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Determination of Antiproliferative Effects of Exopolysaccharides from Six Mushroom Species on Glioma Cells

Şerife Can [1,a], Pınar Öztopcu-Vatan [2,b], Selda Kabadere [3,c,*], Mustafa Yamaç [2,d], Ruhi Uyar [3,e]

[1] *Dr. Burhan Nalbantoglu State Hospital Microbiology Laboratory, Lefkosa, Turkish Republic of Northern Cyprus*
[2] *Department of Biology, Faculty of Science and Letters, Eskisehir Osmangazi University, , Eskisehir, Türkiye*
[3] *Department of Physiology, Faculty of Medicine, Eskisehir Osmangazi University, Eskisehir, Türkiye*
**Corresponding author*

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ABSTRACT

Glial tumors are the largest and danger group of central nervous system tumors. The use of natural products now has been contemplated of exceptional value in the control of cancer. Mushrooms have been used for many centuries, not just as a food, but also to treat many illnesses. Therefore, we aimed to evaluate the effect of exopolysaccharides (EPS) obtained from six different edible mushrooms on the survival of glioma cells. In this study the effects of 0.4, 1, 2, 4 and 6 µg/mL doses of EPSs from six mushroom species *Coprinus comatus*, *Fistulina hepatica*, *Panus neostrigosus*, *Laetiporus sulphureus*, *Polyporus squamosus*, and *Lenzites betulinus* were investigated on the rat glioma cell line (C6) in two different periods by MTT assay. According to our results 0.4 and 1 µg/mL of EPSs from six mushroom species were not effective or less effective, but 2, 4 and 6 µg/mL doses killed glioma cells about 27 to 71 % for 24 hours, 35 to 78 % for 48 hours As a result, these mushroom EPSs showed different cytotoxicity to glioma cells time and dose-dependently. These findings can be suggested that the anti-tumor effects of EPSs can be potential use in clinical applications to treat glioma. Further studies are needed to understand these effects more clearly on glioma.

Keywords: Glioma, In vitro, Mushroom, Exopolysaccharides, Cytotoxicity.

[a] *can.serife@yahoo.com* *https://orcid.org/0000-0003-2265-9124*
[b] *poztopcu@ogu.edu.tr* *https://orcid.org/0000-0003-4339-4129*
[c] *kabadereselda@gmail.com* *https://orcid.org/0000-0002-9589-0063*
[d] *myamac@ogu.edu.tr* *https://orcid.org/0000-0002-7262-0036*
[e] *ruhiuyar66@gmail.com* *https://orcid.org/0000-0002-7608-7531*

Introduction

Evidence of cancer dates back at least to 35.000 BC in a Neanderthal skull and Egyptian and Incan mummies [1] and cancer is one of the major worldwide fatal diseases. Chemotherapy, radiotherapy, and surgical methods exist for the treatment of cancer in modern medicine however, most cancer therapeutic methods severely affect the host normal cells. Glioblastoma multiforme (GBM), also known as glioblastoma or grade IV astrocytoma, is a worldwide and aggressive brain tumor. Although important improvements achieved in the conventional treatment of GBM over recent years, the median survival of GBM patients is still approximately two years [2]. Hence, the use of natural products now has been contemplated of exceptional value in the control of cancer [3].

Edible and medicinal mushrooms have been used since the Neolithic age as a natural bioactive metabolite source [4], and the theoretical and practical background for the use of mushrooms' for medicinal purposes comes from especially traditional eastern medicine. [5]

It is estimated that the fungal kingdom includes 1.5 million species which has a potential natural source for new bioactive metabolite(s). It is well-known that mushrooms contain various bioactive compounds with medicinal properties as cell wall components such as polysaccharides, polysaccharopeptides and polysaccharide-protein complexes or as secondary metabolites such as polysaccharides, phenolic compounds, terpenes, and steroids [6,7]. Polysaccharides are reported as the most important bioactive compounds found in mushrooms which have antioxidant, antidiabetic, antimicrobial, anti-inflammatory, and immunomodulatory activities [6,8], and has reported as potential antitumoral compounds [5].

In various Asian countries, mainly polysaccharides (especially β-glucans) from different mushroom species are used for developing pharmaceutical preparations for clinical and commercial purposes such as GLPS polysaccharide fraction from *Ganoderma lucidum*, grifolan from *Grifola frondosa*, lentinan from *Lentinus edodes*, PSK and PSP from *Trametes versicolor*, and schizophyllan from *Schizophyllum commune* [7,9]. Different clinical studies have also confirmed the cancer inhibitory effects of different mushroom species such as *Agaricus brasiliensis*, *Cordyceps sinensis*, *Ganoderma lucidum*, *Grifola frondosa*, *Flammulina velutipes*, *Hypsizygus marmoreus*, *Lentinus edodes*, *Phellinus linteus*, *Schizophyllum commune*, *Trametes versicolor*, and *Tremella mesenterica* [9]. A lot of compounds from mushrooms have proceeded through phase I, II, and III clinical studies [10], and most of the preparations derived from mushroom polysaccharides have been prescribed and used extensively and successfully in modern clinical practice to treat different cancer types in China, Japan, South Korea, Taiwan, and other Asian countries [5,11].

The biologically active polysaccharides in mushrooms can be found in the fruit bodies, cultured mycelium, and culture broth [9]. Collecting or producing the fruit bodies is a season-depending, time-consuming, and/or labor-intensive process. On the other hand, a submerged culture of mushrooms can be a promising alternative for easy, fast, and efficient production of bioactive metabolites [12]. In this view of point, the anticancer activities of exopolysaccharides (EPS) from submerged culture of six mushroom species, *Coprinus comatus*, *Fistulina hepatica*, *Panus neostrigosus, Laetiporus sulphureus*, *Polyporus squamosus*, and *Lenzites betulinus*, were investigated on the rat glioma (C6) cells in the present study.

Material And Methods

Materials

The used fungal isolates have been stored at 4°C on a potato dextrose agar (PDA) medium. All medium components were purchased from Merck (Darmstadt, Germany). All reagents for cell culture were purchased commercially from Sigma-Aldrich Products.

Inoculum Preparation

The fungal cultures were initially grown on PDA plates at 28 ºC for a week. Five active growing mycelial discs (6 mm diam) from the fungal colony were transferred into 100 mL of potato malt peptone medium (PMP; g/L; potato dextrose broth 24, malt extract 10, peptone 1). After incubation (28 ºC, 100 rpm for 4 days), the fungal biomass was harvested, washed with sterile distilled water (SDW) three times and the total volume was completed with SDW to 100 mL. The inoculants were prepared via homogenization of the cells with a Waring blender (Heidolph Silent Crusher M). The prepared cell suspensions were used as inoculants (4%) for all experimental groups.

Exopolysaccharide Production

Submerged culture studies were performed in a sterilized PMP medium at 28 °C, 100 rpm for 7 days. The fungal cultures were harvested by filtration (Whatman No. 2 filter paper). To precipitate crude EPS, the obtained culture filtrates were mixed with cold ethanol (4:1, v/v), stirred, and stood overnight at 4 °C. The crude EPSs were obtained by centrifugation (7500 rpm, 10 min), discharging of supernatant, and finally, crude EPSs were lyophilized and stored at 4 °C until used.

MTT colorimetric assay

The C6 cells were purchased from ATCC and cultured as described previously [13]. At first, the C6 cells were seeded into $2x10^4$ cells/well in 96 well plates for 24 hours Fungal EPSs were dissolved in DMEM, then diluted further in DMEM again. After the incubation period the cells were then exposed to 0.4, 1, 2, 4 and 6 µg/mL fungal EPSs doses for 24 or 48 hours Control group had only a complete medium containing DMEM supplemented with 10 % fetal calf serum and 1 % penicillin-streptomycin solution.

MTT colorimetric assay was used to evaluate drug cytotoxicity screening [13]. The absorbance was read at 550 nm (Bio-Tek Instruments microplate reader).

Statistical Analysis

All statistical analyses were carried out by one-way analysis of variance (ANOVA) and followed up by Tukey's multiple comparison tests. A p-value less than 0.05 was considered significant.

Results

At the doses of 0.4 and 1 µg/mL, *Coprinus comatus* EPS did not show any significant effect on the viability of C6 cells after 24 or 48-hour incubation but 2, 4 and 6 µg/mL doses reduced the percentages of living cells by 59, 69 and 69 % ($p<0.001$), for 24 hours, and 53, 70, and 69 % ($p<0.001$) after 48 hours, respectively (Figure 1). IC_{50} values of *Coprinus comatus* EPSs were calculated at 1.8 µg/mL for 24 hours and 1.9 µg/mL for 48 hours.

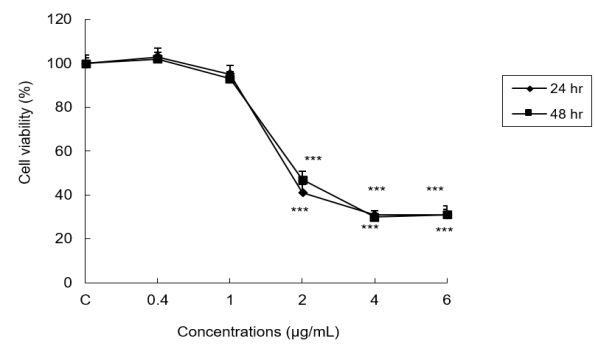

Figure 1. The effects of different doses of *Coprinus comatus* EPS (***: $p<0.001$)

At the doses of 0.4 and 1 µg/mL, *Fistulina hepatica* EPS did not show any significant effect on the viability of C6 cells after 24-hour incubation but 2, 4 and 6 µg/mL doses reduced the percentages of living cells by 35, 37 and 38 % ($p<0.001$), for 24 hours, and 1, 2, 4, and 6 µg/mL doses reduced the percentages of living cells by 39 ($p<0.01$), 44, 45 and 45 % ($p<0.001$) after 48 hours, respectively (Figure 2). Because the effect is lower, IC_{50} values of *Fistulina hepatica* EPSs could not be calculated for 24 and 48 hours.

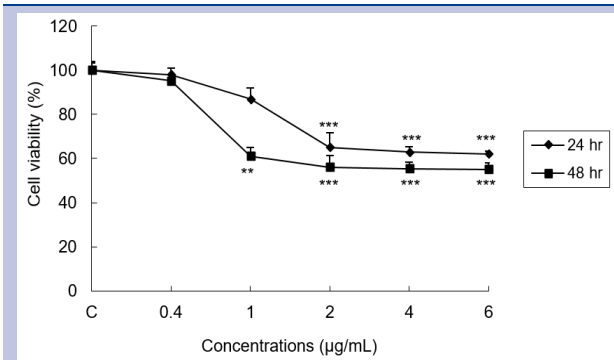

Figure 2. The effects of different doses of *Fistulina hepatica* EPS (**: $p<0.01$; ***: $p<0.001$)

At the doses of 0.4, 1, and 2 µg/mL *Panus neostrigosus* EPS did not show any significant effect on the viability of C6 cells after 24 or 48-hour incubation but 4 and 6 µg/mL doses reduced the percentages of living cells by 27 and 71 % ($p<0.001$) for 24 hours, and 28 and 79 % ($p<0.001$) after 48 hours, respectively (Figure 3). IC_{50} values of *Panus neostrigosus* EPSs were calculated at 4.9 µg/mL for 24 hours and 4.7 µg/mL for 48 hours.

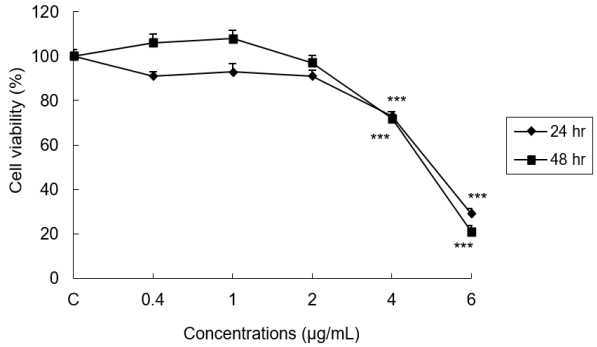

Figure 3. The effects of different doses of *Panus neostrigosus* EPS (***: $p<0.001$).

At the doses of 0.4 and 1 µg/mL, *Laetiporus sulphureus* EPS did not show any significant effect on the viability of C6 cells after 24-hour incubation but 2, 4 and 6 µg/mL doses reduced the percentages of living cells by 36, 54 and 63 % ($p<0.001$) for 24 hours, 1, 2, 4 and 6 µg/mL doses reduced the percentages of living cells by 35, 48, 74, and 78 % ($p<0.001$) after 48 hours, respectively (Figure 4). IC_{50} values of *Laetiporus sulphureus* EPSs were calculated at 2.9 µg/mL for 24 hours and 2.1 µg/mL for 48 hours.

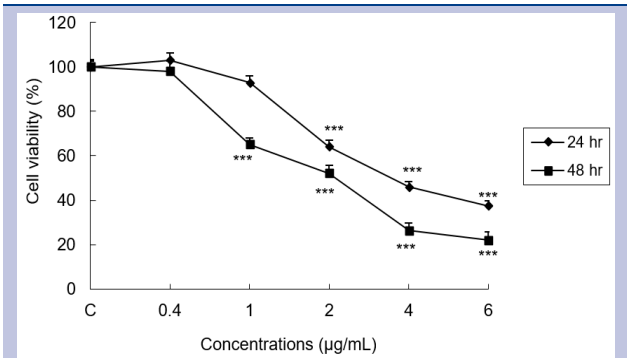

Figure 4: The effects of different doses of *Laetiporus sulphureus* EPS (***: $p<0.001$)

At the doses of 0.4 and 1 µg/mL, *Polyporus squamosus* EPS did not show any significant effect on the viability of C6 cells after 24 incubation but 2, 4 and 6 µg/mL doses reduced the percentages of living cells by 27, 59, and 61% ($p<0.001$) for 24 hours 1, 2, 4 and 6 µg/mL doses reduced the percentages of living cells by 18 ($p<0.01$), 51, 68, and 69 % ($p<0.001$) after 48 hours, respectively (Figure 5). IC_{50} values of *Polyporus squamosus* EPSs were calculated by 3.2 µg/mL for 24 hours and 1.95 µg/mL for 48 hours.

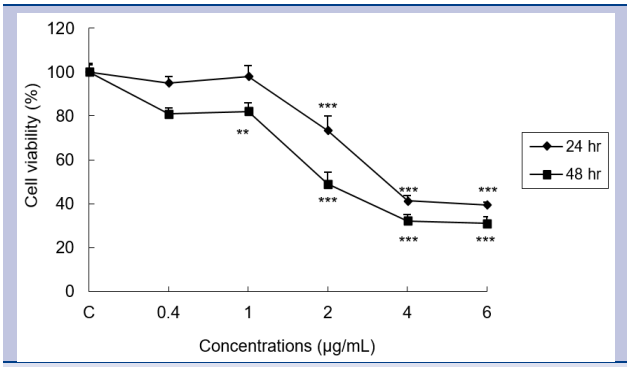

Figure 5. The effects of different doses of *Polyporus squamosus* EPS (***: $p<0.001$).

At the doses of 0.4, 1, and 2 µg/mL *Lenzites betulinus* EPS did not show any significant effect on the viability of C6 cells after 24-hour incubation but 4 and 6 µg/mL doses reduced the percentages of living cells by 42 ($p<0.01$) and 47 % ($p<0.001$) for 24 hours 2, 4 and 6 µg/mL doses reduced the percentages of living cells by 42, 59 and 58 % ($p<0.001$) after 48 hours, respectively (Figure 6). The IC_{50} value of *Lenzites betulinus* EPS was calculated at 2.9 µg/mL for 48 hours.

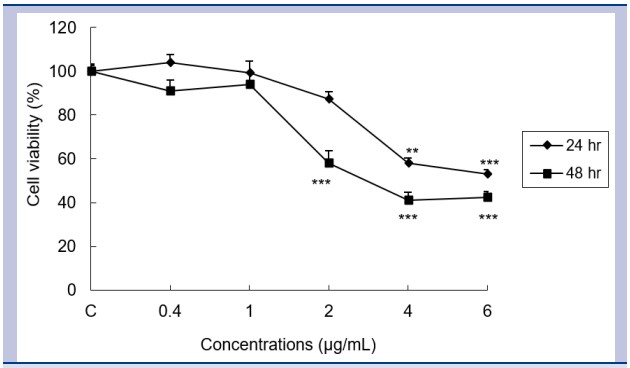

Figure 6: The effects of different doses of Lenzites betulinus EPS (**: $p<0.01$; ***: $p<0.001$).

Discussion

According to Zhang et al, the antitumor activity of the mushroom was reported firstly by Lucas by discovering the inhibitory effect of a substance from *Boletus edulis* against Sarcoma S-180 tumor cells which is also the first report for bioactivity of Basidiomycetes mushrooms [14, 15]. Because of their preventive effect on oncogenesis and tumor metastasis and stimulating effect on the host immune system, mushroom polysaccharides are widely used especially in Asian countries with chemotherapy [5,12]. Other than antimicrobial [16], antioxidant [17] antiviral[18], antiallergic [19], anti-inflammatory [5], antidiabetic [20], hepatoprotective [21], and radioprotective [22] activities and immunological activities of polysaccharides derived from mushrooms are well-documented [23,24,25] and most extensively studied[5].

According to the present results *Coprinus comatus* EPS was found to be the most effective mushroom species on

C6 cells. IC_{50} doses of *Coprinus comatus* EPSs were calculated by 1.8 µg/mL for 24 hours and 1.9 µg/mL for 48 hours and calculations were close to each other depending on time. Similarly, Nowakowski et al [13] mentioned that according to MTT assay results, ethanolic extracts of *Coprinus comatus* were one of the most effective of all the mushroom species they tried on some human glioma cells (U87MG, LN-18 cell lines). In another study, the cytotoxic effects of ethanol and ethyl acetate extracts of *Coprinus comatus* on LNCaP prostate cancer cells were investigated and the IC_{50} value was found to be 28.3 µg/mL. Also, higher concentrations of *C. comatus* extracts killed more than 50% of the cells. In addition, it also had anti-androgenic effects on androgen-dependent LNCaP cells [26]. According to our results, IC_{50} values of *Panus neostrigosus, Laetiporus sulphureus* and *Polyporus squamosus* EPSs were calculated by 4.9, 2.9 and 3.2 µg/mL for 24 hours and 4.7, 2.1 and 1.95 µg/mL for 48 hours. When we compare these three mushroom species in terms of effect for 24 and 48 hours, *Laetiporus sulphureus* EPS (2.9 µg/mL) and *Polyporus squamosus* EPS (1.95 µg/mL) were found to be the most effective on C6 cells, respectively. The IC_{50} value of *Lenzites betulinus* EPS was calculated just in 48 hours and it was found 2.9 µg/mL. Since *Fistulina hepatica* EPS at the dose of 6 µg/mL reduced the percentages of living cells by 45 % ($p<0.001$) after 48 hours, IC_{50} values of *Fistulina hepatica* EPSs could not be calculated for 24 and 48 hours

In different studies, it has been discovered that various polysaccharides obtained from mushrooms have suppressive effects on cell proliferation in cancer cells [27]. It has been determined that they show this effect through apoptosis. Although studies on brain tumors related to the aforementioned fungi are very scarce, studies related to cancer in recent years are increasing. In our study, the proliferation suppressive effect of six different EPSs on glioma cells was investigated. It was determined that the most effective EPSs among them was the *Coprinus comatus* EPS in two different periods. It is thought that there may be different reasons for this difference. The biological effectiveness of the mushroom EPSs can vary depending on their chemical composition, solubility, degree of branching, molecular weight, the charge of polymers, and structure in aqueous media [9,28].

As a result, these mushroom EPSs were time and dose-dependently toxic to glioma cells. Our results can be suggested that the cytotoxic effects of EPS can be potential use in clinical application to treat glioma. Although our data are promising in terms of cancer prevention, there are not enough studies related to this topic. Our future studies will focus on the characterization of polysaccharides and investigation of other cancer cell lines.

Conflicts of Interest

The author declares no conflicts of interest. No competing financial interests exist.

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