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Cytotoxic, Apoptic and Necrotics Effects of *Agaricus sylvaticus* Schaeff on Breast Cancer Cells Mcf-7 and L929

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Abstract

In this study, cytotoxicity, apoptotic and necrotic effects of *Agaricus sylvaticus* Schaeff were investigated. The tissue fragments from taken dry basidiocarps were developed by tissue culture method in malt extract agar. Primer and secondary mycelium were obtained by incubation at 27 °C, darkness for 10 days, respectively. Three pellets were taken from the secondary mycelium and its development completed in 7 days at 140 rpm at 27 °C in nutrient broth. The samples were prepared for cytotoxite, apoptotic and necrotic studies by extracting in 80 °C, 70% ethyl alcohol for 1 hour in a soxlet device. In cytotoxicity studies, sample extract concentrations were applied to MCF-7 and L929 fibroblast cells as 0.5 mg / ml, 0.25 mg / ml, 0.125 mg / ml, 0.0625 mg / ml and 0.03125 mg / ml. The WST-1 solution was added and the absorbance was measured in a microplate reader at a wavelength of 440 nm as a result of the incubation. Apoptosis and necrosis were detected in the same cell line as the double staining experiment. Binary staining solution was prepared and MCF-7 and L929 Fibroblast cells were applied at the same concentrations. At the end of the incubation, measurements were done at 20X magnification in a fluorescence microscope with FITC and DAPI filter. Toxite varies depending on concentrations. At dose 0.125mg/ml, in MCF-7 cancer lines showed 5% cytotoxicity. Cell viability was increased in fibroblast cell lines at all different doses given. Low-grade apoptosis and necrosis have been observed.

Key words: *Agaricus sylvaticus*, cancer, cytotoxicity, apoptotic effect, necrotic effect

1. Introduction

Highly structured fungi (Basidiomycetes) have been used as medicine for many years in the east. The last decade has witnessed the overwhelming interest of western research fraternity in pharmaceutical potential of mushrooms. researches have revealed that fungi are the source of anti-oxidant, anti-cancer, prebiotic, immune modulation, anti-inflammatory, cardiovascular, anti-allergic, hypocholesterolemic, anti-microbial and anti-diabetic molecules (Patel and Goyal, 2012). Mushrooms contain numerous complex substances with therapeutic properties such as polysaccharide-protein / peptide complexes, terpenoids, sterols, lectins, steroids (Popovic et al., 2013). polysaccharides are the best known and most potent mushroom-derived substances with anti-tumor and immunomodulating properties. Polysaccharides (β-(1→3) , β-(1→6)) do not attack cancer cells directly, but produce their antitumor effects by activating different immune responses in the host (Wasser, 2002). Anticancer effects of mushrooms were referred to only immunomodulating effects for a long time. recent studies have shown effects against different pathways of cancer such as antiproliferative, antimetastatic, apoptic, antimutagenic effects (Patel and Goyal, 2012).

In this study, cytotoxicity, apoptotic and necrotic activity of o *Agaricus sylvaticus* on breast cancer MCF-7 cells and L929 normal cells were investigated.

2. Material and Method

2.1. Organism

In this study, *Agaricus sylvaticus* was used. Samples were collected from the Kırıkkale in 2013. The samples are stored in the Protection Biology, Mycology, Molecular Genetics Laboratory of Kırıkkale University Science and Literature Faculty.

2.2 Cultural studies

2.2.1. Studies in solid medium

Tissue fragments obtained from the *Agaricus sylvaticus* strains under aseptic conditions with a scalpel were inoculated on malt extract agar (MEA) medium and primer mycelium developed at 27 °C for 10 d. Mycelium agar pieces from the primer mycelium were inoculated at the center of the MEA and incubated at 27 °C for 10 d to obtain secondary mycelium (Fritsche, 1972).

2.2.2. Studies in broth medium

The three pellet from the secondary cultures, which completed development in the solid culture medium, was grown in nutrient broth in liquid culture medium at 7 d at 27 °C and 140 rpm.

2.3. Preparation of extracts

The samples which completed the development in Nutrient broth were placed in a Soxhlet apparatus and extracted at 80 °C in 70% ethyl alcohol for 1 h. The extract obtained was concentrated under reduced pressure at 75 °C at 40 °C using an evaporator and stored at +4 °C for use in studies.

2.4. Cell cultures for fibroblast and MCF-7 cells

Normal L929 fibroblast cells and MCF-7 cells were placed in flasks containing DMEM with Lglutamine, 10% FCS, and 1% antibiotic and were kept in a CO2 incubator conditioned with 5% CO2 at 37 °C for 24 h. For harvesting cells, the cell culture medium was removed and the cells were treated with trypsin-EDTA (0.5 ml per flask). Cells were then transferred into 15-ml Eppendorf tubes and centrifuged at 2.500 rpm for 2 min. After removing the supernatant the cells were used in the future studies.

2.5. WST-1 assay for cytotoxicity

Fibroblast cells (5 × 103 cells per well) and also MCF-7 cells (5 × 103 cells per well) were placed in 96-well plates containing DMEM and RPMI1640 respectively, with L-glutamine, 10% FCS, and 1% antibiotic. The plates were then kept in a CO2 incubator (37 °C in 5% CO2) for 24 h until the cells attached to the bottom of the plate then the cell culture medium was replaced with fresh medium and different concentrations of *Agaricus sylvaticus* extract (0.5 mg / ml, 0.25 mg / ml, 0.125 mg / ml, 0.0625 mg / ml and 0.03125 mg / ml.) were placed into the wells. Following 24 h incubation under the same conditions, WST-1 (a water-soluble tetrazolium salt) reagent (5 µl) was added into each well (Rzayev et al., 2012). Upon incubation for an additional 4 h, the plates were immediately read in an Elisa Microplate Reader (BioTek, USA) at 440-nm wavelength and the percentage of cell viability of each group was calculated according to definition of control cell viability as 100%.

2.6. Analysis of apoptotic and necrotic cells (live/dead double staining)

Double staining of Hoechst dye with PI is one of the methods that enable the determination and evaluation of the apoptosis and necrosis in culture on the basis of scoring cell nuclei. In this study, the

apoptotic and necrotic effects of *Agaricus sylvaticus* extracts, on fibroblast cells and MCF-7 cells, were examined. Fibroblast cells (5 x 103 cells/ml) and MCF-7 cells (5x103 cells/ml) were cultured in separate 96-well plates in DMEM and RPMI 1640 respectively, supplemented with 10% FCS and 1% antibiotic at 37 ºC and 5% CO2 humidified atmosphere conditions overnight. Then cells were exposed to samples of different concentrations of *Agaricus sylvaticus* (0.5 mg / ml, 0.25 mg / ml, 0.125 mg / ml, 0.0625 mg / ml and 0.03125 mg / ml.) for 24 h. After incubation, the cell culture media were discarded from all wells and 70 µl of the double-staining solution mixture, containing 10 ml of PBS, 500 µl of Hoechst 33342 (200 µg/ml), 100 µl of PI (100 µg/ml) and 100 µl of RNAse A (10 mg/ml), was added on the cells. Then the plate was wrapped with an aluminum foil and incubated for 15 min in incubator. The 33 apoptotic and necrotic cells were visualized under an inverted fluorescence microscope (Leica DMIL, Germany), via DAPI (apoptotic cells were stained with Hoechst (green)) and FITC (necrotic cells were stained with propidium iodide (PI) (red)) filters, respectively. The final data were expressed as percentages of green and red cells, after counting 100 cells from each sample over three randomly chosen areas.

3. Results and Discussion

3.1. Culture studies

3.1.1. Studies on agar media

Agaricus sylvaticus Mycelium began to develop after 48 h. During the development, the air hyphae observed and no pigmentation. After 10 d, colonization was completed (Fig. 1).

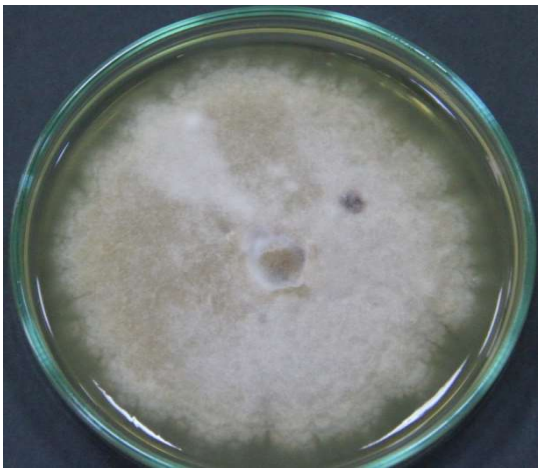


Figure 1. *Agaricus sylvaticus* on agar media.

3.1.2. Studies on broth media

Three pellets from the mycelia that completed the development were collected and cultured in nutrient broth at 27 °C at 140 rpm for 7 d, and no pigmentation was observed (Fig. 2).

Figure 2. *Agaricus sylvaticus* on broth media.

3.2. WST assay for cytotoxicity.

Cytotoxicity was determined at a low rate (cell viability 94.7%) at the concentration of 0.125 mg / mL applied to cancer cells in the cytotoxicity test of *Agaricus sylvaticus* fungus. Increased cell viability was observed at all concentrations applied to L929 Fibroblast cell lines. The highest cell viability was determined to be 0.0625mg / mL (cell viability at 281%) concentration.

Table 1. The absorbance and cell viability results of MCF-7 and L929.

Agaricus sylvaticus	**L929 Fibroblast**		**MCF-7**	
Konsantrasyon (mg/mL)	**Absorbans (440nm)**	**% Canlılık**	**Absorbans (440nm)**	**% Canlılık**
0.5	1.359	150.8	1.421	115.1
0.25	1.702	188	2.29	104.5
0.125	1.922	213.3	1.169	94.7
0.0625	2.539	281	1.691	137.03
0.03125	2.227	247	1.349	109.3
Kontrol	0.901	100	1.234	100

3.3.Analysis of apoptotic and necrotic cells (live/dead double staining)

Apoptosis and necrosis were determined at low indices. Figure 4 and 5 summarize the apoptotic and necrotic cell indexes obtained from the double staining of Hoechst and PI, respectively (Fig. 4, 5).

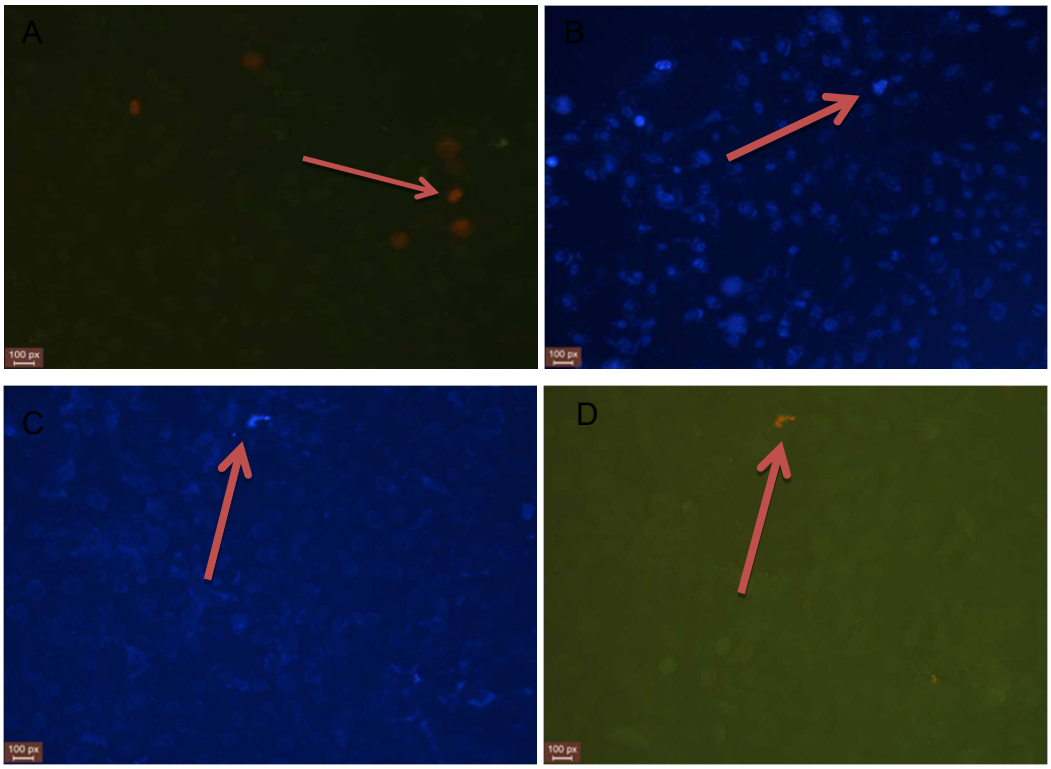
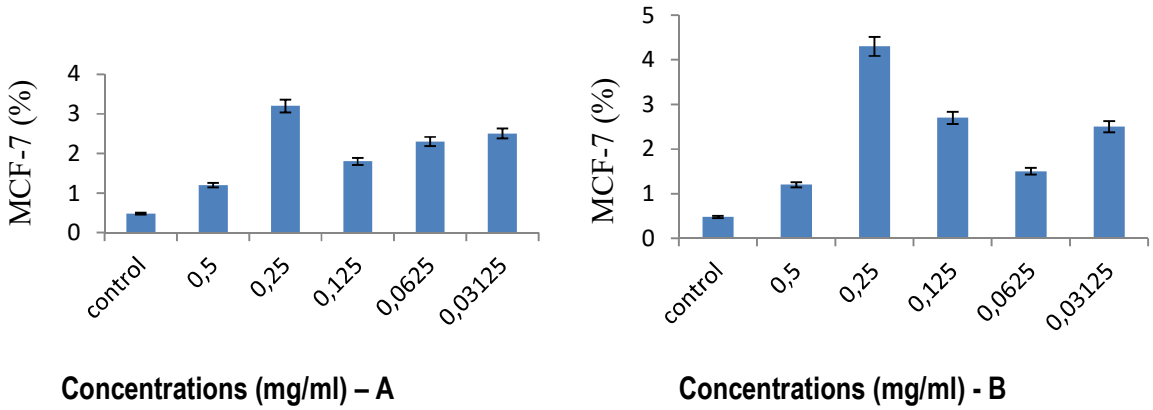


Figure 3. The apoptotic, and necrotic MCF-7 and L929 fibroblast cells under a fluorescent inverted microscope. A) Apoptotic MCF- 7 cells were treated with 0.03125 mg/mL B) Necrotic MCF-7 cells were treated with 0.25 mg/mL extract, C) Apoptotic Fibroblast cells were treated with 0.03125 mg/mL extract, D) Necrotic Fibroblast cells were treated with 0.25 mg/mL extract. Photos were taken by Leica inverted florescent microscope, the scale shows a distance of 100 µm.

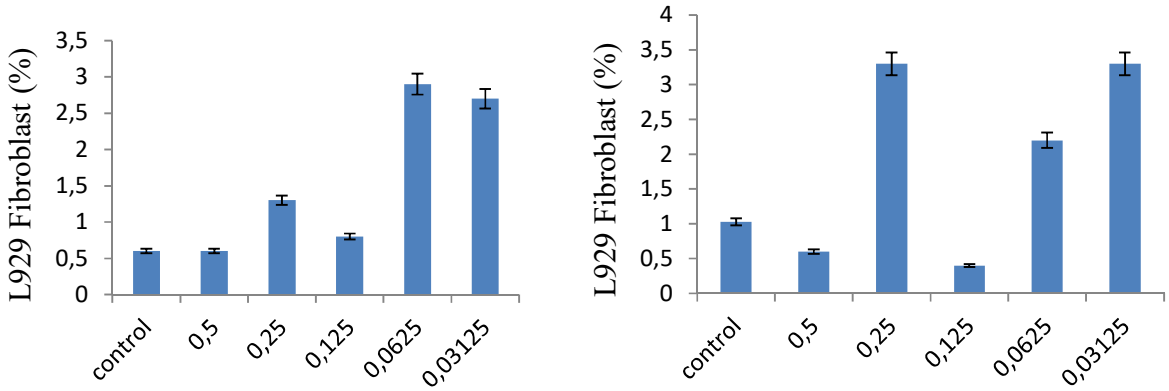
Apoptotic indexes obtained from L929 fibroblast cell and MCF-7 cancer cell cultures following incubation with different concentrations of extracts. Data are expressed as mean ± standard error as calculated from 3 separate experiments. The apoptosis and necrosis states of the MCF-7 cells at different concentrations were determined as shown in the graphs (Fig. 4).



Concentrations (mg/ml) – A **Concentrations (mg/ml) - B**

Figure 4. The apoptosis and necrosis states of the MCF-7 cells at different concentrations. A-Apoptosis; B-Necrosis.

The apoptosis and necrosis states of the L929 Fibroblast cells at different concentrations were determined as shown in the graphs (Fig. 5).



Concentrations (mg/ml) – A **Concentrations (mg/ml) – B**

Figure 5. The apoptosis and necrosis states of the L929 Fibroblast cells at different concentrations. A-Apoptosis; B- Necrosis.

Agaricus sylvaticus is an edible mushroom. researches have shown that Dietary supplementation with *A. sylvaticus* improved nutritional status and reduced abnormal bowel functions, nausea, vomiting, and anorexia in patients with Breast cancer receiving chemotherapy (Valaderes et al., 2013). And supplementation with *A. sylvaticus* produces benefits in hematological and immunological parameters and can reduce glycemia levels in patients with colorectal cancer (Fortes et al., 2008; Fortes et al., 2009)

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