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Toxicological evaluation of submerged liquid culture from *Phanerochaete chrysosporium* mycelium on human blood cells: cytotoxicity, genotoxicity and oxidative damage

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Abstract: Mushrooms produce a variety of bioactive antioxidant secondary metabolites including ectins, polysaccharides, pigments, phenolic compounds, sterols and terpenes. Extracellular and intracellular compounds produced by submerged liquid fermentation are important industrially and economically. *Phanerochaete chrysosporium* (PC) is the model white-rot fungus that easy cultivation on lignocellulose-containing substrates. PC can be used as a bioprotein source. Aims of this study were to determine the *in vitro* antioxidant, cytotoxic and genotoxic effects of hot water extract obtained from PC on human peripheral blood monunuclear cells (hPBMCs). Cytotoxicity was determined by lactate dehydrogenase (LDH) leakeage assay and neutral red (NR). Total antioxidant capacity (TAC) and total oxidant status (TOS) were detected to determine the oxidative damage. Genotoxicity was characterized by micronuclei and chromosome aberrations assays for specify DNA damage. PC (5-75 µg/ml) significantly increased antioxidant capacity and these doses did not cause any significant alterations to cytotoxicity on hPBMCs. The elavated doses of PC (5-250 µg/ml) did not cause increase in genotoxic. Whereas, 250 and 500 µg/ml doses of PC statistically increased TOS levels, NR uptake, LDH release, CA/cell frequency and MN formation however decreased TAC levels. This study is the first time on cytotoxicity, genotoxicity and oxidative damage of PC on hPBMCs. In conclusion, the consumption of PC can be safe for humans, but it has also exposure period and dose-dependent effects on inducing oxidative damage and toxicity on hPBMCs.

Keywords: *Phanerochaete chrysosporium*, human blood cells, cytotoxic, genotoxic, antioxidant

1. INTRODUCTION

A number of studies are performed regarding to the antimicrobial and antioxidant properties of many edible and inedible mushrooms extracts, collected at various habitats and obtained from different types of basidiomycetes [1, 2]. Extracellular and intracellular compounds produced by submerged liquid fermentation are industrially and economically important [3]. Synthetic antioxidants are used at legal limits because they are unhealthy. Natural antioxidants can be nitrogen compounds (amino acids and alkaloids) and phenolic compounds (phenolic acids and flavonoids), pigments represent the main antioxidant components derived from mushrooms [4]. A research had proven the antioxidant activity of PC [5].

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It is fundamental that mushrooms must be professionally inspected and their safety and quality approved before being ingested [6]. The uncontrolled mushroom diet can result in overproduction of reactive oxygen species (ROS), which are associated with oxidative stress [7]. ROS is produced in physiological and metabolic periods, and detrimental oxidative reactions may occur in organisms that remove them via enzymatic and non-enzymatic antioxidative reactions. Antioxidant molecules prevent or inhibit detrimental reactions [8]. Serum concentrations of different antioxidant and oxidants can be evaluated in laboratories respectively, however the measurements are time-consuming, labor-intensive, costly, and require complicated techniques. Because the measurements of oxidant/antioxidant molecules separately are not practical and their effects are additive, the total antioxidant capacity of a sample is measured, and this is called TAC. The total oxidative status of a sample is measured and named as TOS [9, 10].

Customary, the toxic effects of unknown compounds have been measured *in vitro* by counting viable cells after staining with a vital dye. The lactate dehydrogenase (LDH) assay is a means of measuring either the number of cells via total cytoplasmic LDH or membrane integrity as a function of the amount of cytoplasmic LDH released into the medium [11]. In addition, the neutral red (NR) assay system is a means of measuring living cells via the uptake of the vital dye neutral red. This assay is rapid, easy to perform, and suitable for handling of large numbers of cultures simultaneously. It can be used for concentration range-finding pre-experiments [12]. To assess the oxidative stress-mediated DNA damages, the micronuclei (MN) are a small extra nucleus separated from the main one, generated during cellular division by late chromosomes or by chromosome fragments. MN is a rapid, sensitive and simple *in vitro* assay for the detection of micronuclei in the cytoplasm of interphase human lymphocytes [13-15]. Moreover, chromosome aberrations (CAs) assay in hPBMCs is the most widely used cytogenetic marker to define the effects of DNA damaging agents [16].

Mushroom extract supplement or a mushroom ingestion can definitely affect the immune system and have an affect on cancer protection or treatment. Therefore, mushrooms are regarded as functional foods and have been in use for decades in folk medicine. However, what remain uncertain are the effects on blood cells. This study has the aim to determine the antioxidant status and cyto-genotoxic effects of hot-water extract of mycelium of the PC on hPBMCs of healthy donors. In this way, we firstly evaluated the effects of PC extract on the cytotoxicity via LDH and NR assays on cultured hPBMCs. We also appreciated the role of this edible mushroom on antioxidant capacity via TAC and TOS analysis and DNA damage determined by CAs and MN assays after PC treatment in cultured hPBMCs.

2. MATERIAL and METHODS

2.1. Chemicals

All reagents and chemicals were purchased from Sigma Aldrich GmbH (Sternheim, Germany). All other unlabeled reagents and chemicals were of analytical grade.

2.2. Culture and Storage Condition

The PC was obtained from Dr. Abdurrahman Dündar (Mardin Artuklu University, Turkey). The strain was preserved on potato dextrose agar (PDA) and malt extract agar (MEA) slants at 4 °C. The microorganisms were subcultured at regular intervals (15 days) to maintain viability.

2.3. Media Preparation and Fermentation Condition

The PC was initially grown on PDA medium for 10 days at 25 °C. Submerged fermentation was carried out in 500 mL erlenmeyer flasks, containing 200 mL of liquid medium

(Glucose 20 g/L, peptone 2 g/L, yeast extract 3 g/L, KH_2PO_4 1 g/L, $MgSO_4$ 0.5 g/L,). The inoculated flasks were preserved on a rotary shaker at 150 rpm at 25 °C. After 10 days of growth, the mycelium was recovered from the liquid medium by centrifugation at 3500 g for 10 min. Next, the obtained mycelia were washed 3 times with distilled water. Cultured mycelia were then dried using oven until constant weight. The mycelia concentration of 15.25 g/L was obtained for 10 days fermentation.

2.4. Preparation of Mushroom Extract

To obtain hot water extraction, 10 g dry and powdered mycelial biomass was heated with 100 mL deionized water for 1 h, centrifuging at 5000 g-force for 15 min and filtering through Whatman No. 1 filter paper. The dried extract was used directly for analyses of antioxidant components (total phenolic and flavonoid) or redissolved in water to a concentration of 100 mg/ml and stored at 4 °C for further uses.

2.5. Determination of Total Phenolic and Flavonoid Contents

The ingradient of total phenols in extract was estimated with the Folin- Ciocalteu reagent, using gallic acid [17]. The total flavonoid ingradient was calculated from a calibration curve using quercetin (QE) as standard [18].

2.6. Cell Cultures and Treatment

Blood samples were obtained by venipuncture into syringes containing sodium heparin as anticoagulant from four healthy, non-smoking, female donors (age between 22 - 26 yrs) and not exposed to any drug therapy or not known mutagenic agent over the past two years. The blood was diluted with the same volume of phosphate buffer solution (PBS) without Ca^{+2} and Mg^{+2}. Cell-PBS mixture was carefully added onto a Histopaque 1077 (v/v), followed by centrifugation at 400 g for 30 min at 20 °C. The cloudy layer (lymphocytes) were aspirated with a pasture pipette and brought into a new tube. The collected fraction was diluted by 1/4 with PBS, mixed carefully and centrifuged (10 min; 300 g-force; 20 °C). This step was repeated twice. The cell number was adjusted to $2x10^6$/ml. After seperation, cells were routinely cultured in RPMI 1640 Media with 2 mM L-glutamine, and were supplemented with 10 % fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). Cells were maintained in humidified air containing 5 % CO_2 at 37 °C for 24 h before applying the different treatments. Eleven treatment groups were performed. First group, cells were treated with 10 µm final concentration of Mitomycin-C as a positive control (PC) for NR, LDH, MN and CAs assays and 25 µm hydrogen peroxide (H_2O_2) and 10 µm ascorbic acid ($C_6H_8O_6$) for TOS and TAC analysis, respectively, and then washed by medium. Second group, untreated cells named as a negative control (NC). Other ten groups, cells were treated at 5, 10, 15, 25, 40, 75, 100, 250, 500 and 1000 µg/ml concentrations of hot-water extract from mycelium of the PC before incubation for 24 and 48 h (Fig.1). The experiments were repeated six times independently for each assay, and values were represented as means ± standard deviation (SD).

2.7. Cytotoxicity Assesment

2.7.1. NR

Cell viability after 24 and 48 h PC treatment was assessed using NR according to the manufacturer's protocol (Neutral Red based Sigma, USA). Absorbance values at 540 nm for NR (corrected at 690 nm) were measured using a microplate spectrophotometer (Bio-RAD Benchmark Plus). An increase or decrease in the number of cells or their physiological state results in a concomitant change in the amount of neutral red dye incorporated by the cells in the culture. This indicates the degree of cytotoxicity caused by PC.

2.7.2. LDH Release

LDH released from damaged cells in culture was quantified using the producer's method (Cell Biolabs CytoSelect™, USA). The rate of NAD^+ reduction was determined as an increase in absorbance at 490 nm. The rate of NAD^+ reduction was directly proportional to LDH activity in the culture.

2.8. Oxidant/Antioxidant Status

2.8.1. TAC and TOS Analysis

TAC and TOS assays were carried out in plasma samples obtained from cell cultures for 2 h by trading available kits (Rel Assay Diagnostics, Turkey).

2.9. Genotoxicity Assesment

2.9.1. CAs

Blood cell cultures were incubated at 37 °C for 72 h. Test materials were added after 24 and 48 h of culture initiation, and colchicine (0.06 ppm) was added to each culture at 2 h before reaping. Cells were cured with different doses of PC. A negative control (untreated group, in PBS) and a positive control (10 μM mytomycin C, Sigma-Aldrich) were also used for testing the correctness of the assays. The CA test was performed as described by Evans, (1975). 1000 metaphases were investigated for the CA assay per donor (totally 400 metaphases). The mean frequency of abnormal cells and the number of CAs per cell (CA/cell) were computed. The MI (MI: number of metaphases/total interphases and metaphases) was scored by recording the number of metaphases in 1000 cells from each group. MI was evaluated according to the OECD [19].

2.9.2. MN

The MN assay was performed using the standard procedure recommended by Fenech [20], with slight modifications according to Surrallés [21]. After 24 and 48 h PC treatment, blood cultures were set up. The test material was added 24 h after phytohaemagglutinin stimulation. Cytochalasin B (Cyt-B), at a final concentration 6 μg/ml was added at 44 h after the cultures were established, to arrest cytokinesis of dividing cells. Binucleated lymphocytes were then harvested 72 h after culture setting. The cells were collected by centrifugation and washed with hypotonic solution (0.075 M KCl) at room temperature, centrifuged and fixed with a methanol/acetic acid (3:1) solution. This step was repeated twice. Air-dried preparations were made and the slides were coded prior to scoring.

2.10. Statistical Analysis

Statistical analysis was performed using SPSS software (version 21, USA). Dunnett t-test (two tailed) was performed in order to compare the frequency of micronucleus cells between negative and positive control. One–way ANOVA and Duncan's test was used to detect whether any treatment significantly differed from each other or controls. Statistical results were made with a significance level of 0.05.

3. RESULTS

3.1. Total Phenolic and Flavonoid Contents of PC

In present study, PC were used for quantitative evaluation of the total phenolic contents as gallic acid equivalent (GAE) per gram extract and total flavonoid quantification as quercetin (QE) per gram extract were defined by a colorimetric method. The total phenolic and flavonoid content were found as 4.6 ± 0.22 mg GAE/g extract and 1.2 ± 0.1 mg QE/g extract, respectively.

3.2. Oxidant/Antioxidant Status

Table 1 presents the comparison of oxidant–antioxidant profiles of PC extracts on cultured hPBMCs. As seen from the Table, 10, 15, 25 and 40 μg/ml doses of mushroom extracts caused important increases of TAC levels and decreases of TOS levels as compared to untreated culture. Likewise, mushroom extracts (75 and 100 μg/ml) did not cause any changes in TAC and TOS levels on hPBMCs. On the other hand, mushroom extracts caused statistically significant increases TOS levels and decreases TAC levels at concentrations 250, 500 and 1000 μg/ml in comparison with control values on hPBMCs.

Table 1. TAC and TOS analysis in lymphocytes treated with hot-water extract from mycelium of *PC*

Test Substances	**Treatment** **Concentration (μg/mL), 24 hours**	**Oxidant/Antioxidant status** **TOS (mmol H_2O_2 equiv./L)**	**TAC (mmol Trolox equiv./L)**
Negative Control	Untreated cultures	10,22 b ± 1.54	5.02c ± 0.44
Positive Control	25 μm H_20_2 for TOS, 10 μm L-Ascorbic acid for TAC	33,5d ± 4.52*	12.22a ± 1.26*
Doses of PC (μg/ml)	5	10.21b ± 1.55	5.15c ± 0.51
	10	9,63a ± 1.74	7.14b ± 0.74*
	15	9,58a ± 1.51	7.51b ± 0.43*
	25	9,44a ± 1.68	7.23b ± 0.55*
	40	9,25b ± 1.58	7.72bc ± 0.38
	75	9,16b ± 1.35	7.64c ± 0.42
	100	10,33b ± 1.65	6.55c ± 0.46
	250	11,52bc ± 2.41*	4.52cd ± 0.26*
	500	12,08c ± 2.22*	3.91 ± 0.38*
	1000	14.36 ± 2.56*	3.08 ± 0.29*

The results are given as the means ± SD from six independent experiments; means in the figure followed by different letters (a, b, c, d) present significant differences. *$p<0.05$ was considered significant according to the control group. Positive control: Ascorbic acid (10 μM) and hydrogen peroxide (25 μM) in TAC and TOS analysis, respectively.

3.3. Cytotoxicity

Lactate dehydrogenase (LDH) assays and neutral red (NR) were used to quantify cell viability in response to PC on hPBMCs (Figures 1 and 2). Cells exposed to 5, 10, 15, 25, 40, 75, 100 and 250 μg/ml concentrations of hot-water extract from mycelium of the PC didn't show any important alterations in cell viability for 24 and 48 h, as determined by NR and LDH assays. But, 500 and 1000 μg/ml doses of mushroom extracts showed cytotoxic effect on hPBMCs.

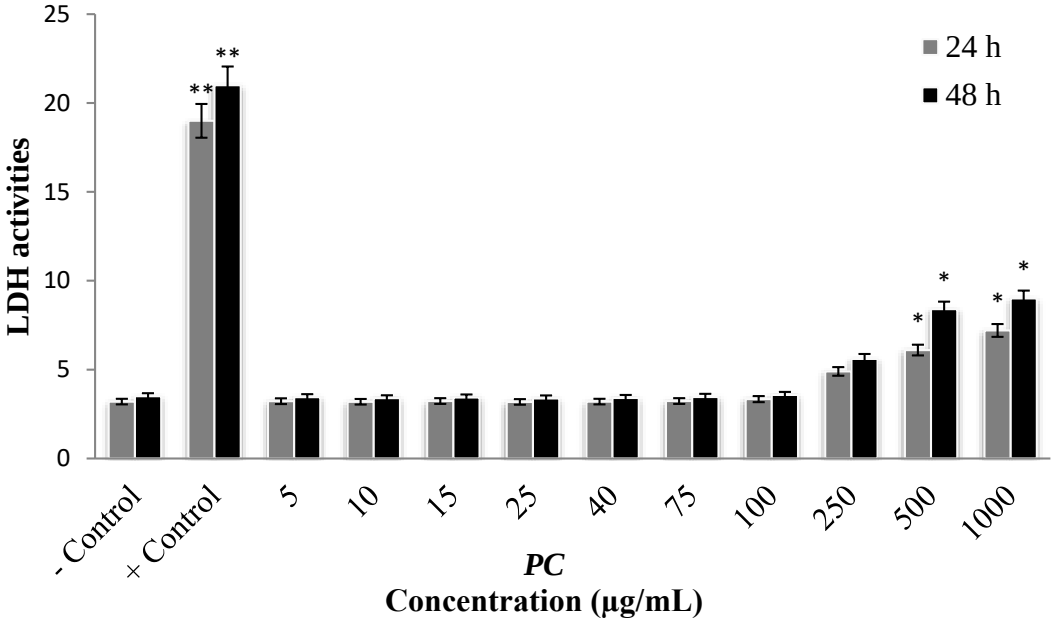

Figure 1. Lactate dehydrogenase (LDH) activity cultured hPBMCs treated with 0-1000 µg/mL of hot-water extract from mycelium of the PC for 24 and 48 hours (n = 6). *P < 0.05, **P < 0.01 vs. control. (–) Control: untreated cultures, (+) Control: 10 µM mitomycin C. The bars shown by different letters are significantly different from each other at a level of 5% (n = 6).

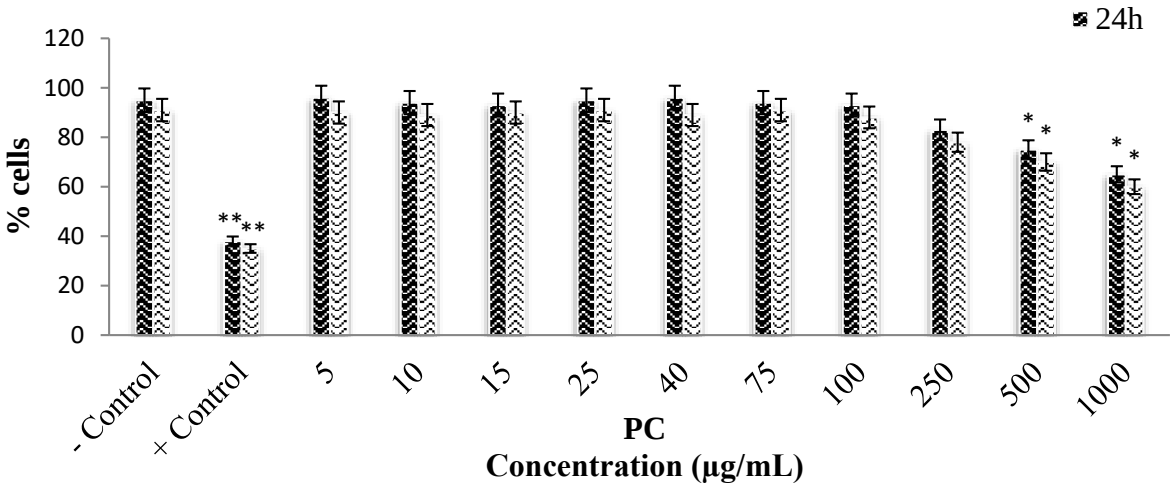

Figure 2. Effects of PC extracts on the viability of hPBMCs at different concentrations (n = 6) using neutral red (NR) assay. *P < 0.05, **P < 0.01 vs. control. (–) Control: untreated cultures, (+) Control: 10 µM mitomycin C. The bars shown by different letters are significantly different from each other at a level of 5% (n = 6).

3.4. Genotoxicity

The hot-water extract from mycelium of the PC at concentrations of 0–250 µg/ml did not affect significantly the CAs frequency, compared with the negative control (Table 2). The untreated (negative control) cultures did not show any difference, compared with the controls; this result suggested that PBS has no genotoxic effects. Positive control indicated a significant eleviation of CAs when compared to the negative control. Chromatid and chromosome breaks, sister unions, fragments, chromatid exchanges, dicentric chromosomes and polyploidies were researched in this study and any aberrations were not observed at all concentrations. The

conclusions showed that PC decreased the MI % at 500 and 1000 µg/ml doses in depending on time. On the other hand, 0-250 µg/ml concentrations did not affect the MI signicantly.The effects of PC exposure on the frequency of MN formation are shown in Table 2. MN results did not show any statistically significant differences between control and the first eightdoses of mushroom (0 - 250 µg/ml). On the contrary, the higher doses of mushroom (500 and 1000 µg/ml) caused increases of MN rates.

Table 2. Results of the CAs and the frequencies of MN in lymphocytes treated with hot-water extract from mycelium of PC.

Test Substances	Treatment	Chromosome aberrations							
	Concentration (µg/mL), 24 hours	CB	CB'	CE	DC, F, P, SU, T	CA/ Cell ± SD	Abnormal Cell % ± SD	MI % ± SD	MN / 1000 cells
-C	Untreated cultures	1	4	-	-	0.08 ± 0.03	5.60 ± 1.41	9.12 ± 1.57	6. 63
+C	10 µM MMC	8	30	2	-	0.64 ± 0.24	65.26 ± 5.86*	4.02 ± 0.51*	25.63*
Doses of PC (µg/ml)	5	1	3	-	-	0.07 ± 0.02	5.15 ± 1.15	9.01 ± 1.63	6.56
	10	1	4	-	-	0.08 ± 0.04	5.19 ± 1.1	9.46 ± 1.4	6,21
	15	1	3	-	-	0.07 ± 0.04	5.08 ± 1.32	9.65 ± 1.39	5.45
	25	1	4	-	-	0.08 ± 0.03	5.19 ± 1.22	9.18 ± 1.57	6.62
	40	1	6	-	-	0.08 ± 0.03	5.43 ± 1.4	9.41 ± 1.7	5.77
	75	1	5	-	-	0.07 ± 0.04	5.68 ± 1.2	9.33 ± 1.84	6.44
	100	1	3	-	-	0.07 ± 0.04	5.44 ± 1.4	9.15 ± 0.88	5.81
	250	1	6	-	-	0.09 ± 0.05	6.24 ± 1.91	8.79 ± 0.57	6,15
	500	2	7	1	-	0.14 ± 0.08	9.16 ± 2.35*	8.2 ± 0.81*	8,32*
	1000	3	10	1	-	0.18 ± 0.1	12.54 ± 3.24*	7.5 ± 0.76*	10.45*
	Concentration (µg/mL), 48 hours								
-C	Untreated cultures	1	6	-	-	0.9 ± 0.05	7.04 ± 2.12	8.56 ± 1.02	8,35
+C	10 µM MMC	14	45	3	-	0.86 ± 0.3	81.32 ± 7.92*	3.08 ± 0.4*	30,46*
Doses of PC(µg/ml)	5	1	6	-	-	0.08 ± 0.02	7.1 ± 1.76	8.70 ± 1.05	8,47
	10	2	4	-	-	0.08 ± 0.03	7.23 ± 1.74	8.91 ± 1.14	8,34
	15	2	5	-	-	0.08 ± 0.02	7.41 ± 1.8	8.80 ± 1.25	8,17
	25	1	5	-	-	0.09 ± 0.03	7.24 ± 1.55	8.77 ± 1.31	8,76
	40	2	4	-	-	0.08 ± 0.02	7.56 ± 1.65	8.81 ± 1.14	8,25
	75	2	4	-	-	0.09 ± 0.03	8.33 ± 2.49	8.76 ± 1.25	8,52
	100	1	5	-	-	0.09 ± 0.03	8.41 ± 2.61	8.69 ± 1.13	8.44
	250	1	5	-	-	0.1 ± 0.05	8.27 ± 2.38	8.53 ± 1.14	9,04
	500	2	8	1	-	0.17 ± 0.07	10.40 ± 2.84*	7.85 ± 1.37*	10,45*
	1000	4	11	2	-	0.25 ± 0.1	15.67 ± 3.7*	6.15 ± 0.58*	13.11*

Abbreviations: Negative control (-C), positive control (+C), mitomycin (MMC), chromosome break (CB), chromatid break (CB'), chromatid exchange (CE), dicentric (DC), fragment (F), polyploidy (P), sister union (SU), translocation (T), standard deviation (SD), mitotic index (MI). Significantly different from the negative control: *p≤ 0,05.

4. DISCUSSION AND CONCLUSION

Phenolic compounds are known as major components of antioxidant in mushrooms. These secondary metabolites also exhibit a wide range of biological effects including antimicrobial, anti-inflammatory, anti-cancer and antihyperglycemic [22; 23; 24]. This study presents the amount of phenolic and flavonoid compenents of hot water extract from mycelia of PC. The total flavonoid and phenolic content were determined as 4.6 ± 0.22 mg GAE/g extract and 1.2 ± 0.1 mg QE/g extract, respectively. According to previous studies at different species, Lee [25] reported that hot water extract from mycelia of *P. citrinopileatus* had 7.85 mg GAE/g. González-Palma [26] reported that hot water extract from mycelia of PC as 4.09 mg GAE/g and 0.192 mg QE/g. This study showed that hot water extract from mycelia of PC has the high amounts of phenolic and flavonoid compenents when compared by above studies. Many studies have confirmed that *Pleurotus* mushrooms have been valued as nutritional foods and for their medicinal features [27]. Different extracts and bio-materials extracted from oyster mushroom have been used against chronic diseases such as diabetes [28]. According to the literature, *in vitro* effects of PC extract have still not been studied on hPBMCs of healthy donors. The other aim of the present study was to explore the antioxidant/oxidant, cytotoxic and genotoxic effects of mycelia extract of PC on blood cell cultures. NR and LDH assays were used to evaluate the effects on cell viability and cytotoxicity of PC applications in cultured hPBMCs. The assesment of NR results revealed that PC did not effect to cell viability of blood cells at all doses, in a time and dose dependent, except for 250, 500 and 1000 µg/ml. Similarly, LDH leakage demonstrated that PC exhibited cytotoxic effects on human blood cells at the mentioned doses. *P. abalonus* (500 µg /ml) and *Auricular*ia *auricula-judae* (250-500 µg/ml) reduced cell viability [29]. Our results showed that the cytotoxic effect of hot water extract from mycelia of PC is in good agreement with mentioned studies. Moreover, the low doses of hot water extract of PC (0-250 µg/ml) didn't cause any changes in cell proliferation relative to control in this study.

There are several studies reported antioxidant capacity of *Pleurotus cornucopiae* [30], *P. pulmonaris* [31], *P. citrinopileatus* [32] and *P. djamor* [33]. Findings of this study were in accordance with previous studies on antioxidant properties of other edible mushrooms, since TAC method showed that treatments with mycelium extracts of PC also supported the antioxidant capability of the blood cells *in vitro*. In present study, the increasing concentrations of PC (10, 15, 25, 40, 75 and 100 µg/ml) significantly increased antioxidant capacity in blood cultures. Also, studied doses of PC did not cause an increase in oxidative stress on hPBMCs. Moreover, the applied concentrations of mushroom significantly decreased TOS levels. It is known that phenolic compounds of edible mushrooms can act as free radical scavengers, hydrogen donators and singlet oxygen quenchers [34]. On this context, phenolic and flavonoid-rich hot water extract from mycelia of PC could decrease oxidative stress and protect lymphocytes and monocytes against reactive oxygen species. It is reported that ROS generation decreases mitochondrial membrane potential and increases LDH release due to membrane distribution [35]. In our study, the high doses of PC 500 and 1000 µg/ml) increased TOS levels and also induced cell injury in a dose-dependent manner, as above indicated by decreased cell viability and increased LDH release.

The MN and CAs could be used to determine the genotoxic effects of the test substances according to previous studies [36; 37]. We have determined the genotoxic effect of PC on hPBMCs using MN and CAs assays. This study is the first reported that PC (0–250 µg/ml) has no genotoxic or mutagenic effects on hPBMCs since the observed mean values of the frequency of CAs and MN per cell was not found significantly different from values of the untreated cultures on both cells. In parallel to our findings, a recent study has revealed that edible mushroom such as ethanol extract of *Agaricus braziliensis* (50 µg/ml) has no genotoxic effects

[38]. However, the high doses of ethanol extract of this mushroom (250-500 µg/ml) have slightly genotoxic effects on human lymphocytes. As it is supported by this study, the high doses of PC (500 and 1000 µg/ml) caused genotoxic effects.

In conclusion, these findings provide evidence on the lack of oxidant/antioxidant status and cyto-genotoxicity of hot water extract from mycelia PC under our *in vitro* conditions. As a result of this study revealed that PC at low doses (0-250 µg/ml) on human blood lymphocytes is not genotoxic and also significantly increases the antioxidant capacity of blood. On the other hand, based upon our results, we conclude that PC, if used at higher concentrations (500 and 1000 µg/ml) have cytotoxic and genotoxic effects and cell damage as due to increased oxidative stress. Hence, the overconsumption of this traditional edible mushroom should be considered. Otherwise, it may cause serious toxic side effects in terms of human health.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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