higher proliferative activity and phagocytic function, along with stimulation of cytokine production (inclu-ding such interleukin-1, -2, and -6) (Quinn, 1990). Increased generation of these cytokines activates immune effector cells, leading to an improvement in anti-infectious and, in particular, anti-cancer immunity (Vannucci et al., 2013). β-Glucan therapy has been shown to be effective against a variety of cancers in both human and animal models (Sima et al., 2019). Two key β-glucan receptors, lactosylceramide and receptor 3 (CR3), mediate its anti-cancer properties. Immune boosting, cancer-prevention and direct tumor suppession behaviors are all related to anti-cancer effects of β-glucan (Choromanska et al., 2015). Both soluble and particulate β-glucans isolated from *S. cerevisae* inhibited the growth of B16 melanoma and breast carcinoma cells, and also increased the longevity of mice implanted with subcutaneous tumors (di Luzio et al., 1979).

Lentinula edodes-derived β-glucan was shown to have good antitumor activity against sarcoma 180 *in vivo* studies (Zhang et al., 2005). In the treatment of human neuroblastoma xenografts, it was shown that,3-1,4-beta-glucan synergized with anti-GD2 monoclonal antibody (MoAb) 3F8 (mouse IgG3) (Cheung et al., 2002). Relapse -free survival was 80-10% at 24 months in a phase 1 study of a bivalent gangliosides vaccine in combination with β -glucan. The vaccine and β -glucan were well tolerated (Kushner et al., 2014). Several works have demonstrated the anti-cancer effects of β-glucan on a variety of cancer cell lines yet the mechanisms involved in anti-cancer effect of β-glucan against neuroblastoma remains unknown. The goal of this research was to investigate the cytotoxic activity of β-glucans on the SH -SY5Y cell line, as well as the mechanisms involved in this activity.

Materials and Methods

Cell culture and cell lines

SH-SY5Y cell line (neuroblastoma) and L929 cell line (mouse fibroblast) were purchased from the American Type Culture Collection (ATCC, USA). SH-SY5Y and L929 cell lines were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) and placed in humidified atmosphere of 5% CO_2 incubator at 37 ^{o}C. 10% fetal bovine serum (FBS) (Sigma-Aldrich) and 1% antibiotic mixtures of penicillin and streptomycin were added to this medium. Before the procedure, the *L. edodes*–derived β–glucan (lentinan) (Sigma-Aldrich) was dissolved in DMSO (dimethyl sulfoxide) and diluted in the culture medium to a final DMSO concen-tration of less than 0.1 percent.

Examination of 8-oxo-dG, cleaved caspase 3, BCL-2, cleaved PARP and Bax levels

The human ELISA kits of 8-hydroxy-desoxyguanosine (8-oxo-dG) (BT Lab, catalog #E1436HU), cleaved caspase 3 (BT Lab, catalog # E6970HU), BCL-2 (BT Lab, catalog #E1832HU), cleaved PARP (BT Lab, catalog #E6971HU) and Bax (BT Lab, catalog #E1825HU) were used to assess the levels of 8-oxo-dG, cleaved caspase 3, BCL-2, cleaved PARP and Bax in β-glucan-treated and untreated SH-SY5Y cells. In short, SH-SY5Y cells were cultivated into a 6-well plate and treated with 125 µg/mL β-glucan for 24 hours. SH-SY5Y cells that had been treated with β-glucan and those that had not were

Box 1: XTT assay

Principle

XTT assay is a colorimetric assay for the nonradioactive quantification of cellular proliferation, viability, and cytotoxicity.

Requirements

Dulbecco's modified Eagle's medium; β-Glucan; Microplate reader (Thermo Fisher Scientific, UK); SH-SY5Y and L929 cell line; XTT (sodium 3´-[1- (phenylaminocarbonyl)-3,4-tetra-zolium]-bis(4-methoxy6-nitro) benzene sulfonic acid hydrate)

Preparation of solutions

XTT mixture: The assay kit consists of two reagents, XTT reagent and electron coupling reagent. These reagents were mixed in a ratio of 50:1 respectively.

β-Glucan: 5 mg of β-glucan was weighed and dissolved in 100 µL of DMSO. After that the resulting solution diluted up to 1 mL with DMEM. Then, 100 µL of this stock was taken and diluted up to 1 mL with DMEM. The obtained concentration was 500 µg/mL. Other concentrations were obtained by serial dilution on this concentration.

Procedure

Step 1: The two cell lines were cultivated at a concentration of 1×10^4 cells per well and incubated overnight before the addition of β-glucan.

Step 2: After that the different concentrations (62.5, 125, 250 and 500 µg/mL) of β-glucan were applied to cells for 24 hours. Cells that had not been treated were used as a control.

Step 3: After incubation, 50 µL of XTT mixture was supplemented to each well.

Step 4: Following 4-hours incubation, the cells were shaken.

Step 5: The absorbance was measured using a microplate reader at 450 nm.

Calculation

Cell viability was evaluated as a percentage of live cells versus untreated cells after each experiment was done three times.

Reference

Taskiran and Ergul, 2020

gathered and diluted in PBS. Then they were frozen and thawed three times. Following that, the quantities of 8-oxo-dG, cleaved caspase 3, BCL-2, cleaved PARP and Bax in cell lysates were measured following the manufacturer's directions. Bradford protein assay kit (Merck Millipore, Germany) was used to assess the total protein quantities in β-glucan-treated and untreated SH -SY5Y cells (Ergul and Bakar-Ates, 2021).

Total antioxidant status and total oxidant status measurement in SH-SY5Y cells

The total antioxidant status assay kit (Rel Assay Diagnostics, Turkey) and the total oxidant status assay Kit (Rel Assay Diagnostics, Turkey) were used to examine total antioxidant status and total oxidant status levels in β-glucan-treated and untreated SH-SY5Y cells, respectively. SH-SY5Y cells were treated with 125 µg/mL β-glucan for 24 hours and the manufacturer's directions were implemented. For total antioxidant status and total oxidant status, the data were expressed as mmol Trolox Equiv./L and mol H_2O_2 Equiv./L, respectively (Taskiran and Ergul, 2021).

Statistical analysis

The laboratory findings were stated as mean ± standard error. The results obtained from the cell viability tests were examined using the one way ANOVA test with post hoc test. The results obtained from 8-oxo-dG, cleaved caspase 3, BCL-2, cleaved PARP and Bax, total antioxidant status and total oxidant status levels tests were examined using Independent Samples t Test. A p-value of less than 0.01 was used to accept statistically significant differences. GraphPad Prism 8.0 software (USA) was used for data analysis and graphical presentations.

Results

Cytotoxic effect of β-glucan on SH-SY5Y cells

In the beginning, the cytotoxic activity of β-glucan was investigated in SH-SY5Y cells. At 62.5 µg/mL and higher doses, β-glucan significantly inhibited the growth of SH-SY5Y cells as compared to control ($P < 0.01$). The IC_{50} value of β-glucan in SH-SY5Y cells was determined to be 94.6 µg/mL for 24 hours (Figure 1a). In addition, the cytotoxic activity of β-glucan was tested in L929, a non-cancerous cells, and the results demonstrated that β-glucan did not produce cytotoxic effect on L929 cells (Figure 1b).

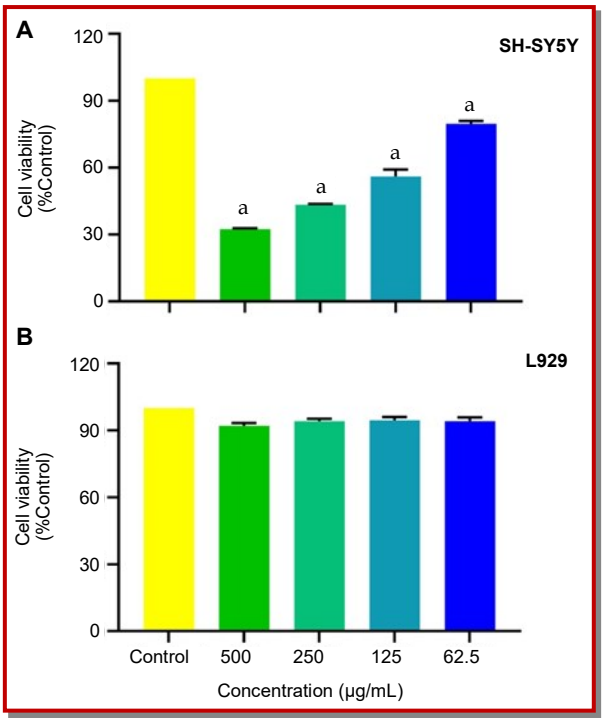

Figure 1: The antiproliferative effects of β-glucan on SH-SY5Y cells (a) and L929 cells (b). The findings are calculated as a percentage of viable cells versus control. The results are presented as the mean ± SEM of three samples. [a]p<0.01 as compared to the control group

Effect of β-glucan on Bax, cleaved caspase 3, BCL-2 and cleaved PARP levels in SH-SY5Y cells

The ELISA technique was used to determine the protein

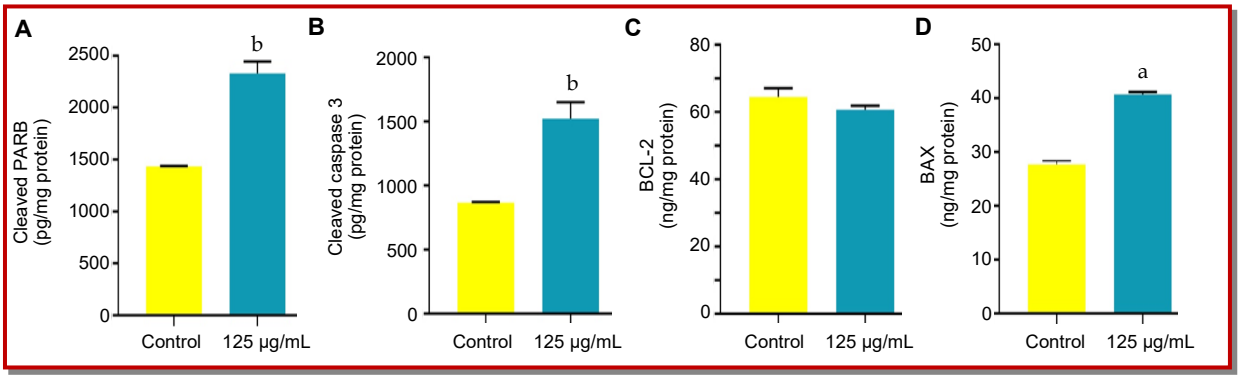

Figure 2: β-glucan (125 µg/mL) enhanced apoptosis of SH-SY5Y cells. The cleaved caspase 3, BCL-2, cleaved PARP and Bax levels were calculated using the ELISA kits. Results are represented as mean ± SEM of three samples. [a]p< 0.01 and [b]p<0.05 as compared to the control group

expressions in SH-SY5Y cells, such as Bax, cleaved caspase 3, cleaved PARP, and BCL-2, all of which are involved in apoptosis (Figure 2). Treatment with 125 µg/mL of β-glucan for 24 hours significantly increased the levels of Bax ($p<0.01$), cleaved PARP ($p<0.05$) and cleaved caspase 3 ($p<0.05$). β-glucan, on the other hand, did not alter the BCL-2 level ($p>0.05$).

Effect of β-glucan on 8-oxo-dG level in SH-SY5Y cells

The ELISA technique was also used to determine 8-oxo-dG expressions in SH-SY5Y cells in order to assess the DNA-damaging effects of β-glucan. Treatment with 125 µg/mL of β-glucan for 24 hours significantly increased the level of 8-oxo-dG ($p<0.01$) (Figure 3).

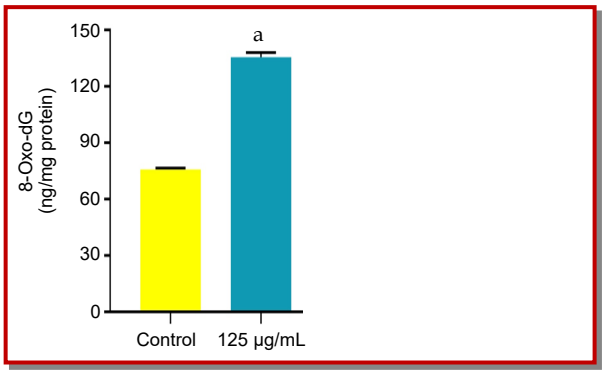

Figure 3: β-glucan (125 µg/mL) enhanced DNA damage of SH-SY5Y cells. The 8-oxo-dG level was calculated using the ELISA kit. Results are represented as mean ± SEM of three samples. [a]$p<0.01$ as compared to the control group

Effect of β-glucan on total antioxidant status and total oxidant status levels in SH-SY5Y cells

The effect of β-glucan on total antioxidant status and total oxidant status in SH-SY5Y cells was assessed using total antioxidant status and total oxidant status assay kits. β-glucan administration significantly increased total oxidant status level ($p<0.05$).

On the other hand, total antioxidant status level did not change in β-glucan-treated cells ($p>0.05$). Total antioxidant status was determined as 0.48 ± 0.02 in the control group and 0.47 ± 0.00 in 125 µg/mL β-glucan-treated cells. Additionally, total oxidant status was found to be as 26.96 ± 5.46 in the control group and 57.20 ± 3.86 in 125 µg /mL β-glucan-treated cells (Figure 4).

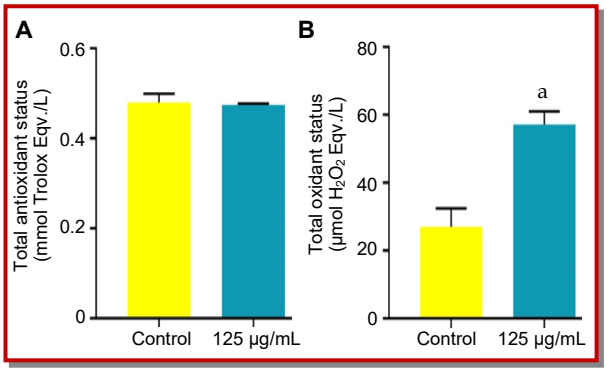

Figure 4: β-glucan at dose of 125 µg/mL didn't not cause a significant change in the total antioxidant status level (A), but increased the total oxidant status level (B) of SH-SY5Y cells significantly. Results are represented as mean ± SEM of three samples. [a]$p<0.05$ as compared to the control group

Discussion

β-Glucan had a concentration-dependent cytotoxic effect on SH-SY5Y cells. It significantly suppressed SH-SY5Y cell reproduction in a concentration-dependent way, with an IC_{50} value of 94.6 µg/mL after 24 hours. β-Glucan cytotoxic effects on non-cancerous L929 cells were studied in this context, and the findings showed that β-glucan had no significant cytotoxic effects on L929 cells.

Apoptosis is well understood to play a key role in the cellular pathogenesis of tumors and to impact the result of pharmacological treatments (Chen et al., 2018). The levels of cleaved PARP, cleaved caspase 3, Bax and BCL-2 were measured using ELISA to confirm the apoptotic effect of β-glucan on SH-SY5Y cells. One of the defense mechanisms against the growth and progression of tumors is the initiation of cell apoptosis, which is a major focus of cancer treatment (Tang et al., 2018). Pro-apoptotic and anti-apoptotic proteins are involved the BCL-2 protein family, and the ratio of pro-apoptotic versus anti-apoptotic proteins is widely used to determine cell fate (Ergul and Bakar-Ates, 2020). Apoptosis-inducing protein Bax interrupts the mitochondrial membrane, releasing cytochrome c, which generates the apoptosome complex. This complex triggers apoptosis by activating effector caspases. BCL-2, on the other hand, maintains membrane stability, prevents apoptosis, and inhibits cytochrome c release (Baig et al., 2016).

Caspases are a group of enzymes that are essential apoptosis effectors, and their stimulation is a hallmark of the process. They are generated as zymogens, that can be split to produce active enzymes when apoptosis is induced (Jin and El-Deiry, 2005). Caspases 3 is an executioner caspase that cleaves a variety of cellular substrates, resulting in a variety of morphological and biochemical alterations in apoptotic cells (Elmore, 2007).

Furthermore, PARP is a critical component of DNA repair pathways, especially in the repair of base excisions, and its cleavage or suppression causes cell death via exploiting a DNA repair defect (Sachdev et al., 2019). This compound is among the most well-studied activated caspase substrates (Tang et al., 2018). In this research, 125 µg/mL β-glucan treatment significantly increased pro-apoptotic Bax, cleaved

caspase 3, and cleaved PARP protein expressions, while not alerting anti-apoptotic BCL-2 expression. The 8-oxo-dG ELISA method was used to look at DNA fragmentation in SH-SY5Y cells after 24 hours of β-glucan treatment to see if the cytotoxic effect of β-glucan is linked to DNA damage.

8-oxo-dG is a well-known biomarker for DNA oxidative damage (Guo et al., 2016). β-Glucan treatment at 125 µg/mL significantly increased 8- oxo-dG quantities in SH-SY5Y cells, implying that β-glucan has cytotoxic and apoptotic properties.

Most anti-cancer drugs work by causing oxidative stress in tumor cells, which is thought to be the cause of most macromolecular alterations throughout the cell. Proteins, membrane lipids, and DNA are among the macromolecules that reactive oxygen species can target (Matos et al., 2019). The total oxidant status is one of several parameters used to estimate oxidative stress and therefore is commonly used to assess the overall oxidative status of cells. The total antioxidant status is also used to assess the overall antioxidant status of cells (Ergul and Bakar-Ates, 2020). In this context, we examined whether β-glucan could cause cytotoxicity by raising total oxidant status levels. In comparison to untreated cells, exposure to β-glucan for 24 hours increased total oxidant status levels; but, there was no significant variation in total antioxidant status levels. Treatment with β-glucan caused a rise in total oxidant status but no alteration in total antioxidant status levels, indicating that oxidative stress was induced in the β-glucan-treated SH-SY5Y cells. Furthermore, the damage of mitochondrial membrane and excessive increase of reactive oxygen species can activate the intrinsic apoptosis pathway (Lee et al., 2019).

In light of our mitochondrial membrane potential results, it can be conclude that glucosamine sulfate promotes apoptosis via the mitochondrial route. In agreement with our results, It was shown that β-glucan isolated from *L. edodes* had a remarkable inhibition rate of 75% on S-180 cell viability, which was even higher than that of the positive control of cytoxan (54%). β-glucan facilitated the accumulation of immune cells into tumors, which was followed by cell apoptosis and inhibition of cell viability. β-Glucan also down-regulated PARP1 and the anti-apoptotic protein Bcl-2 expressions in cancer cells, while up-regulating the tumor suppressor p53 and pro-apoptotic proteins Bax and caspase 3/9 (Xu et al., 2016). β-Glucan from oats had antitumor activity and triggered oxidative stress in human lung cancer cell lines. Normal cells, on the other hand, showed no signs of toxicity (Choromanska et al., 2018). It was demonstrated that pulmonary exposure to CSBG (a soluble cell wall β-glucan derived from *C. albicans*) significantly increased apoptosis and raised the accumulation of 8-OHdG in the lung parenchyma (Inoue et al., 2009). Zhang et al. (2006) also used a water -soluble β-glucan derived from *Poria cocos* mycelia (PCM3-II). The growth-inhibitory activity of PCM3-II was investigated further *in vitro* using human breast cancer MCF-7 cells. MTT analysis revealed that PCM3-II decreased MCF-7 cell viability in a dose-dependent manner. At 400 µg/mL PCM3-II, cancer cell viability was reduced by 50% compared to the control. The PCM3-II also caused a reduction of the anti-apoptotic Bcl-2 protein, but not the proapoptotic Bax protein, resulting in an increase in the Bax/Bcl-2 ratio in breast cancer cells (Zhang et al., 2006). Breast cancer cells have also been shown to be inhibited by LNT. Further exploration discovered that LNT adminis-tration induced the cleavage of Poly (ADP-ribose) polymerase (PARP) (Zhang et al., 2020). β-Glucan showed cytotoxic effects on colon cancer cells by using MTT assay. At 200 µg/mL dose, the viability of cancer cells was reduced by about 50% (Kim et al., 2009). Lentinan (1-6,1-3)- β-glucan) suppressed the viability of C6 glioma cells by inducing apoptosis, blocking the cell cycle, increasing the rate of cells in the G0/G1 stage, and decreasing the rate of cells in the S-stage (Atiq and Parhar, 2020). It was also demonstrated that lentinan showed synergistic effects with oxaliplatin in inhibiting NF-kB, STAT3, and survivin expression in Hep-G2 cells (human hepatocyte carcinoma) in mice with H22 tumors (Zhang et al., 2016). Lentinan treat-ment stimulates autophagy and programmed cell death in human osteosarcoma cells by inhibiting MAPK/ERK signaling and up-regulating miR-340 in MG63 cells. LNT raises Beclin-1, LC3B-II/LC3B-I, and caspase-3, -9 amounts while decreasing cyclin D1 amounts (Xu et al., 2018). It was recently discovered that schizophyllan (-1,3 β-glucan) therapy inhibits the growth of rat CNS-1 glioma cells by inhibiting cell cycle and programmed cell death by p53-mediated inhibition (Atiq and Parhar, 2020). As a result, natural compounds would be effec-tive anti-glioblastoma drugs, either alone or in combi-nation with other anti-cancer drugs to improve their sensitivity to glioblastoma. However, due to the exis-tence of blood-brain barrier, the most significant obstacle in the treatment of GBM is insufficient drug delivery. The role of modified formulations such as liposomes, nanoparticles and drug conjugates in over-coming the BBB and improving drug delivery for the management of glioblastoma multiforme was high-lighted (Atiq and Parhar, 2020).

Conclusion

Fungi derived β-glucan significantly suppressed SH-SY5Y cell reproduction in a concentration-dependent way without causing significant cytotoxic effects on L929 cells. β-glucan treatment significantly increased pro-apoptotic Bax, cleaved caspase 3, and cleaved PARP protein expressions. β-Glucan treatment also significantly increased 8- oxo-dG quantities in SH-SY5Y cells and thus the cytotoxic effect of β-glucan may be

linked to DNA damage. Treatment with β-glucan caused a rise in total oxidant status supporting its cytotoxic effects.

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Conflict of Interest

Authors declare no conflict of interest

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