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Pleurotus ostreatus (Jacq.) P. Kumm. Extract Alters the Expression of Some Apoptosis Related Genes

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Abstract: Mushrooms have been used for food and medicinal purposes since ancient times. Especially mushrooms with therapeutic effects attract the attention of many research groups. Besides, it is thought that the active compounds derived from fungi could potentially be a valuable source of new anticancer agents. This study aims to evaluate the effects of methanolic extract of *Pleurotus ostreatus* (Jacq.) P. Kumm. on the expression levels of some genes important in the intrinsic pathway in apoptosis. For this purpose, after the mushroom samples were dried without sunlight, extracts were prepared via Soxhlet apparatus by using methanol. Cytotoxic effects of the extracts were evaluated with the MTT test. Real-time PCR was performed to evaluate the expression levels of the four apoptotic genes (*Hrk*, *Bax*, *Apaf1* and *casp3*). The results of the MTT assay showed that the extracts obtained show a cytotoxic effect in a dose and time-dependent manner. Also, methanolic extracts from *P. ostreatus* were found to cause upregulation in expression levels of genes which related apoptotic cell death. In conclusion, this study shows that *P. ostreatus* has a potential therapeutic effect on colorectal cancer and is compatible with other studies of different types of cancer and cell lines. This study is a pioneering study for future studies that will continue to identify the active substances in the extract and find the molecular pathways of cell death.

Key words: cDNA, MTT, RT-PCR, Oyster mushroom, Turkey.

Pleurotus ostreatus (Jacq.) P. Kumm. Ekstraktı Bazı Apoptotik Genlerin İfadesini Değiştirir

Öz: Mantarlar, eski çağlardan beri yiyecek ve tıbbi amaçlarla kullanılmıştır. Özellikle tedavi edici etkisi olan mantarlar birçok araştırma grubunun ilgisini çekmektedir. Aynı zamanda, mantarlardan türetilen aktif bileşiklerin potansiyel olarak yeni antikanser ajanların değerli bir kaynağı olabileceği düşünülmektedir. Bu çalışmanın amacı, *Pleurotus ostreatus* (Jacq.) P. Kumm'un metanolik ekstraktının apoptozda intrinsik yolda önemli olan bazı gen bölgelerinin ekspresyon seviyeleri üzerindeki etkilerini değerlendirmektir. Bu amaçla mantar örnekleri güneş ışığı görmeden kurutulduktan sonra Soxhlet cihazı ile metanol kullanılarak ekstraktlar hazırlandı. Ekstrelerin sitotoksik etkileri MTT testi ile değerlendirildi. Gerçek zamanlı PCR ile, dört apoptotik gen bölgesinin (*Hrk*, *Bax*, *Apaf1* ve *casp3*) ekspresyon seviyelerini değerlendirildi. MTT testinin sonuçları, elde edilen ekstrelerin doza ve zamana bağlı bir şekilde sitotoksik bir etki gösterdiğini göstermiştir. Bununla birlikte *P.ostreatus*'tan elde edilen metanolik özütlerin apoptotik hücre ölümüyle ilişkili gen bölgelerinin ekspresyon seviyelerinde upregülasyona neden olduğu bulundu. Sonuç olarak, bu çalışma ile, *P. ostreatus*'un kolorektal kanser üzerinde potansiyel bir terapötik etkiye sahip olduğunu ve bu durumun farklı kanser türleri ve hücre dizileri ile ilgili diğer çalışmalarla uyumlu olduğunu göstermektedir. Bu çalışma, ekstraktaki aktif maddeleri tanımlamaya ve hücre ölümünün moleküler yollarını bulmaya devam edecek gelecekteki çalışmalar için öncü bir çalışmadır.

Anahtar kelimeler: cDNA, MTT, RT-PCR, Kavak Mantarı, Türkiye.

Introduction

Nature has been an important material and source of inspiration for medicine since ancient times. Throughout evolution, it produces, among other things related to cancer treatment, a wide variety of biologically active substances with therapeutic potential (Blagodatski et al. 2018). Recently, mushrooms have come to the fore as an excellent antiinflammatory, antioxidant, antidiabetic, anticancer, antimicrobial, prebiotic and immunomodulatory resources (Barros et al. 2007; Kim et al. 2007; Sarıkurkcu et al. 2008; Synytsya et al. 2009).

Cancer is one of many types of diseases that cause death and can occur in humans regardless of age group, gender or race. Current anticancer drugs on the market highlight the urgent need for new, effective and less toxic therapeutic approaches, leading to various side effects and complications in the clinical management of various types of cancer. In this context, the fungal treatment of cancer is a hopeful scientific area dealing with antitumor agents produced from fungi and has been a complementary part of traditional medicine since ancient times (Xu et al. 2012). *P. ostreatus* is one of the medicinal mushrooms and has various benefits such as antioxidant, anticancer, blood pressure lowering and cholesterol (Wasser, 2002; Lindequist et al. 2005; Fan et al. 2006). Various studies have demonstrated the antiproliferative and proapoptotic effects of *P. ostreatus* on leukaemia, breast, cervical, colon and prostate cancer cell lines (Lavi et al. 2006; Gu &Sivam, 2006; Polyakov et al. 2007; Jedinak and Sliva, 2008; Ekowati et al. 2017).

Apoptosis is programmed cell death and the applied extract or active substance is desired/expected to direct the cancer cells to programmed cell death. This process is controlled by intracellular and extracellular pathways. The Bcl-2 family is the oncoprotein group, which consists of antiapoptotic and proapoptotic members and has the most important role in regulating apoptosis (Altunkaynak & Özbek 2008). Bcl-2 family consists of two groups with opposite effects. One of these groups is antiapoptotic (such as Bcl-XL and Bcl-2), the other is pro-apoptotic (such as Hrk, Bid, Bax, Apaf1, Puma and Noxa). The sensitivity of cells to apoptotic stimulation depends on the balance between pro-apoptotic and antiapoptotic Bcl-2 proteins (Burlacu, 2003). The aim of this study was to evaluate the effects of *P. ostreatus* methanolic extract on expression levels of Hrk, Bax, Apaf1 and casp3 genes, which are important in the intrinsic pathway in apoptosis, on the DLD1 cell line.

Material and Method

Material

In our study, the studied mushroom samples were provided by Dr Sinan AKTAŞ. Samples were dried without sunlight at room temperature. The dried material was pulverized and prepared for extraction process.

Preparation of extracts

P. ostreatus were extracted in absolute methanol for 6-8h via Soxhlet apparatus then the extract was filtered. The extract was concentrated using a rotary evaporator. The extracts were stored at -20 °C until use. The extract coded as POE.

Cell line and culture

DLD1 (human colorectal adenocarcinoma) cells were obtained from ATCC and maintained in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1 % (v/v) penicillin-streptomycin. Cells were incubated at 37 °C under 5 % CO_2 conditions.

Cell Viability Assay

Cell viability was determined via MTT assay. Cells were seeded into a 96-well plate and treated with *P. ostreatus* extracts (0–5 mg/ml) and then the plates were incubated for 24 -48 hours. The optical density of the plates was measured using the Elisa microplate reader at 540 nm. Each experiment was performed three times and mean values were taken into consideration.

RNA isolation and Real-Time PCR

The DLD1cells (1 × 10^6 cells/mL) were seeded in six-well plates and treated with different concentrations of *P. ostreatus* extracts (0-1mg/ml) for 24h at 37°C and 5% CO_2. Total RNA was isolated using Bio-Rad Aurum Total RNA Isolation kit and cDNA was synthesized using Bio-Rad cDNA synthesis kit. The expression levels of apoptotic genes (*Hrk*, *Bax*, *Apaf1* and *casp3*) were evaluated via Real-Time PCR. Amplifications were

performed using the Bio-Rad CFX Connect system. The data were analysed by the comparative CT method and the fold change was calculated by $2^{-\Delta\Delta Ct}$ method. To confirm the specificity of PCR products melting curve analysis was performed. Results were determined by three independent experiments and graphs were created using mean values.

Evaluation of data

For evaluation of the data, multiple comparisons were made using one-way variance analysis followed by Dunnett's test for post hoc analysis. The differences in $p < 0.05$were considered statistically significant.

Results

Cytotoxicity results

In this study, the cytotoxic activity of *P. ostreatus* methanol extracts were determined by using MTT test in DLD1 cell line exposed to 0.3125-5 mg/ml extracts in two 24- and 48-hours incubation periods. Cell survival analysis showed that *P. ostreatus* extract caused cell death of DLD1 cells in a dose and time-dependent manner. The graphics of the MTT assay were given in **Figure.1.** After 48 hours incubation, the extract showed the most cytotoxic activity at the highest concentration (5000 µg / ml) with 98% inhibition of cell growth. The IC_{50} dose for 48h was calculated as 1263 µg /ml. These results show that methanolic extracts of *P. ostreatus* have a cytotoxic effect on colon cancer cells and suppress the proliferation.

Gene expression results

The mRNA levels of four genes involved in different steps of apoptosis were studied by Real-Time PCR. Gene expression changes were given in **Table 1** and **Figure 2**. First, when the expression level of the Hrk gene located at the beginning of apoptosis was evaluated, no difference was observed at low doses compared to the control, while a 1.2 and 2-fold increase was observed at 0.5mg and 1mg / ml doses, respectively. Another gene, Bax, was evaluated, a concentration-dependent increase in Bax gene expression was observed at all doses administered. Bax expression increased 2.41-7.54-fold in the applied dose range. Looking at the Apaf1 gene expression, it was found that the Apaf1 gene expression reached its highest value at a dose of 0.5 mg/ml and this increase was approximately 6.8-fold. Finally, when the casp3 gene expression in the last step of apoptosis was evaluated, a concentration-dependent increase was observed like in the Bax gene expression. This increase was calculated as 1.35-4.05-fold respectively. In general, we can clearly say that POE induces apoptosis-related gene expressions and the optimum concentration for gene expressions is 0.5mg / ml.

Table.1. Gene expression fold changes by $2^{-\Delta\Delta Ct}$ calculation (st: stable)

POE extract conc.	Gene expression fold change			
	Hrk	**Bax**	**Apaf1**	**casp3**
1mg/ml	2.05	7.54	2.31	4.05
0.5mg/ml	1.2	6.38	6.81	2.5
0.25mg/ml	st	2.78	st	2.18
0.125mg/ml	st	2.41	st	1.35

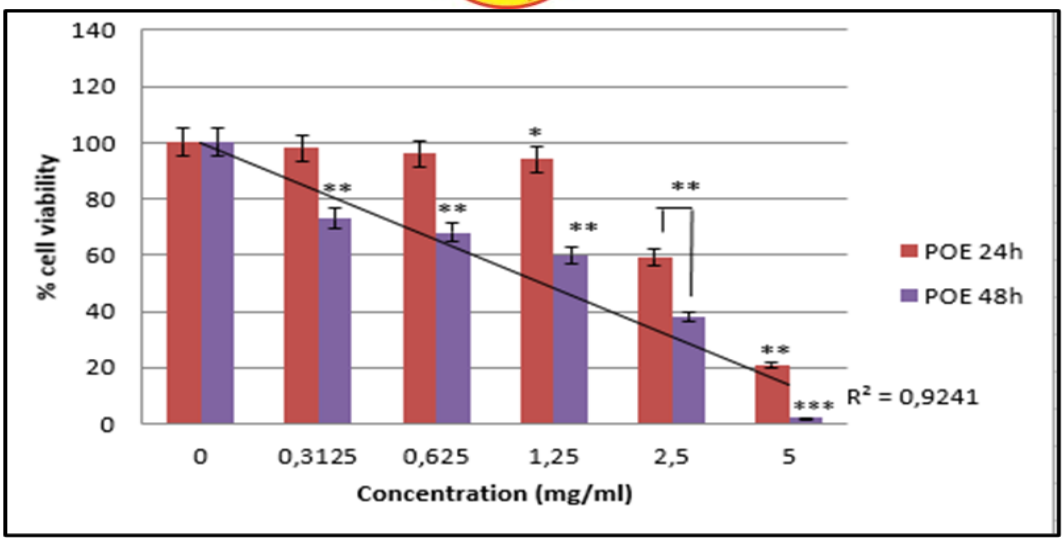

Figure 1. The viability plot based on MTT assay results (*p <0.05, **p <0.01 and ***p <0.001)

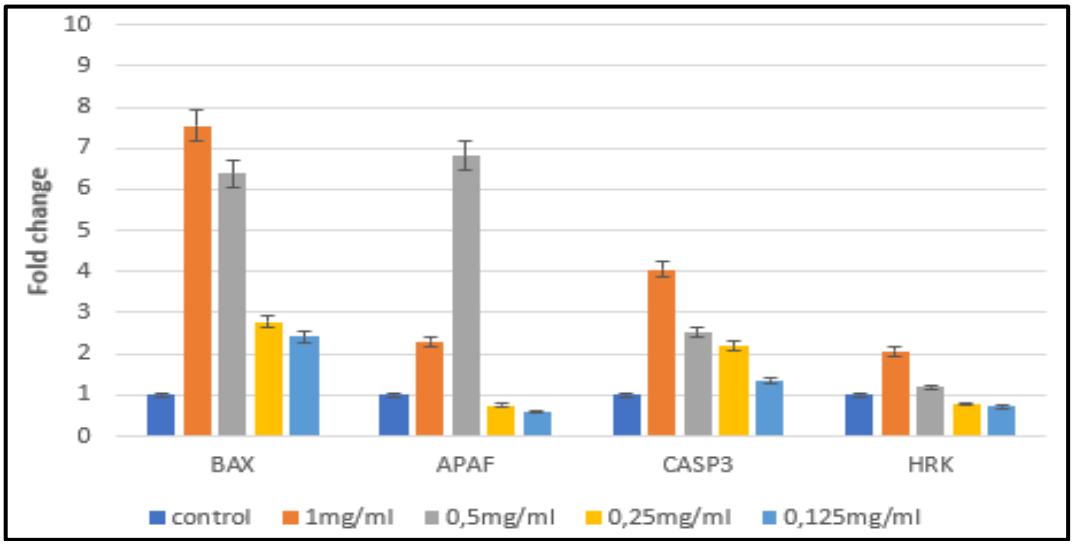

Figure 2. Relative expression levels of apoptosis-related genes

Discussion

Triggering apoptosis in cancer cells is a valuable therapy and it has been recognized that natural substances derived from various sources can induce apoptosis in various tumour cells.In this context, mushrooms play a very important role as antitumour agents. Edible mushrooms have been used for several centuries as healthy nutritional supplements and as a complementary therapy in chemotherapy and radiation therapy against cancer side effects (Mizuno et al. 1995). This study aims to determine the effects of methanolic extract of *P. ostreatus* on the expression levels of some genes that are important in the intrinsic pathway in apoptosis. POE significantly induces the expression of the four apoptotic genes in a concentration-dependent manner. Our results show that the treatment of cancer cells with POE leads to changes in the expression pattern of genes associated with apoptosis.

Expression changes in pro-apoptotic Bcl-2 members and mitochondrial status of tumour cells are an important indicator of their response (Czabotar et al. 2014; Montero et al. 2015). Hrk is an important component in the regulation of apoptosis in tumor cells, contains BH3 and also regulates apoptosis by interfering with antiapoptotic Bcl-xL and Bcl-2 proteins and blocking their functions (Inohara et al.1997). In this study, POE upregulated Hrk expressions compared to untreated cells. This may indicate that Bcl-2 gene expression is suppressed. It is

desirable that the Bax / Bcl-2 ratio, which is the key to apoptosis, shifts to Bax. Bax is a pro-apoptotic gene homologous to Bcl-2 (Oltvai et al. 1993). However, Bax works as an apoptosis enhancer unlike Bcl-2, which has antiapoptotic properties. In our study, Bax expression levels increased depending on the concentration, indicating that apoptosis was triggered in the intrinsic pathway and correlated with Hrk gene expression. In previous studies, it has been reported that extracts obtained from *P. ostreatus* using different solvents increase Bax expression, but the cell lines used (HT-29, COLO-205 and KG-1) and the preparation method of the extracts are different, our results are consistent with these studies in terms of triggering Bax expression (Lavi et al. 2006; Arora &Tandon 2015; Ebrahimi et al. 2018). In the mitochondrial pathway of apoptosis, the main soluble receptor is Apoptotic protease-activating factor 1 (Apaf1) (Fulda& Debatin 2006). Apaf1 is the central component of the apoptosome, which activates procaspase-9 following cytochrome c release from mitochondria in the intrinsic pathway of apoptosis (Gortat et al 2015). In our study, Apaf1 expression was upregulated, indicating that the apoptotic process is irreversible. The last gene caspase3 is a ruling caspase that is activated by the promoter caspases and impairs the survival and integrity of proteins. Death signals lead to the establishment of the Bcl-2 family of apoptosis proteins, particularly Bax. These proteins cause cytochrome c to be released out of the mitochondria. The release of mitochondrial cytochrome c into the cytoplasm and its subsequent association with the Apaf-1 protein is thought to be an absolute requirement for the activation of caspase-9, the apical caspase in the mitochondrial pathway of apoptosis (Nagata 2000; Spanos et al. 2002). According to the results of this study, the caspase3 expression up-regulated concentration-dependent manner. This suggests that POE reduces mitochondrial membrane potential and increases caspase-3 gene expression. As a general conclusion, when analysing the results of RT-PCR, it was found that the methanolic extracts from *P.ostreatus* caused an increase in the expression levels of the genethat regulate apoptosis at four different apoptotic stages. Although this study reveals differences at the gene level, future studies at protein level are needed. Many clinical studies are confirming the efficacy of fungi and their extracts from fungi as components of modern anticancer therapy. However, the complex mechanisms of action and molecular pathways and the precise structures of the active ingredients obtained from these fungi still need to be studied in more detail. As a conclusion, although strong progress has been made in the field of medicinal mushroom research in anticancer drug development, more research is needed in this area. Our studies will focus on elucidating the molecular targets of medicinal mushrooms, gene expression as well as changes in protein level and determination of active substance or substances.

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