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ORIGINAL ARTICLE

Extract of *Calvatia gigantea* inhibits proliferation of A549 human lung cancer cells

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Abstract In this study, in order to investigate the anticancer mechanism of *Calvatia gigantea* extract, edible mushroom species, which belong to Lycoperdaceae family, changes of CCND1, CCND2, CDK4, p21, Akt, Bax, Bcl-2, p53, caspase-3 and caspase-9 were evaluated in A549 lung cancer cells. Cytotoxic effect of *C. gigantea* extract was evaluated by using XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5 carboxanilide). The *C. gigantea* extract was treated in a time and dose dependent manner within the range 25 μg/ml–2 mg/ml to determine the IC_{50} dose. IC_{50} dose for *C. gigantea* extract was detected as 500 μg/ml for 72 h. According to expression results, while CCND1, CCND2, CDK4, Akt and Bcl-2 expression clearly decreased, *Bax, p53, caspase-3* and *caspase-9* expression clearly increased in the dose group cells (A549 cells treated with 500 μg/ml dose of *C. gigantea* extract for 72 h). However, there was no change in p21 expression. *C. gigantea* extract induced cell cycle arrest and apoptosis by decreasing the CCND1, CCND2, CDK4, Akt and Bcl-2 expression and by increasing Bax, p53, caspase-3 and caspase-9 expression in A549 cells. Mushrooms are eukaryotic organisms heavily used because of their supposedly anticancer effect. Many mushroom species have been used for medical purposes, as a result of also having many effects such as antibiotic, antiviral and anticancer effects. It is thought that the *C. gigantea* extract may be a significant agent for treatment of lung cancer as a single agent or in combination with other drugs.

Keywords Apoptosis · *Calvatia gigantea* · Cell cycle · Lung cancer cell line

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Introduction

Cancer is caused by genetic changes following the failure of homeostatic mechanisms. Most chemotherapeutic agents are used for the treatment of cancer. These agents can destroy tumors and arrest cancer progress to some extent, but they may damage healthy cells and tissues. Thus, new anticancer drugs obtained from natural products are thought to be less toxic and safer agents to inhibit cancer (Hann et al. 2002). Increasing cancer-associated molecular advances lead to less toxic treatments. Dysregulation in molecular signaling is usually associated with altered cell growth, cell cycle progression, and corrupted apoptotic responses in cancer. The dysregulation in

signaling pathways results from an overexpression and loss/gain of functional mutations in growth factor receptors and/or in regulatory proteins (Yamasaki et al. 2007). In recent years, proto-oncogenes and tumor-suppressor genes have been used as markers in the diagnosis of cancer. Apoptosis is the process of programmed cell death and includes features such as morphological changes, chromatin condensation and caspases activation, a family of cysteine-aspartic acid proteases (Taylor et al. 2008). Cell-cycle progression in eukaryotes depends on consecutive activation of cyclin dependent kinase (CDKs), whose activation is related to cyclin (Pan et al. 2002). CDKs bind to cyclin at specific intervals during cell cycle progression, and these complexes are activated (Ahmad et al. 2001). Cyclin D1 is overexpressed as a result of dysregulated growth factors or gene amplification in many types of cancers (Chen et al. 2008).

Lung cancer is the most common cancer type for both men and women in the world. Lung cancer also has a high incidence of recurrence and is difficult to treat (Khuri et al. 2001; Li et al. 2004; Molina et al. 2006). Non-small cell lung cancer (NSCLC) accounts for 75–80 % of total lung cancer. Despite the fact that lung cancer treatment methods are constantly improving, this cancer still ranks as number one cause for cancer related deaths (Siegel et al. 2014). Most common method of treatment used for patients with advanced NSCLC is either cisplatin or platinum-based chemotherapy. However, these drugs have extremely toxic effects with low survival profiles (De Petris et al. 2006).

Approximately 2000 mushrooms species are safe for human consumption as food and approximately 650 of these mushrooms have medicinal characteristics (Rai et al. 2005). Medicinal mushrooms constitute a large group of organisms that are heavily used because of antiviral, antimicrobial, anti-inflammatory, antihyperglycemic, and anticancer effects (Jiao et al. 2013). Mushrooms are heavily used because of their supposedly anticancer effect. *C. gigantea* belonging to the Lycoperdaceae family is an edible mushroom species. It was reported that *C. gigantea*, which is one of the puffballs, could be used in cases of ambustion due to its anesthetic effect. Puffball, belonging to Agaricomycetidae, Agaricales, Lycoperdaceae, is a giant-type fungus. It was shown in several studies that many active compounds were found in puffball (Kahlos et al. 1989; Nam et al. 2001). Many studies demonstrated that some proteins and peptides isolated from the puffball have anti-tumor activity. Anticancer factor calvacin was first isolated from *C. gigantea*, which belongs to the basidiomycota phylum and which has also long been used as a medicinal food, by Roland et al. (1960).

In a study, it was reported that CULP protein, isolated from *C. caelata,* had an anti-proliferative effect on breast cancer cells (Lam et al. 2001). Moreover, it was reported that some chemicals or biological agents in mushrooms inhibit cancer progression by inducing cell cycle arrest and apoptosis (Hseu et al. 2014).

In this study, we aimed to investigate the anticancer mechanism of *C. gigantea* extract by evaluating the expression chances of CCND1, CCND2, CDK4, p21, Akt, Bax, Bcl-2, p53, caspase-3 and caspase-9 in A549 human lung cancer cell line. We also demonstrated that the *C. gigantea* extract could down regulate the expression of CCND1, CCND2, CDK4, Akt and Bcl-2, and upregulate expression of Bax, p53, caspase-3 and caspase-9.

Materials and methods

Macrofungi material and preparation of *C. gigantea* extract

Fruit bodies of the *C. gigantea* samples were collected in the forest around Gireniz Valley, located in the western part of Denizli, Turkey during the field works between 2010 and 2012 years. Identification and classification of macrofungus were carried out at the laboratory of the Mushroom Research and Application Center, Pamukkale University, Denizli, Turkey.

Fresh mushrooms were randomly divided into three samples of 75 g and then dried in an oven at 40 ± 0.1 °C before analysis. Approximately 25 g of the dried mushroom samples were weighed and then extracted by stirring with 250 ml of methanol at room temperature at 150 rpm for 24 h and filtering through Whatman No. 4 filter paper. The residue was then, extracted with two additional 250 ml of methanol as described above. The combined methanolic extracts were then rotary evaporated at 40 ± 0.1 °C to obtain dry extract, re-dissolved in methanol to a concentration of 10 mg/ml and stored at 4 °C for further use.

Cell culture and chemicals

A549 human lung cancer cell line (obtained from ATCC, Manassas, VA, USA) was used in this study. Trizol reagent was purchased from Invitrogen

(Carlsbad, CA, USA). The following chemicals were purchased from Biological Industries (Kibbutz, Beit Haemek, Israel): DMEM medium, penicillin/streptomycin mixture, phosphate buffered saline (PBS), heat-inactivated fetal bovine serum (FBS), XTT reagent-cell proliferation kit. Transcriptor first strand cDNA synthesis kit was purchased from Roche Diagnostics (Mannheim, Germany). Trypan blue solution, sodium dodecyl sulfate (SDS), 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS) was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

A549 cells were grown in DMEM medium supplemented with 2 mM L-glutamine, penicillin (20 units/ml), streptomycin (20 μg/ml), and 10 % (vol/vol) heat-inactivated fetal bovine serum at 37 °C in a saturated humidity atmosphere containing 95 % air and 5 % CO_2. A549 cells were treated with 25, 50, 100,150, 200, 250, 300, 400, 500, 750 μg/ml, 1 and 2 mg/ml *C. gigantea* extract during 72 h, considering a time and dose dependent manner.

Cytotoxicity assay

Cytotoxicity assays and determination of IC_{50} dose of *C. gigantea* extract in A549 human lung cancer cells were performed by using trypan blue dye exclusion test and XTT assay as indicated in the manufacturers' instruction. Cells were seeded in 96-well tissue plates at a number of 1×10^4 cells/well and incubated for 24 h without reagent. After 24 h of incubation, the cells were treated with various concentrations (0–2 mg/ml) of extract of *C. gigantea* and incubated for 24, 48 and 72 h. After addition of reagents, cell viability was assessed by using XTT mixture (0, 1 ml activation solution and 5 ml XTT reagent) as recommended by the supplier. Formazan formation was quantified spectrophotometrically at 450 nm (reference wavelength 620 nm) using a microplate reader. The experiment was repeated three times. Viability was calculated using the background-corrected absorbance as follows:

$$\text{Viability } (\%) = \text{absorbance value of experiment well} / \text{absorbance value of control well} \times 100$$

RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated from the cells exposed to IC_{50} dose (500 μg/ml for 72 h) of *C. gigantea* extract in 500 μl Trizol Reagent according to the manufacturer's instructions. The first-strand cDNA synthesis was performed by using anchored-oligo (dT) primer and protector RNase inhibitor according to the manufacturer's protocol (Roche Diagnostics). Appropriate cycles were chosen to ensure the termination of PCR amplification.

Semi-quantitative PCR products were analyzed by 2 % agarose gel electrophoresis and visualized by ethidium bromide staining and photographed under u.v. light. In this study, *CCND1, CCND2, CDK4, p21* and *Akt* gene expressions were presented with the comparison of the yield of PCR products with the housekeeping gene *GAPDH*. The experiment was repeated three times.

Western blot analysis

The cells were washed with PBS and then were lysed under ice-cold condition. Briefly, total protein was extracted from A549 cells and the dose group using RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % SDS) supplemented with protease inhibitor cocktails. Protein samples (100 μg protein) were separated on 8.5 % polyacrylamide gels. Proteins were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA), and bands were visualized and recorded using GelQuant image analysis software in a DNR LightBIS Pro image analysis system (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel). The experiment was repeated three times.

Statistical analysis

Analyses have been evaluated using the ''Student t test'' with the SPSS 17.0 statistical analysis program.

Results

Anti-proliferative effect of *C. gigantea* extract in A549 cell line

A549 cell death upon treatment with *C. gigantea* extract was assessed using the XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2h-tetrazolium-5 carboxanilide) assay. A cells dose-dependent decrease in the

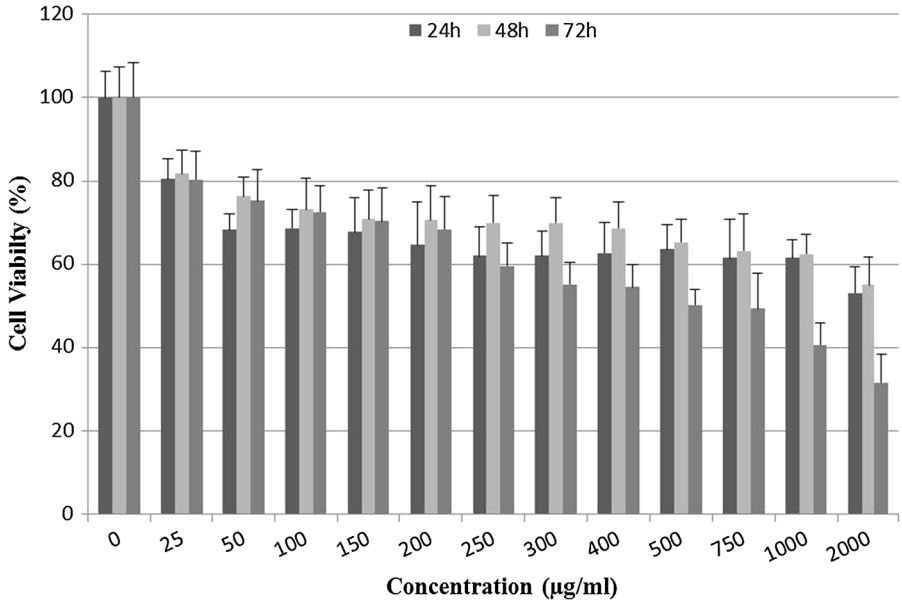

Fig. 1 Concentration and time dependent inhibitory effects of *C. gigantea* extract on cell viability in A549 cells. Cell viability was determined by XTT assay and was reported as the percentage of viable cells relative to the control. Data are the average results of three independent experiments and expressed as mean ± SD

susceptibility of A549 cells was found. In this study, IC_{50} dose of the *C. gigantea* extract in the A549 cells was found to be 500 µg/ml of the 72nd hour (Fig. 1).

Semi-quantitative RT-PCR

In this study, the expression changes of the *CCND1, CCND2, CDK4, p21* and *Akt* genes were evaluated by using semi-quantitative PCR method for A549 cells exposed to *C. gigantea* extract. mRNA levels were analyzed in cells treated with *C. gigantea* extract and this was compared with the levels of mRNA in untreated cells. While *CCND1, CCND2, CDK4* and *Akt* gene expression decreased in the dose group, there were no expression changes in the p21 gene compared with control cells (Fig. 2).

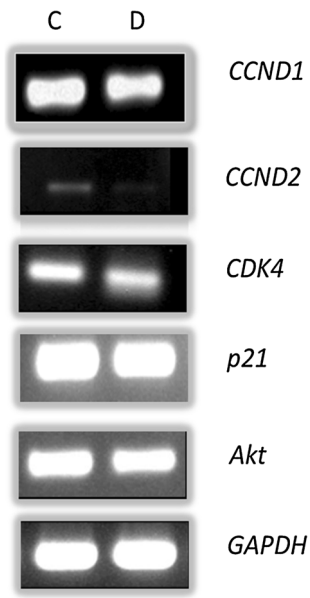

Fig. 2 mRNA expression changes in control and dose groups (500 µg/ml for 72 h). *CCND1, CCND2, CDK4* and *Akt* gene expression clearly decreased in the dose group cells. However, there was no change in p21 expression. (*C* control group, *D* dose group)

Western blot analysis

Based on the results obtained by semi-quantitative PCR, we analyzed CDK4, Akt, Bax, Bcl-2, p53, caspase-3 and caspase-9 protein expression in cells treated with the *C. gigantea* extract and compared these results with the levels of proteins in untreated cells. These protein changes were determined by Western blot analysis. Western blot analysis results indicated that CDK4, Akt and Bcl-2 protein expression decreased and pro-apoptotic protein Bax and cell cycle checkpoint protein p53 expression increased in

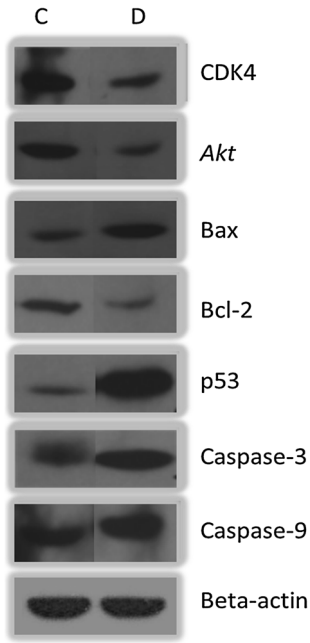

Fig. 3 Effect of *C. gigantea* extract (500 µg/ml for 72 h) on the expression of CDK4, Akt, Bax, Bcl-2, p53, Caspase-3 and Caspase-9 proteins in A549 cells. Western blot was performed to detect the protein expressions (*C* control group, *D* dose group)

the dose group after 72 h of incubation with *C. gigantea* extract (Fig. 3).

Discussion

Several studies reported that different mushroom extracts have antitumoral activity. Furthermore, several studies related to antibacterial and antioxidant activities of *C. gigantea* were conducted. Ren et al. showed that DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging percentages of *C. gigantea* extract was less than 40 % at 10 mg/ml (Ren et al. 2014). There are studies that are related to the effects of individual compounds (such as calvacin) of *C. gigantea* in cancer cells (Roland et al. 1960). However, there is no information on effects of *C. gigantea* extract in cancer cells within our research. The present study is important in terms of analysing the effect of *C. gigantea* extract in A549 cells.

Cancer continues to be one of the highest death cases among various diseases. Many different types of anti-cancer drugs have been developed for clinical use against many different types of cancer, however, cancer chemotherapy can cause adverse effects, which can even be a cause of death. For instance, drugs like sorafenib and sunitinib which are tyrosine kinase inhibitors are associated with cardio toxicity (Chintalgattu et al. 2013). At the same time, the cure rate of chemotherapy depends on the development of drug resistance (Meng et al. 2014). Cancer chemotherapeutic agents may change the regulation of cell cycle during different processes of the cell cycle, thereby it causes a decrease in proliferation and induction of apoptosis in cancer cell. The fact that natural compounds are an alternative approach for the treatment of cancer has been highlighted in recent studies (Surh 2003). Some studies reported that some of macrofungi had a strong antibiotic effect and they should be consumed for health reasons (Jayakumar et al. 2006; Solak et al. 2006). Furthermore, it was reported that extracts obtained from these macrofungi slowed down the development of cancer and tumor cells and some of them completely stop development of cancer and tumor cells (Sarangı et al. 2006; Kurashıge et al. 1997; Wang et al. 1996).

Meng et al. reported that White-line-inducing principle (WLIP), a lipodepsipeptide isolated from *Lasiosphaera fenzlii* Reich that belongs to the same family as *C. gigantea* inhibited the growth of K562 human leukemia cells by inducing cell cycle arrest through PPAR-γ (peroxisome proliferator-activated receptor) (Meng et al. 2014). In another study, it was indicated that the ubiquitin-like peptide isolated from *C. caelata* had a cytotoxic effect on human breast carcinoma (MDA-MB-231) cells (IC_{50} = 100 nM) (Lam et al. 2001). It was pointed out that *Calvacia lilacina* (Fam. Lycoperdaceae) affected cell viability by inducing many apoptosis-related events, such as mitochondrial transmembrane potential reduction, mitochondrial cytochrome c release, Bax over-expression, glutathione depletion, and reactive oxygen species (ROS) production in several human colon cancer cell lines, such as SW480, Colo250, Colo320DM, and DLD-1 cells. It was indicated that the concentration of 75–150 µg/ml of *Calvatia lilacina* could induce marked apoptosis in SW480 cells (Tsay et al. 2009). It is known that calvacin obtained from *C. gigantea* has anticancer effect. Calvacin is a compound purified from an aqueous extract from the fruit bodies of *C. gigantea*. It was reported that this substance had antitumor effects on experimental animals (Roland et al. 1960). However,

the anticancer mechanism of the *C. gigantea* extract is still unclear. We found that *C. gigantea* extract might affect the expression of genes of the anti- and pro-apoptotic super family.

Activation of CDKs and cyclins plays an important role in cell cycle progression by leading to complex formation (Bloom and Cross 2007). Cyclin D is a member of the cyclin protein family which plays a key role in regulating cell cycle progression. The synthesis of cyclin D starts at G1 stage and regulates the G1/S phase transition. Cylin—CDK complexes have a sequential role in cell cycle and most of these active oncogenes are inhibited by tumor suppressors. p21 is a potent cyclin-dependent kinase inhibitor, and thus functions as a regulator of cell cycle progression at G_1 and S phase (Chen et al. 2015).

Activation of apoptosis occurs via a separate mitochondrial and/or death receptor signalling cascade (Hellwig et al. 2011). Mitochondrial dysfunction due to potential releasing of cytochrome c into the cytosol from the mitochondria is important in apoptosis (Fulda and Debatin 2006). The release of cytochrome c is associated with Bax and Bcl-2, and it causes the activation of caspase-9 and caspase-3, which are closely related to apoptosis (Miyazawa et al. 2008). Procaspase-9 is activated by cytosolic cytochrome c, and this leads to the activation of caspase-3, and finally apoptosis occurs (Jiang and Wang 2004). In the present study, we found that *C. gigantea* could induce caspase-3 and caspase-9 activation in A549 cells. It is thought that the Bcl-2 gene family contains a number of anti-apoptotic proteins that play an important role in resistance against traditional cancer treatments. Pro-apoptotic proteins such as Bax, Bak and Bad are members of the same gene family and have critical importance as they cause apoptosis (Huang et al. 2012).

In the present study, cytotoxic effects of *C. gigantea* extract in A549 cells were detected in a time and dose dependent manner by using XTT and the IC_{50} dose of *C. gigantea* extract in A549 cells was found to be 500 µg/ml for the 72nd hour. In this study, we demonstrated that the treatment with *C. gigantea* can decrease CCND1, CCND2, CDK4, Akt *and* Bcl-2 expression levels and can increase Bax, p53, caspase-3 and caspase-9 expression level, suggesting that the *C. gigantea* extract can induce cell cycle arrest and apoptosis in A549 cell.

In conclusion, *C. gigantea* may inhibit the proliferation of human lung cancer cells by downregulating the *CCND1, CCND2, Akt* and *CDK4* genes that play a significant role in cell cycle arrest in G1/S phase. At the same time, *C. gigantea* extract induces apoptosis by increasing Bax, p53, caspase-3 and caspase-9 that have a role in apoptosis, and decreasing antiapoptotic protein Bcl-2 expression. Finally, *C. gigantea* extract may be a significant agent for treatment of lung cancer as a single agent. Thus more studies should be designed to find a safe dose with the best effects of the *C. gigantea*.

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